Supplementary Data

accompanying the manuscript

Target site cleavage by the monomeric restriction enzyme BcnI requires translocation to a random DNA sequence

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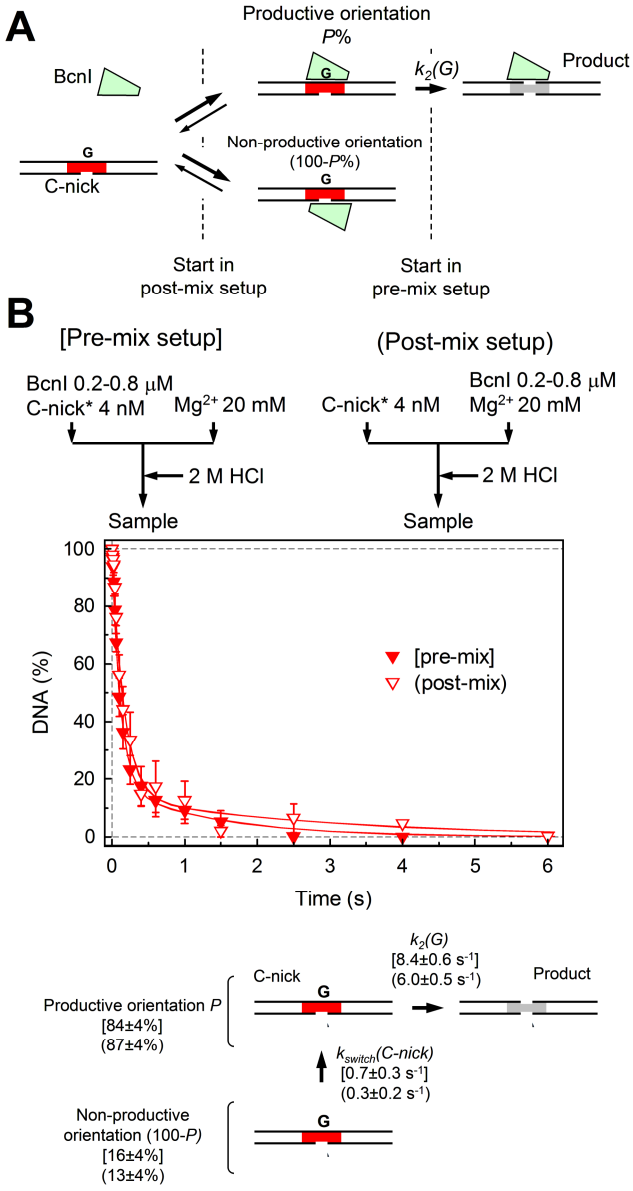
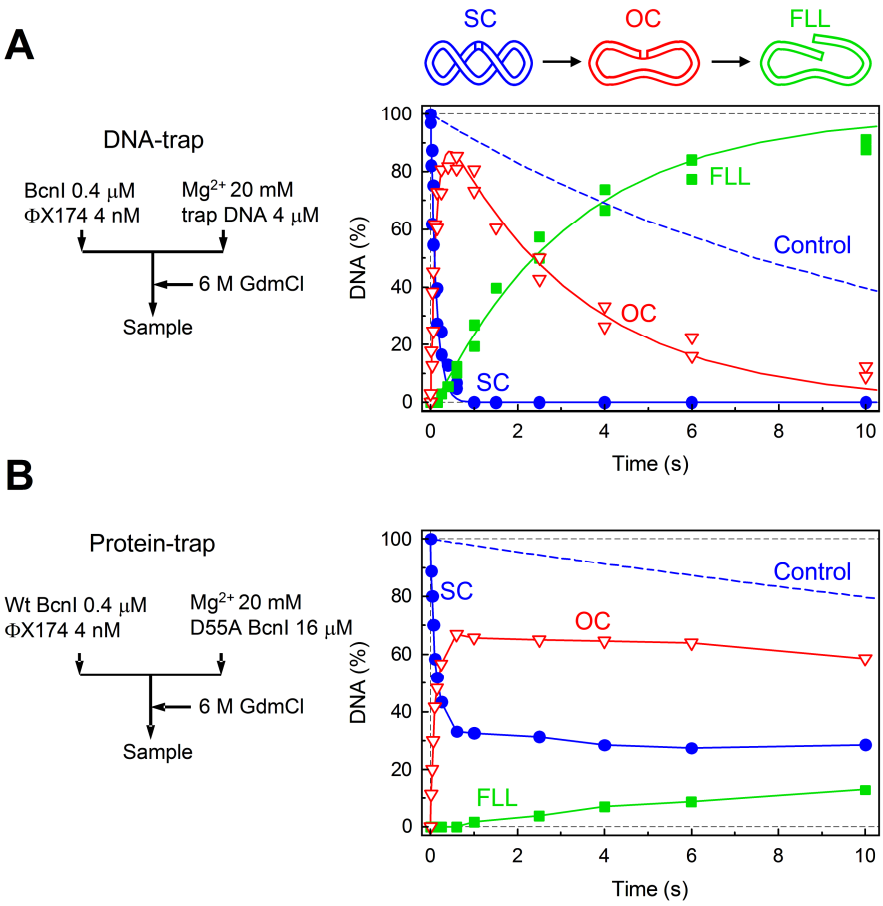
Supplementary Figures



Supplementary Figure S1. BcnI cleavage of the C-nick intermediate.
(A) Scheme for BcnI reaction on the C-nick intermediate. At the initial moment of the reaction, BcnI may bind the nicked DNA in either of two alternative orientations. In the productive orientation, the catalytic center is positioned at the scissile phosphodiester bond in the intact G–strand to proceed with the cleavage (rate constant $k_2(G)$). In the alternative orientation, the catalytic center of BcnI is placed against the nicked DNA strand C, thus the enzyme has to dissociate and then re-bind in the opposite orientation before cleavage can occur. This rearrangement is described by the rate constant $k_{switch}(C\text{-}nick)$. The fraction of substrate bound in the productive orientation is designated by P (%).
(B) Cleavage of the C-nick intermediate in the pre-mix (filled triangles) and post-mix (open triangles) reactions. Flow diagrams schematically depict the two types of experiments performed in a quench-flow device. All data points are presented as mean values from 5-6 independent experiments ±1 SD. Solid lines represent the fit of the reaction scheme in panel A to experimental data, assuming that the enzyme binding steps are fast and do not affect the observed DNA cleavage rates. Determined rate constants for the pre-mix and post-mix reactions are provided below the graph in square brackets and parentheses, respectively. Parameter $k_{switch}(C\text{-}nick)$ for the post-mix experiment could not be determined reliably due to the low fraction of enzyme that binds substrate in the non-productive orientation and high data scattering.



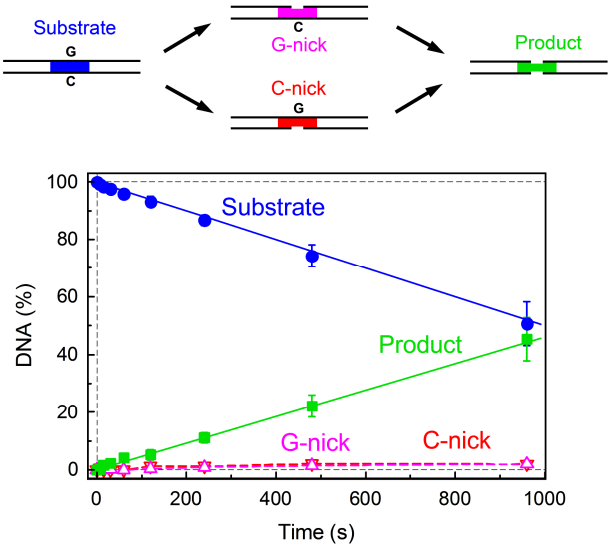
Supplementary Figure S2. BcnI reactions on supercoiled ΦX174 DNA. The 5386 bp ΦX174 DNA has a single BcnI recognition sequence 5′-CCSGG-3′. Three DNA forms were resolved by electrophoresis through agarose: supercoiled DNA SC (corresponds to the intact substrate; filled circles); open-circular DNA OC (corresponds to the nicked intermediate; open triangles); linear DNA FLL (corresponds to the final product with a double-strand break; filled squares).

(A) DNA-trap experiment. The flow diagram schematically depicts the experiment performed in a quench-flow device. Continuous lines are the best fit to the reaction equation

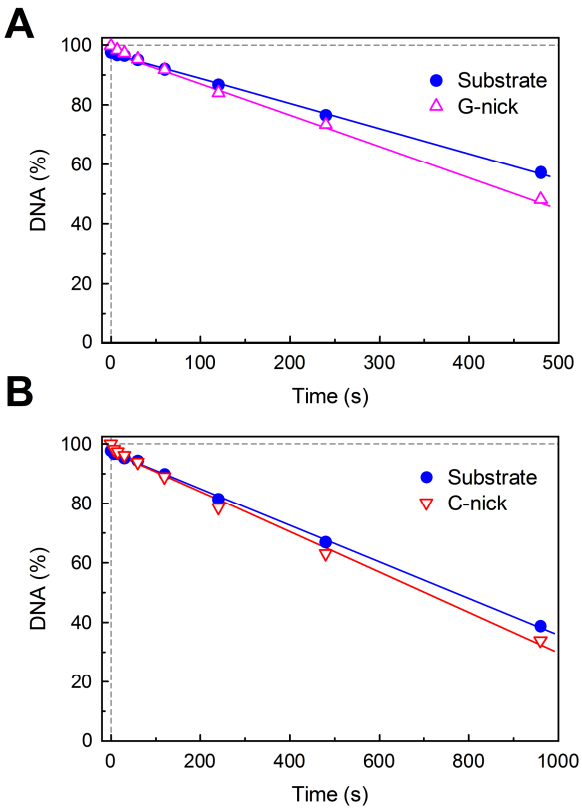
SC $-k_1\rightarrow$ OC $-k_2\rightarrow$ FLL

that gave k_1 = 7.5±0.4 s^{-1} (the rate constant for nicking of the first DNA strand) and k_2 = 0.31±0.02 s^{-1} (the rate constant for cleavage of the second DNA strand). The excess of unlabeled oligonucleotide duplex has no effect on the formation and cleavage of the nicked intermediate OC (cf. Figure 2A in the main text). The dashed line depicts SC DNA cleavage in a control experiment where reaction was initiated by adding wt BcnI to the mixture of ΦX174 and excess of oligoduplex DNA.

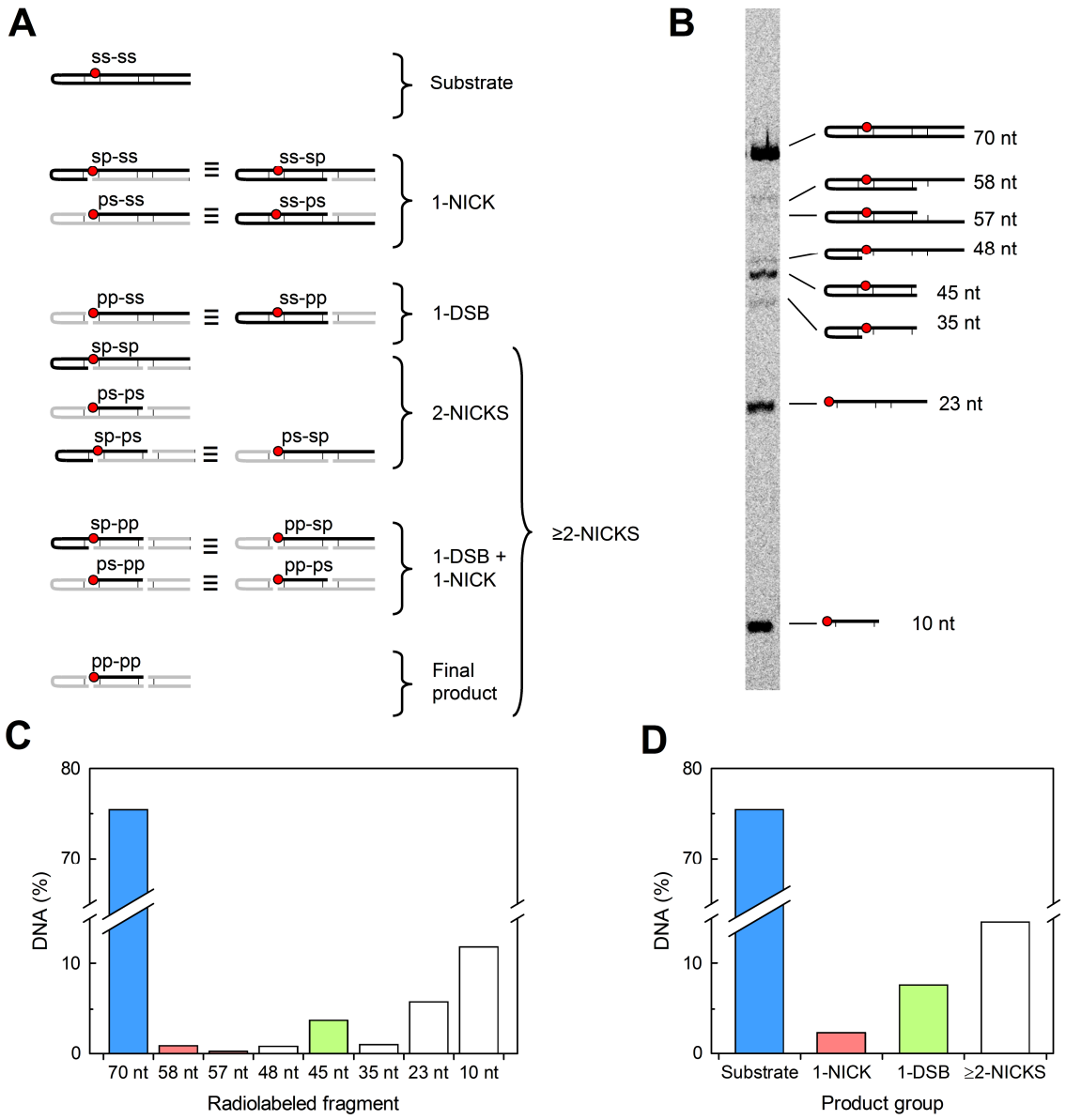
(B) Protein-trap experiment. The flow diagram schematically depicts the experiment performed in a quench-flow device. The excess of D55A mutant inhibits cleavage of the nicked intermediate OC (cf. Figure 2A in the main text). The blue dashed line depicts SC DNA cleavage in a control experiment where reaction was initiated by adding a mixture of wt and D55A BcnI to ΦX174 DNA.



Supplementary Figure S3. Steady-state reactions on the one-site oligoduplex substrate HP. Reactions contained 1 nM BcnI and saturating substrate concentration (100 nM). The amounts of four DNA forms are shown: intact DNA (filled circles), G-nick intermediate (up triangles), C-nick intermediate (down triangles) and final reaction product (filled squares). All data points are presented as mean values from 2-6 independent experiments ±1 SD. Solid lines are linear fits to the intact substrate depletion and final product accumulation data. The determined initial rate of substrate cleavage is 0.050±0.008 nM/s, and the k_{cat} equals 0.050±0.008 s^{-1}. The initial rate of the final reaction product formation equals 0.046 nM/s, indicating that BcnI converts 92% (ratio 0.046/0.050) of the substrate into the final product during a single binding event.



Supplementary Figure S4. Steady-state reactions on a mixture of intact and nicked DNA.
(A) BcnI reaction on a mixture of intact oligoduplex substrate HP and G-nick intermediate. The reaction contained 1 nM wt BcnI, 50 nM HP DNA (filled circles), and 50 nM G-nick DNA (up triangles). The initial cleavage rates of the intact substrate (0.042 nM/s) and G-nick intermediate (0.053 nM/s) were determined by linear regression (solid lines).
(B) BcnI reaction on a mixture of intact oligoduplex HP and C-nick intermediate. The reaction contained 1 nM wt BcnI, 50 nM HP DNA (filled circles) and 50 nM C-nick DNA (down triangles). As in (A), the initial cleavage rates of the intact substrate (0.031 nM/s) and C-nick intermediate (0.034 nM/s) were determined by linear regression.
In both experiments the enzyme cleaves intact DNA and nicked intermediates with comparable rates, indicating that under steady-state reaction conditions BcnI has no preference for the nicked intermediate over substrate. Efficient formation of the final reaction product with a double strand break under steady state reaction conditions therefore must be due to processive action of the enzyme on both DNA strands.



Supplementary Figure S5. Analysis of the GG substrate cleavage data. Two BcnI target sequences are marked by rectangles, internal ^{33}P radiolabel is designated by a red circle, radioactive DNA fragments visualized by phosphorimaging are black and unlabeled fragments are grey.
(A) BcnI reaction on the GG oligonucleotide substrate ('ss-ss') may yield 14 intermediates with 1, 2 or 3 nicks and the final product ('pp-pp'). The pairs of intermediates that occupy two columns of the same row are equivalent due to the symmetry of the GG substrate.
(B) Eight radiolabeled DNA fragments are separated by denaturing gel electrophoresis. The fragments of the GG DNA substrate that correspond to each radiolabeled band are depicted.
(C) Raw data from the gel lane in panel (B).
(D) Processed data from the gel lane in panel (B). Amounts of the intact DNA, '1-NICK', '1-DSB' and '≥2-NICKS' were calculated as described in Supplementary Methods.

Supplementary Methods

DNA substrates

The HP substrate (Table 1) was 5′-labeled with [γ-^{33}P]ATP (Hartmann Analytic, Braunschweig, Germany) and T4 polynucleotidyl kinase (PNK) (Fermentas, Vilnius, Lithuania) or 3′-labeled with [α-^{32}P]cordycepin triphosphate (PerkinElmer) and terminal deoxynucleotidyl transferase (Fermentas). The G-nick and C-nick oligoduplexes were assembled by annealing the 5′-labeled 30 nt oligonucleotide (top strand) and two complementary oligonucleotides (nicked bottom strand). To mimic the nicked products from BcnI reactions, the 5′-termini of the short fragments facing the nick were phosphorylated with ATP and PNK. Prior to annealing with the complementary strands, radiolabeled and phosphorylated oligonucleotides were precipitated by ethanol in the presence of glycogen and EDTA. This procedure eliminated divalent metal ions from the radiolabeled oligonucleotide substrates. Annealing was performed in the pH 7.9 buffer (33 mM Tris-acetate, 66 mM potassium acetate, 0.5 mM EDTA).

The radiolabeled hairpin GG was prepared from the 47 nt and 23 nt oligonucleotides 5′-TGCGTTATTGCCCGGCGTTGCCCGGCGTACTGCGTTAGCAGTACGCC-3′ and 5′-GGGCAACGCCGGGCAATAACGCA-3′. After phosphorylation of the 5′-terminus with [γ-^{33}P]ATP and PNK, the 23 nt oligonucleotide was annealed to the 47 nt strand to assemble a nicked DNA hairpin which was subsequently sealed with T4 DNA ligase. The resultant 70 nt oligonucleotide was gel-purified and annealed in the pH 7.9 buffer mentioned above. The radiolabeled hairpin GC was prepared in a similar way from the 12 nt and 58 nt oligonucleotides 5′-TGCGTTATTGCC-3′ and 5′-GGGCGTTGCCCGGCGTACTGCGTTAGCAGTACGCCGGGCAACGCCCGGCAATAACG CA-3′. For steady state experiments the radiolabeled GG and GC hairpins were used together with excess of unlabeled oligoduplexes that are identical to the GG and GC hairpins except for the lack of the hairpin loop.

Analysis of double stranded DNA cleavage data

Single turnover pre-mix DNA cleavage experiments (Figure 3B) were performed with enzyme (200-400 nM BcnI) in large excess over the HP substrate (2 nM). Reaction rates at both BcnI concentrations tested (200 nM and 400 nM) were identical, suggesting that the observed reaction rates are not affected by enzyme-DNA association but instead reflect just the conversion of enzyme-bound substrate to product via the G-nick and the C-nick intermediates (Figure 3A). Therefore, the single-turnover pre-mix reactions of BcnI on the double-stranded DNA substrate HP were fitted to

the equations (1-3) that take into account only the DNA hydrolysis reactions (simplified reaction scheme in Figure 3B):

$$SS(t) = G \times e^{-k_1(G)\cdot t} + (100 - G) \times e^{-k_1(C)\cdot t} \quad (1),$$

$$Gnick(t) = G \times \frac{k_1(G)}{k_1(G) - k_{obs}(C)} \times \left(e^{-k_{obs}(C)\cdot t} - e^{-k_1(G)\cdot t} \right) \quad (2),$$

$$Cnick(t) = (100 - G) \times \frac{k_1(C)}{k_1(C) - k_{obs}(G)} \times \left(e^{-k_{obs}(G)\cdot t} - e^{-k_1(C)\cdot t} \right) \quad (3),$$

where *SS(t)*, *G-nick(t)* and *C-nick(t)* are the concentrations (in %) at time *t* of the DNA substrate and both nicked intermediates, $k_1(G)$ and $k_2(C)$ are the rate constants for nicking of the G- and C-strands, $k_{obs}(C)$ and $k_{obs}(G)$ are the rate constants for cleavage of the C- and G- strands in the second stage of the reaction, and *G* denotes the initial fraction (in %) of the DNA substrate carrying BcnI in the orientation that places the catalytic center close to the G-strand.

In the post-mix reactions starting with the enzyme and DNA in separate solutions, DNA cleavage must be preceded by the enzyme-DNA association step. However, the observed reaction rates with both enzyme concentrations tested (200 and 400 nM) were also identical, indicating that under these reaction conditions BcnI-DNA association is a fast process that does not limit the observed DNA cleavage rate. Therefore, post-mix BcnI reactions were also fitted to the equations (1-3) (Figure 3C).

Analysis of nicked DNA cleavage

Single turnover pre-mix DNA cleavage experiments on the G-nick intermediate (Figure 4B) were performed with enzyme (200-400 nM) in large excess over the G-nick oligonucleotide (2 nM). Reaction rates at both BcnI concentrations tested (200 nM and 400 nM) were identical, suggesting that the observed reaction rates are not affected by enzyme-DNA association but instead reflect just the conversion of enzyme-bound DNA into the product. Therefore, we approximated the reaction steps that lead to the switch in enzyme orientation by a single first-order rate constant $k_{switch}(G\text{-}nick)$. This converted the reaction mechanism for G-nick DNA cleavage (Figure 4A) into the simple two-step scheme (Figure 4B). Equation (4) for the mechanism in Figure 4B

$$Gnick(t) = P \times e^{-k_2(C)\cdot t} + (100 - P) \times \frac{\left(k_{switch}(Gnick) \times e^{-k_2(C)\cdot t} - k_2(C) \times e^{-k_{switch}(Gnick)\cdot t} \right)}{\left(k_{switch}(Gnick) - k_2(C) \right)} \quad (4)$$

was fitted to the pre-mix G-nick cleavage data, yielding the rate constant $k_2(C)$ for the C-strand hydrolysis, rate constant $k_{switch}(G\text{-}nick)$ for the switch in enzyme orientation, and the initial fraction P (%) of the G-nick substrate carrying BcnI in the productive orientation that places the catalytic center close to the C-strand.

In the post-mix reactions starting with BcnI and G-nick DNA in separate solutions, the observed reaction rates were also identical at both enzyme concentrations tested (200 nM and 400 nM), indicating that under these reaction conditions BcnI-DNA association is a fast process that does not limit the observed DNA cleavage rate. Therefore, post-mix G-nick cleavage reactions (Figure 4B) were also fitted to the equation (4).

Single turnover pre-mix and post-mix reactions on the C-nick oligonucleotide (Supplementary Figure S1) were fitted to the analogous equation (5):

$$Cnick(t) = P \times e^{-k_2(G)\cdot t} + (100 - P) \times \frac{\left(k_{switch}(Cnick) \times e^{-k_2(G)\cdot t} - k_2(G) \times e^{-k_{switch}(Cnick)\cdot t} \right)}{\left(k_{switch}(Cnick) - k_2(G) \right)} \quad (5),$$

where $k_2(G)$ is the rate constant for the G-strand hydrolysis in the C-nick intermediate, $k_{switch}(C\text{-}nick)$ is the rate constant for the switch in enzyme orientation, and P (%) denotes the initial fraction of the C-nick DNA carrying BcnI in the productive orientation with the catalytic center close to the G-strand.

Interpretation of the double-site substrate cleavage data

BcnI reaction on the two site substrate GG (Table 1) may yield 14 reaction intermediates with 1, 2 or 3 nicks and the final reaction product cleaved at all four phosphodiester bonds (Supplementary Figure S5A). The two BcnI recognition sites in the GG substrate are identical, therefore some reaction products are equivalent (for example, products ‘pp-ss’ and ‘ss-pp’, bearing a double-strand break either at the first or at the second recognition site, Supplementary Figure S5A).

Depending on the position and the number of cleaved phosphodiester bonds, the internal radiolabel may reside in one of the eight DNA fragments separated on high resolution denaturing polyacrylamide gel (Supplementary Figure S5B). Assuming that equivalent reaction products

should be formed in equal quantities, amounts of eight DNA forms could be unambiguously deduced from the denaturing electrophoresis data (equations 6-10):

$$[\text{ss-ss}] = [70\ \text{nt}] \quad (6),$$

$$[\text{ss-sp}] = [\text{sp-ss}] = [58\ \text{nt}] \quad (7),$$

$$[\text{ss-ps}] = [\text{ps-ss}] = [57\ \text{nt}] \quad (8),$$

$$[\text{sp-sp}] = [48\ \text{nt}] - [\text{sp-ss}] \quad (9),$$

$$[\text{ss-pp}] = [\text{pp-ss}] = [45\ \text{nt}] \quad (10).$$

Amount of the intact substrate ‘ss-ss’ was determined using equation (6) (Supplementary Figure S5C and D); the combined amount of all cleavage products bearing a single nick (‘1-NICK’) was calculated using equation (11):

$$[\text{1-NICK}] = [\text{ss-sp}] + [\text{sp-ss}] + [\text{ss-ps}] + [\text{ps-ss}] = 2\times[58\ \text{nt}] + 2\times[57\ \text{nt}] \quad (11).$$

The amount of DNA fragments with a single double-strand break (‘1-DSB’) corresponds to the sum (12):

$$[\text{1-DSB}] = [\text{ss-pp}] + [\text{pp-ss}] = 2\times[45\ \text{nt}] \quad (12).$$

The total amount of reaction products bearing two or more cleaved phosphodiester bonds distributed between the two sites (‘≥2-NICKS’) was determined using equation (13):

$$[\geq\text{2-NICKS}] = 100\% - [\text{ss-ss}] - [\text{1-NICK}] - [\text{1-DSB}] \quad (13).$$

A similar approach was used to determine the amount of ‘ss-ss’, ‘1-NICK’, ‘1-DSB’ and ‘≥2-NICKS’ products in the GC DNA cleavage experiments.