Concerted action at eight phosphodiester bonds by the BcgI restriction endonuclease

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ABSTRACT

The BcgI endonuclease exemplifies a subset of restriction enzymes, the Type IIB class, which make two double-strand breaks (DSBs) at each copy of their recognition sequence, one either side of the site, to excise the sequence from the remainder of the DNA. In this study, we show that BcgI is essentially inactive when bound to a single site and that to cleave a DNA with one copy of its recognition sequence, it has to act in trans, bridging two separate DNA molecules. We also show that BcgI makes the two DSBs at an individual site in a highly concerted manner. Intermediates cut on one side of the site do not accumulate during the course of the reaction: instead, the DNA is converted straight to the final products cut on both sides. On DNA with two sites, BcgI bridges the sites in cis and then generally proceeds to cut both strands on both sides of both sites without leaving the DNA. The BcgI restriction enzyme can thus excise two DNA segments together, by cleaving eight phosphodiester bonds within a single-DNA binding event.

INTRODUCTION

Most restriction–modification (R–M) systems display two activities that act in response to a specific DNA sequence: a modification methyltransferase (MTase) that methylates a specific base within the sequence; and a restriction endonuclease (REase) that cleaves the DNA provided neither strand is modified (1). R–M systems fall into four main Types: I–IV (2,3). The classical Type I systems feature an oligomeric protein with separate subunits for DNA specificity (S), methylation (M) and cleavage (R), in a R2M2S arrangement (4,5). The REase activity predominates at unmodified sites and the MTase at hemi-methylated sites. The standard Type II systems contain two separate proteins, which independently recognize the same DNA sequence (6): a dimeric REase that cuts both DNA strands (7,8); and a monomeric MTase whose main reaction is to transfer one methyl group onto DNA already methylated in one strand (9). However, some Type II systems carry both REase and MTase activities in the same polypeptide, and have a separate subunit—akin to an S subunit from a Type I system—for DNA recognition (10,11). Others carry all three functions in one polypeptide (12–15), as do certain Type I enzymes (16). In the standard Type II systems, the REase generally requires only Mg²⁺ and the MTase AdoMet (S-adenosyl methionine) as co-factors (6–8) but some REases from the fused systems need both Mg²⁺ and AdoMet (10,12,13,15).

Type II REases cleave DNA at fixed positions at or near their recognition sites (6). Their recognition sequences are often 4–8 bp palindromes, usually continuous but in some cases interrupted by a fixed length of non-specific DNA (3). The dimeric enzymes that recognize palindromic sites cut a specified phosphodiester bond in one strand, normally within the sequence, and the symmetrically equivalent bond in the other strand (7,8). However, the Type II nucleases can be categorized into several subtypes on the basis of, among other criteria: where they cut the DNA; their mode of action; and, as noted above, their genetic organization and subunit assembly (2).

The REases from one subtype, the Type IIS systems, recognize asymmetric sequences and cut both DNA strands set distances downstream of the site (17–19). In another group, the Type IIB systems (20), the endonucleases make two double-strand breaks (DSBs) at each site, both at specified positions distant from the recognition sequence, one upstream and one downstream of the site. With some exceptions (21,22), Type IIB recognition sequences are discontinuous and asymmetric, with upstream and downstream sites of cleavage at fixed positions...
equidistant from the sequence. For example, BcgI cleaves DNA as follows:

\[ 5'-\text{N} \downarrow \text{N}_{10}\text{-CGA}\text{-N}_{6}\text{-TGC}\text{-N}_{12} \downarrow \text{N}-3' \]
\[ 3'-\text{N} \uparrow \text{N}_{12}\text{-GCT}\text{-N}_{6}\text{-ACG}\text{-N}_{10} \uparrow \text{N}-5' \]

where N is any nucleotide (nt) and arrows mark cleavage points (23). The Type IIB systems thus excise from the DNA a short fragment containing the recognition sequence: in the case of BcgI, a 34-nt long in each strand (this will be noted as the 34-mer). The excision reactions of the Type IIB enzymes are reminiscent of the transposases that operate by cut-and-paste mechanisms and the dicer enzymes that cleave RNA duplexes to fragments of defined length (24,25).

Most REases cleave DNA substrates with two specific sites more rapidly than DNA with one site (26). The Types I, III and IV systems display this behaviour (4,5), as do numerous Type II nuclease (27–30). The latter include most of the IIS and virtually all of the IIB enzymes (18,31). For the Type II enzymes, faster cleavage of a two-site substrate is often taken as a diagnostic that the enzyme needs to interact with two sites at the same time (32). This conclusion is based on the fact that proteins that bind two DNA sites at the same time normally have higher affinities for sites in cis over sites in trans, simply because two loci in the same chain are almost always closer in 3D space than sites in separate molecules (26). Consequently, these enzymes often cleave plasmids with two target sites more rapidly than one-site plasmids. Rates on one-site plasmids can, however, be enhanced by adding an oligoduplex that carries the cognate sequence (28,33): the enzyme on the plasmid can form a synaptic complex more readily with the duplex than with a second plasmid (26,27).

The Type II enzymes that need two sites fall into two subtypes, IIE and IIF (2). The IIE REases bind two or more copies of the recognition sequence, at catalytic and at allosteric loci, but only the DNA at the catalytic locus is cleaved (28,33,34). In contrast, the IIF enzymes have two identical DNA binding surfaces, both of which have to bind the sequence before the enzyme becomes active: both sites are then usually cut before the DNA is released (27). The Type IIF enzymes are thus said to act in a concerted manner. For example, SfiI, the archetype of IIIE (26,27,34–38). The Type IIF enzymes act as tetramers with each pair of subunits resembling a standard dimeric Type II REase at a solitary site (36–38).

Most of the Type IIB REases act like Type IIF enzymes in that they cleave DNA with two cognate sites more rapidly than substrates with one site and that the initial product from a two-site substrate is usually the DNA cut at both sites (20,31). However, the Type IIB systems cut both sides of each site, cleaving a total of eight phosphodiester bonds on a two-site DNA. To cut all eight bonds within the lifetime of a protein–DNA complex, the enzyme will need eight active sites if each is used once per turnover. The BcgI R–M system, the archetypal IIB REase (11,39), is an oligomeric protein composed of A and B subunits, in a 2:1 ratio (10,40). The A subunit contains a nuclease active site near its N-terminus while its C-terminus resembles an adenine MTase (10,40). The B subunit is similar to an S subunit from a Type I R–M system and is responsible for DNA recognition (11,41). The minimal assembly for BcgI, the A2B1 heterotrimer, thus contains two nuclease active sites and so might be expected to cleave just two phosphodiester bonds per turnover.

**MATERIALS AND METHODS**

**Protein**

*Escherichia coli* XL1-Blue MRF® Kan (Stratagene) was transformed with pARG3–BcgI (gift from New England Biolabs) and the colonies grown at 37°C on LB agar containing the relevant antibiotics. This plasmid carries the complete R–M system for BcgI (bcgIA and b cgIB) expressed from a tac promoter (personal communication, H. Kong). The strain was grown in LB at 37°C to an OD600 of ~0.6 before adding IPTG to 1 mM. After 4 h, the cells were harvested by centrifugation and stored at −20°C. In subsequent steps, all solutions were at ≤4°C. Cells from each litre of culture were suspended in 10 ml 0.3 M NaCl in Buffer A [20 mM Tris–HCl, pH 7.4, 1.0 mM EDTA, 10% (v/v) glycerol] with protease inhibitors (Roche). The suspension was fed through a Constant Systems Ltd cell disruptor and debris removed by centrifugation. The supernatant was appropriately diluted in Buffer A and then subjected to chromatography on Heparin–Sepharose followed by Blue-Sepharose and then Mono-Q (GE Healthcare). All three columns were developed with linear gradients of 0.075–1.0 M NaCl in Buffer A, and fractions analysed by SDS–PAGE and endonuclease assays (42). The fractions from the Mono-Q that contained pure BcgI protein, as judged by SDS–PAGE, were pooled and stored at −80°C in the buffer in which it eluted (0.34 M NaCl in Buffer A). Concentrations of pure BcgI were evaluated from A280 readings using a calculated extinction coefficient (43), 101320 M−1 cm−1; M denotes the molarity of the A2B1 form of BcgI. Immediately before each experiment, BcgI was dialysed to the requisite concentration in 20 mM Tris–HCl, pH 8.4, 0.1 mM EDTA, 10 mM β-mercaptoethanol, 1 mM spermine, 10% glycerol and 0.2% (v/v) Triton X-100.

**DNA**

Transformants of *E. coli* HB101 with either pUC19 or pDG5 (31) were grown in M9 minimal media containing [methyl-3H]thymidine. The monomeric supercoiled (SC)
form of the plasmid was purified by CsCl density gradient centrifugations (27,29).

Oligodeoxyribonucleotides were purchased as HPLC- or gel-purified samples from Eurofins MWG Operon or Sigma Genosys. Oligoduplexes (Figure 1) were usually formed by mixing two complimentary oligonucleotides, both ~20 μM, in 20 mM Tris–HCl, pH 8.0, 100 mM NaCl and heating the mixture to 95°C before cooling slowly to room temperature. To make the duplex BIO-61 (Figure 1), the 5'-end of the bottom strand was 32P-labelled, with polynucleotide kinase and [γ-32P]ATP (44,45), and then mixed with an excess of the biotinylated top strand before annealing.

The DNA noted as the 235-mer substrate was generated by using standard PCR methods (46) to amplify a 235-bp segment of pDG5 that spanned one of its BcgI sites (31). The PCR product was purified by electrophoresis through agarose followed by extraction from the gel using a QIAEX II kit (Qiagen).

DNA concentrations were evaluated from A260 readings: for double-stranded DNA, an A260 of 1 was taken to denote 50 μg/ml DNA (46): for oligonucleotides, extinction coefficients were derived from www.basic.northwestern.edu/biотools/oligocalc.html.

Reactions

Reactions to measure plasmid cleavage were carried out in 200 μl volumes at 37°C in Buffer R (20 mM Tris–HCl, pH 8.4, 66 mM NaCl, 10 mM MgCl2, 1 mM DTT, 20 μM AdoMet, 200 μg/ml BSA, 10% glycerol) containing 20 nM BcgI, 5 nM SC plasmid (3H-labelled) and, in some instances, the requisite oligoduplex at 10 nM. A zero-time point was taken before initiating the reaction with BcgI, and further samples (10 μl) removed at intervals thereafter. The samples were mixed immediately with 5 μl Quench Mix (100 mM EDTA, 40% (w/v) sucrose, 100 mM Tris–HCl, pH 8.0) supplemented with 100 μg/ml bromophenol blue and, after heating briefly to 67°C, analysed by electrophoresis through agarose [containing 0.5 μg/ml ethidium bromide (EtBr)] under conditions that separated the following from each other (32): the SC substrate; the open circle (OC) DNA with one or more single-strand break(s) (SSBs); the linear DNA (LIN) with one (or more) DSB(s) at a single site; and, on substrates with two BcgI sites, the linear products (L1 and L2) with at least one DSB at both sites. The segments of the gels containing each DNA were assessed by scintillation counting (42). (Note that the OC form is not separated from the dimeric plasmid so a fraction of the counts assigned to the OC form originate from the dimer).

Reactions to measure the release of the 34-mer product from plasmid substrates were in Buffer R* [as Buffer R but with 5% (w/v) Ficoll 400 in place of the glycerol]. Aliquots (20 μl) were taken from the reactions at various times and quenched in 5 μl Quench Mix that also contained 250 μg/ml proteinase K and the 42NS duplex (20 nM: to act as a normalization standard to quantify the 34-mer). After 30 min at 37°C, one portion (10 μl) of each quenched sample was loaded onto an agarose gel and processed as above. A second 10 μl portion was subjected to electrophoresis through 20% polyacrylamide in TBE (46). After electrophoresis, the polyacrylamide gels were stained with SYBR Safe DNA gel stain (Invitrogen) in TBE for 20 min. The fluorescence from the DNA was then recorded in a Typhoon PhosphorImager (Molecular Dynamics), using its 488-nm laser and 520 BP 40 emission filter, and quantified in ImageQuant.

Cleavage of the 235-mer was monitored by taking 50 μl aliquots from 600 μl reactions containing 5 nM 235-mer and 20 nM BcgI REase in Buffer R at 37°C, and adding these to Quench-Mix (10 μl). The samples were treated with proteinase K and the DNA precipitated with ethanol (46). The pellets were suspended in 10 μl 10 mM Tris–HCl, pH 8.0, 1 mM EDTA followed by 10 μl Quench Mix, and applied to 8% polyacrylamide in TBE (46). After electrophoresis, the gels were stained with 0.5 μg/ml EtBr in TBE.

![Figure 1. Oligoduplexes. The oligoduplexes named on the left have the sequences indicated. HEX denotes hexachlorofluorescein: duplex 42NS (data not shown) has the same nucleotide sequence as HEX-42NS but lacks the HEX label. The recognition sequence for BcgI is in bold and underlined: in HEX-42SM, both were replaced by N6-methyladenine. To construct the duplex BIO-61, the 5'-end of the bottom strand was 32P-labelled before annealing to the top strand: the latter carries at its 5'-end a biotin moiety (indicated as BIO). The arrows mark the scissile bonds on the bottom strand, that give 32P-labelled products of 48 and 14 nt from left and right cleavage loci, respectively.](image-url)
for 20 min, washed and the fluorescence recorded in the PhosphorImager. Images were analysed in ImageQuant to determine the relative concentrations of the substrate and of all of the various products.

Streptavidin-coated magnetic beads (Promega) were attached to 32P-labelled BIO-61 (Figure 1) as described previously (37,38,44,45), at a 1:125 ratio of biotinylated duplex to streptavidin. The washed beads were suspended in 200 µl Buffer R and BcgI protein added, to give reactions at 37°C containing 1 nM BIO-61 and 20 nM BcgI; some also contained 5 nM HEX-42S (Figure 1). Aliquots (15 µl) were taken from the reactions at timed intervals and added to 10 µl Formamide Loading Mix (45). The quenched samples were heated to 95°C for 10 min, cooled on ice and analysed by denaturing gel electrophoresis through 12% polyacrylamide in a TBE-urea buffer at 55°C: a series of 32P-labelled oligonucleotides, 12- to 61-nt long, were run as markers. The gels were held overnight in PhosphorImager screens, which were then scanned to evaluate the extent of cleavage of the radiolabelled strand of BIO-61 and the amounts of the labelled products.

RESULTS

BcgI reactions in trans

The BcgI endonuclease is less reactive on DNA with one cognate site than DNA with two sites: the minimal enzyme concentration needed to cleave all of a one-site plasmid is higher than that for a two-site plasmid (40) and, at an enzyme concentration sufficient to complete the reactions on both one- and two-site substrates, the one-site DNA is cleaved at a slower rate (31). Complete cleavage of a one-site plasmid is achieved only when the BcgI protein is at a higher concentration than the DNA (40). All of the reactions in this study contained a fixed level of BcgI in excess of DNA sites.

Reactions on a SC DNA with two BcgI sites yielded virtually none of the nicked OC form of the DNA with SSBs at one or both sites and a relatively low level of the linear DNA cleaved at one site (31). Instead, the initial product from the two-site plasmid was mainly the linear fragments with at least one DSB at each site. Furthermore, a catenane composed of two interlinked rings of DNA, with a BcgI site in each ring, was converted directly to a LIN form, without liberating any of the nicked OC species (Figure 5), but at a relatively slow rate for a reaction containing enzyme in excess of substrate.

The addition of a 42-bp duplex with the specific sequence (HEX-42S: Figure 1) enhanced the rate of cleavage of the one-site plasmid (Figure 2): optimal enhancement was observed with 10 nM duplex (data not shown). This concentration was therefore used in subsequent experiments. At this optimum, the rate approached that of BcgI reactions on a two-site plasmid (Figure 6).

Two 42-bp duplexes with the same sequence as HEX-42S apart from the recognition site were also tested: in one case (HEX-42NS), the recognition site was ablated by two base pair substitutions, one in each segment of the bipartite site, to give a non-specific sequence; in the other (HEX-42SM), the target adenines for the MTase of the BcgI R–M system were both replaced by N6-methyladenine, to give a fully modified duplex resistant to the nuclease (10). Neither of these duplexes had any effect on plasmid cleavage (Figure 2). Further studies (data not shown) employed hemi-methylated derivates of HEX-42S, modified in either top or bottom strand: neither had any effect on plasmid cleavage. However, as in previous studies (39), the BcgI protein readily converted the hemi-methylated duplexes to the fully methylated form while failing to methylate the unmodified duplex.

Figure 2. BcgI reactions in trans. Reactions at 37°C contained 20 nM BcgI enzyme and 5 nM 3H-labelled pUC19 (initially 90% SC) in Buffer R, and either no oligoduplex (white circles) or one of the following duplexes at 10 nM: HEX-42S (white squares); HEX-42NS (white triangles); HEX-42SM (black triangles); HEX-18S (black squares). Samples were withdrawn from the reactions at the times indicated and analysed as in the ‘Materials and Methods’ section to evaluate the residual concentrations of the SC form of pUC19. Each data point is the mean from three repeats and each set is fitted to a single exponential decay.
DNA molecules were too far apart for interactions moieties on the beads, so that in most cases the individual employed a low ratio of biotinylated DNA to streptavidin proximal or distal to the label (Figure 1). The attachment depending on whether the bottom strand is cut at the bond initial products radiolabelled species of either 14 or 48 nt [Figure 3]. These reactions contained a 20-fold molar excess of HEX-18S or HEX-42S (≥100 nM) inhibited plasmid cleavage (data not shown), presumably due to BcgI interacting with two molecules of the duplex in preference to one duplex and one plasmid.] The second DNA must carry an intact unmodified recognition sequence but not necessarily the loci for DNA cleavage. As with other Type IIE and IIF REases (28), the second DNA need not be a true substrate. But it has yet to be seen if BcgI can form a complex in trans with two molecules of pUC19. Two molecules of a SC plasmid are less able to form a synaptic complex than one SC and one linear DNA, as the linear DNA can readily penetrate the domain of the SC DNA, especially if it is a short duplex (26,27,32). Hence, the difficulty of synapsing two SC DNA molecules may prevent BcgI from acting in trans during its reaction on pUC19 alone, so that the rate then reflects the activity of BcgI bound to a solitary site.

**BcgI reactions on isolated DNA**

To see if the BcgI REase had any activity when bound to a solitary site, an oligoduplex, the 61-bp DNA BIO-61 (Figure 1), was immobilized on the surface of a streptavidin-coated bead (37,45): the duplex has a biotin unit on the 3'-end of one strand and a 32P-label on the 5'-end of the other. BcgI can cut this DNA to give as initial products radiolabelled species of either 14 or 48 nt depending on whether the bottom strand is cut at the bond proximal or distal to the label (Figure 1). The attachment employed a low ratio of biotinylated DNA to streptavidin moieties on the beads, so that in most cases the individual DNA molecules were too far apart for interactions in trans (37,38,44,45). Hence, the cleavage of the immobilized DNA provides a measure of the activity of the enzyme at a solitary site. However, if a second duplex is added to the solution, the enzyme may cut the immobilized DNA at an enhanced rate, due to interactions in trans between the free and the immobilized duplexes (37,44).

When BcgI was added to beads carrying the BIO-61 substrate, without any other DNA present, virtually none (<3%) of the substrate was cleaved after 30 min (Figure 3). These reactions contained a 20-fold molar excess of BcgI enzyme over BIO-61 on the beads, a stoichiometry that would allow for every DNA molecule to bind enzyme. Moreover, increasing the level of enzyme to a 100-fold excess over duplex did not increase the extent of cleavage of the immobilized DNA (data not shown). The BcgI REase thus has essentially no activity when bound to a solitary recognition site under conditions that largely exclude interactions with a second site in trans.

When the HEX-42S duplex was added to the suspension of the beads holding the BIO-61 DNA, BcgI cleaved the immobilized DNA much more rapidly than in the absence of the duplex (Figure 3). When this strategy had been applied to other Type IIE REases—namely Bse634I, Eel18kI and SfiI (37,38,44)—it had been found that, though these enzymes were more active upon adding a second substrate, they still cleaved the immobilized one-site DNA at readily detectable rates. Under comparable conditions to those used here, all three of these enzymes cleaved about 50% of their isolated DNA substrates within 30 min. In contrast, BcgI cleaved practically none of its immobilized substrate after 30 min. The trace level of cleavage in the absence of the second duplex may be due to the fact that the ratio of biotinylated DNA to biotin binding sites on the beads used here (125:1) will occasionally leave two DNA molecules sufficiently close for them to be spanned by the BcgI protein. BcgI activity thus seems to be totally dependent on binding two recognition sites. Hence, it must cleave pUC19 by means of a synaptic complex in trans, spanning sites on two separate molecules of the SC plasmid.

If BcgI cuts the DNA on one side of its site at a time, with equal probability for left- and right-hand loci, the 61-nt 5'-radiolabelled strand of BIO-61 will give rise initially to the 48- and the 14-nt radiolabelled products in equal yield (Figure 1). The 48-nt product still possesses
the right-hand target so it should subsequently be cut to give the 14-nt species as the only radiolabelled product. Yet even at the start of the reaction in the presence of the second duplex, only low levels of the 48-nt product were generated (Figure 3). Instead, the majority of the immobilized DNA was converted directly to the 14-nt product.

The inequality between the initial yields of the 48- and 14-nt products could arise if BcgI acts on both sides of its target site in a highly concerted manner, to give rise directly to the final product cut on both sides: the 14-nt species would then be the primary radiolabelled species. As with the SfiI restriction enzyme (27,35), concerted action is taken to denote the cleavage of multiple phosphodiester bonds by a single enzyme–substrate complex rather than each bond being cleaved in a separate DNA binding event (26). It should, however, be noted that no reaction is ever likely to be 100% concerted since this would require the enzyme to remain bound to the DNA until all of the target bonds had been cleaved. In particular, the BcgI enzyme would have to have an extraordinarily high affinity for DNA to remain bound to the DNA for long enough to cleave all of its numerous target bonds. It is instead more likely that a fraction of the enzyme–DNA complexes will dissociate before eight bonds at two recognition sites are all cleaved: this could account for the formation of the low level of the 48-nt intermediate seen here. Nevertheless, an alternative explanation for the lack of the 48-nt intermediate is that the left-hand locus in BIO-61 is more resistant to BcgI than the right-hand locus.

**Concerted action on both sides of one BcgI site**

To distinguish between these possibilities, in particular to see if the BcgI REase can act concertedly on both sides of its recognition site, the enzyme was tested against a 235-bp linear DNA substrate with a single site. The substrate, noted as ABC (Figure 4A), was designed so that the DNA fragments from cleaving on the left (A and BC), on the right (AB and C) and on both sides of the site (A, B and C) were all sufficiently distinct from each other in size to allow for their separation by electrophoresis through polyacrylamide (Figure 4B).

The reaction on the 235-mer DNA employed BcgI in molar excess over the DNA, in order to observe the conversion of the enzyme–substrate to the enzyme–product complexes as opposed to the accumulation of the free products left after multiple turnovers: the latter would have been the case if these reactions had contained a large excess of substrate over enzyme. A reaction that produced a DSB on just the left of the site should give rise to equal concentrations of A and BC. A second reaction would then be needed to convert the BC intermediate into the final products, B and C. Similarly, a DSB on just the right-hand side generates as initial products equal concentrations of AB and C. The yield of the AB + C products may however differ from those for A + BC, if one side is cleaved preferentially to the other. Conversely, if BcgI can introduce DSBs on both sides of its site, one immediately after the other, the ABC substrate will be converted directly to the final products, without liberating the AB and BC intermediates.

BcgI readily cleaved the 235-mer (Figure 4C), presumably by acting *in trans* across two molecules of the DNA.
However, the levels of the BC and AB intermediates formed during the reaction were considerably lower than their partner products, A and C, respectively. During the initial phase, coincident with the formation of the BC and AB intermediates, a lag phase was observed in the production of B (Figure 4C, yellow circles), after which the level of this product, the excised 34-mer, matched those of the terminal products, A and C. Hence, during a single DNA binding event, the BcgI REase usually makes two DSBs at each site, one on either side of the site, and only rarely dissociates from the DNA after making just one DSB. The latter happens in about 10% of the reactions, as judged by the yields of the AB and BC intermediates. Once liberated, the AB and BC intermediates do not progress through to the end products, A + B and B + C, respectively.

Strikingly, the same level of an intermediate cut on one side of the site was observed during the cleavage of the immobile BEO-61 duplex (Figure 3): in the presence of free duplex, about 10% of this DNA was converted to the 48-nt product cleaved only on the left of the site and this species was not cleaved again at the right-hand locus. Furthermore, the sites of DNA cleavage by BcgI were initially identified by using primer extension to incorporate a radiolabel into the DNA and then cleaving the resultant DNA with the REase (23). Though the amounts of the DNA products cut on one or both sides of the site were not quantified, visual inspection of the autoradiograph published from this experiment indicate that the molar yield of product cut on one side of the site appears to be about one-tenth of that of the product cut on both sides [Figure 2 in (23)]. These pioneering studies are thus fully consistent with the quantitative analysis presented here, and provide further support for the view that BcgI acts in a highly concerted manner, with ~90% of the enzyme-substrate complexes cleaving all of the target bonds before falling apart.

The initial lag phase in the production of B shows that the two DSBs are made separately, one after the other, but both within the lifetime of the DNA-protein complex. Since the AB and BC intermediates are formed in equal yield (Figure 4), the first DSB occurs with equal probability on the left or the right of the site.

**Concerted action on both sides of two BcgI sites**

The ability of the BcgI REase to excise its recognition sequence from DNA was examined further with plasmid substrates that carried one or two BcgI sites (Figures 5 and 6, respectively). To date, BcgI reactions on plasmids have been monitored by using agarose gels to separate the intact DNA substrate from the cleaved products (31,40), but such experiments fail to distinguish the kilobase-sized fragments cleaved on one side of the recognition site(s) from those cleaved on both sides, as these differ by just 34 bp, while the 34-mer itself is not retained in the gel. To monitor in parallel plasmid cleavage and the release of the 34-mer, samples were taken at intervals from reactions containing BcgI and a plasmid substrate: one portion of each sample was applied to agarose, to measure the amount of DNA with at least one DSB break at each recognition site, while a second portion was subjected to PAGE, to record the 34-mer. The samples also contained a known amount of a 42-bp duplex, 42NS (Figure 1), from which the concentration of the 34-mer was determined.

This strategy was first applied to a SC plasmid with one BcgI site, pUC19 (Figure 5), since this might be analogous to the formation of the 34-mer from a linear DNA with one site (Figure 4). However, unlike the linear substrate, a SC DNA might reveal transient SSBs in the DNA, as nicked OC forms. Moreover, the excision of the 34-mer from a 2.7 kb SC DNA could potentially proceed by a different mechanism from the highly concerted scheme seen with the 235-bp DNA. The BcgI enzyme should
bridge two molecules of a relatively short linear DNA more readily than two molecules of a SC plasmid (26,27), which might allow for the former to be cleaved concertedly while the latter follows a sequential pathway.

The agarose gel (Figure 5A) showed that BcgI generated as an initial product from the one-site plasmid, the LIN species, with at least one DSB at the recognition site: none of the nicked OC form accumulated during the reaction (Figure 5C). The parallel analysis on PAGE (Figure 5B) revealed that the production of the 34-mer closely followed the LIN species. The full-length linear form with just one DSB break at the site is thus processed directly to the final product in which the specific segment has been excised by two DSBs (Figure 5C). Two separate assays on topologically different substrates (Figures 4C and 5C) have thus shown that BcgI acts at an individual recognition site to cleave the DNA predominantly on both sides of the site in a highly concerted manner, without liberating intermediates cut on just one side.

The BcgI REase cleaves plasmids with two copies of its recognition sequence more efficiently than one-site plasmids (31,40). On two-site substrates, it usually acts at both sites concurrently, to give as its initial product mainly the two linear fragments with at least one DSB at each site. But whether the concurrent action results in one or two DSBs at both sites has yet to be established. The above strategy of parallel analyses on agarose and polyacrylamide was therefore applied to BcgI reactions on a two-site plasmid, pDG5 (Figure 6).

The agarose gels (Figure 6A) showed that, as in previous studies (31), the two-site plasmid was cleaved about five times faster than the one-site plasmid. A small fraction (~20%) of it gave rise initially to the LIN product cut at one BcgI site, while the majority was converted directly to the two linear products (L1 and L2) cut at both sites (Figure 6B). Each molecule of the two-site plasmid can potentially give rise to two molecules of the 34-mer. The LIN product may have been cleaved on both sides of one site, to liberate one molecule of the 34-mer, or it may have been cut on just one side of the site and so still carry the 34-mer: the same applies to the L1 and to the L2 species, which together can yield two 34-mers. Hence, to correlate the DSB(s) at each site from the agarose gels with the liberation of the 34-mer observed on the polyacrylamide gels (Figure 6C), the yield of the 34-mer was compared to the sum of the concentrations of all of the DNA species observed in the agarose gel that can potentially give rise to the 34-mer, [LIN + L1 + L2] (black inverted triangles, solid line).

Figure 6. Excision of BcgI sites from a two-site plasmid. Reactions, and the subsequent processing of the aliquots, were identical to that in Figure 5 except that they contained pDG5 (>85% SC) in place of pUC19. (A) One portion of each aliquot was analysed by electrophoresis through agarose containing EtBr. The times of withdrawal of the aliquots are indicated above the gel and the electrophoretic mobilities of the following forms on the left: OC, nicked DNA with at least one SSB; LIN, DNA cut at one BcgI site; SC, the intact substrate; L1 and L2, the linear products from cutting at both BcgI sites. (B) The concentrations for DNA species observed in the agarose gel were measured by scintillation counting: SC, black squares; OC, white circles; LIN, black triangles; the average of [L1] and [L2], white diamonds. (C) A second portion of each aliquot was analysed by electrophoresis through polyacrylamide: the gel image is labelled with the reaction times (above) and the mobilities of both the 34-mer product excised during the reaction and the 42-bp duplex (42NS) present in the Quench Mix (on the left). (D) The concentration of the 34-mer formed during the reaction (inverted white triangles, dashed line) is compared to the total concentration of all of the DNA species observed in the agarose gel that can potentially give rise to the 34-mer, [LIN + L1 + L2] (black inverted triangles, solid line).
L1 and the L2 species (Figure 6D). This comparison showed that the 34-mer is formed shortly after the linear DNA species were produced. The first DSB at each site must therefore be followed directly by the second, without releasing the intermediates cut on one side only. The BcgI REase can thus bind to both copies of its recognition sequence on a two-site plasmid and cleave the DNA on both sides of both sites within the lifetime of the complex.

**DISCUSSION**

The experiments described here show that the BcgI REase is essentially inactive at a solitary recognition site on a DNA held in isolation from other DNA molecules and that it is only active after binding two copies of its recognition sequence at the same time (Figure 3). The two sequences are preferably located in cis, on the same molecule of DNA ([31]; see also Figure 6], but BcgI can act in trans, spanning sites in separate DNA molecules (Figure 2). The enzyme usually cuts both sites on a two-site DNA in a highly concerted process, largely bypassing the intermediates cut at just one site (Figure 6). Moreover, at each site, the enzyme makes two DSBs, one directly after the other (Figures 4-6). All of these reactions employed enzyme in excess of the DNA, yet they yielded only low levels of the intermediates cleaved on just one side of the site (Figures 3 and 4). Hence, the two DSBs at each site, one either side of the recognition sequence, must both occur within the lifetime of the complex bridging the sites. The BcgI REase can thus cleave a total of eight phosphodiester bonds on the DNA with two BcgI sites, both strands on both sides of both sites, without dissociating from the DNA.

The SfiI REase binds two copies of its recognition sequence and then cleaves its four target bonds, two in each site, in random order but with the same rate constant at all four phosphodiester bonds: all four are usually cut before the DNA is released (47). If BcgI acted similarly and cleaved a SC plasmid with two recognition sites with equal rate constants at all eight scissile bonds, and if the first reaction occurs with equal probability at any one of the eight bonds, essentially all of the SC DNA would be converted first to its nicked OC form: the subsequent conversion of the OC DNA to the LIN form would be very much slower as the next reaction has only a 1:7 chance of occurring opposite the nick site. In complete contrast to this expectation, virtually none of the OC form was observed during BcgI reactions. Hence, the lack of accumulation of intermediates cut at fewer than eight bonds implies that the rate-limiting step in the reaction pathway of BcgI is the assembly of the synaptic complex spanning two recognition sites, after which all eight bonds are cleaved in rapid succession. DNA released from those few synaptic complexes that disassemble before all eight bonds are cut does not progress through to the final products (Figures 3 and 4).

The reaction of BcgI at an individual recognition site, namely the precise excision of a DNA segment 34-nt long in both strands, is reminiscent of the action of the RNaseIII enzymes that cleave double-stranded RNA into fragments of defined lengths, a central process in the RNAi and the CRISPR pathways (47,48). It had once been suggested that RNaseIII acts by assembling two dimers immediately adjacent to each other in tandem repeat along the RNA; each dimer was thought to make one DSB and so liberate a pre-set length of RNA duplex (49). However, BcgI can excise two distant segments at the same time so is likely to possess a more complicated arrangement of subunits than just a dimer of dimers. In particular, for BcgI to hydrolyse eight phosphodiester bonds while remaining bound to two DNA loci, the nuclease needs either to have eight active sites or to use its active sites more than once. The latter can be achieved by cutting both strands in the same active site via a hairpin intermediate, as seen with several recombination enzymes (24), but BcgI has the PD...EXK motif characteristic of a conventional nuclease (11). Alternatively, the active sites need to move either from one strand to the other or from one side of the site to the other. The movement of an active site between strands requires the protein to be rotated through 180° perpendicular to the helical axis of the DNA, to accommodate the anti-parallel strands, but at present this sort of event has been seen only with Bii1, which is in many ways an atypical REase (50). The movement from one side of the recognition site to the other is also improbable as the cleavage loci are about 115 Å apart in standard B-DNA, which is perhaps too far to be spanned by a protein conformational change.

The BcgI protein, like many but not all other Type IIB systems (20), is composed of two subunits, A and B, in a 2:1 ratio (10). The A subunit carries the catalytic centres for both REase and MTase activities while the B subunit recognizes the target sequence (11), much like an S subunit from a Type I R–M system (41). BcgI is active only after binding two copies of its cognate sequence, presumably via two B subunits, so the active enzyme is likely to consist of at least a dimer of the A2B1 protomer. A dimer of A2B1 units might be able to excise the 34-mer from a single recognition site as it possesses four catalytic centres for phosphodiester hydrolysis, one in each A subunit: these could perhaps act as in the model for RNaseIII. But it seems unlikely that this dimer could excise two 34-mers from two separate DNA loci. Thus the cleavage of eight phosphodiester bonds in one DNA–protein complex may well require a tetramer of A2B1 units bound to two recognition sites. Such an assembly would possess more B subunits than is necessary to bind two copies of the recognition sequence, and more MTase centres in the A subunits than needed to methylate these sites, as this requires only one methyl group per site on a hemi-methylated substrate.

Other REases also seem to contain an excess of DNA recognition units: the Type IIS REase BspMI is a homotetramer that binds two copies of an asymmetric sequence, presumably via two of its subunits, but then cuts four phosphodiester bonds (both strands downstream of both sites), presumably via active sites in all four subunits (51,52). The heterotetrameric Type III systems also seem to have more DNA recognition/methylation units than needed for one DSB (5). Moreover, the FokI endonuclease bound to DNA has to recruit a second FokI protein to
give a dimeric assembly with two catalytic domains, to cut two DNA strands, but also with two DNA binding domains, only one of which is used on a one-site substrate (53,54). It seems likely that the BcgI REase bound to its recognition site also need to recruit additional catalytic domains to cut all eight of its target bonds.

The only Type IIB REases whose reactions at both sides of their recognition sites have been reported to date are HaeIV (13) and NmeDI (22). In contrast to BcgI, both of these first cut the DNA on one side of their site (with no preference for any particular side) and then, in a separate reaction, make the DSB on the other side of the site. While NmeDI cuts the DNA on both sides of its recognition site, it differs radically from the other Type IIB R–M systems (20). Instead of a single protein with both REase and MTase activities, NmeDI is organized like a standard Type II system, with separate REase and MTase domains; and instead of the usual asymmetric bipartite site, it recognizes a continuous palindromic sequence, again like a standard Type II system (22). NmeDI thus cannot be compared directly to BcgI. On the other hand, HaeIV is clearly a *bona fide* Type IIB enzyme though it differs from BcgI in terms of its genetic and structural organization (13).

While several Type IIB enzymes are like BcgI in containing two polypeptides (21,55), one for DNA recognition and one for both REase and MTase activities, HaeIV belongs to a group that carry all three functions in a single polypeptide (13,14,20,41). Unlike BcgI which possesses two nuclease centres per DNA recognition unit, the single-polypeptide systems have only one and might thus be unable to cleave the same number of phosphodiester bonds in one complex as those with the A₂B₃ organization. It has, however, yet to be shown whether HaeIV needs to interact with two copies of its recognition site for activity. Conversely, another single-polypeptide Type IIB enzyme, AloI, is known to need two recognition site to cut DNA (31) but it is not yet known whether it cuts both sides of its sites concurrently, as with BcgI, or sequentially, as with HaeIV. Gel filtration studies indicate that HaeIV exists in solution as a dimer where AloI forms a tetramer (13,14). But in neither case is it known how many bonds these single-polypeptide enzymes cleave while bound to DNA. The dimeric structure of HaeIV suggests is should be capable of cutting at least two bonds, perhaps making one DSB on one side of one site, while the AloI tetramer could potentially cut four bonds, possibly either all four bonds at one site or one DSB at each site. A BcgI tetramer of A₂B₃ units has double the number of nuclease domains over the AloI tetramer, which may account for why BcgI can cut up to eight phosphodiester bonds in a single DNA–protein complex.

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