The *SalGI* restriction endonuclease

Mechanism of DNA cleavage

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The cleavage of supercoiled DNA of plasmid pMB9 by restriction endonuclease *SalGI* has been studied. Under the optimal conditions for this reaction, the only product is the linear form of the DNA, in which both strands of the duplex have been cleaved at the *SalGI* recognition site. DNA molecules cleaved in one strand at this site were found to be poor substrates for the *SalGI* enzyme. Thus, both strands of the DNA appear to be cleaved in a concerted reaction. However, under other conditions, the enzyme cleaves either one or both strands of the DNA; the supercoiled substrate is then converted to either open-circle or linear forms, the two being produced simultaneously rather than consecutively. We propose a mechanism for the *SalGI* restriction endonuclease which accounts for the reactions of this enzyme under both optimal and other conditions. These reactions were unaffected by the tertiary structure of the DNA.

Type II restriction endonucleases provide a convenient system for the study of protein–nucleic acid interactions. These enzymes recognize short nucleotide sequences in DNA and catalyse the cleavage of one phosphodiester bond in each strand of the duplex. In principle, a double strand cleavage reaction could occur by two distinct mechanisms; either by cleavage of both strands of the DNA in a single event or by cleavage of each strand in separate events. In the latter case, intermediates cut in one strand only may be observed; the appearance of such intermediates would provide some insight into the reaction mechanism. As a consequence of the symmetry of the DNA sequences at the recognition sites for many restriction endonucleases, an attractive hypothesis for DNA cleavage has been the simultaneous binding of two identical subunits of a dimeric protein to each half of the recognition site and the concomitant cleavage of both strands (Kelly & Smith, 1970). The *HindIII* restriction endonuclease reacts principally by the concerted cleavage of both strands of the duplex (Smith & Wilcox, 1970). It was suggested that the enzyme either cleaves both strands within the same enzyme–DNA complex or that the activity of the enzyme towards the DNA was much greater following the first single strand scission. Likewise, Halford *et al.* (1979) reported that both the *BamHI* and *SalGI* enzymes cleave DNA by a similar mechanism, as no intermediates cut in one strand only were detected in these reactions. However, the *EcoRI* restriction endonuclease may cleave DNA either by a con-

certed mechanism (Rubin & Modrich, 1978) or by a mechanism involving two single strand scissions (Ruben *et al.*, 1978; Halford *et al.*, 1979). Rubin & Modrich (1978) suggested that the mode of DNA cleavage by the *EcoRI* enzyme varies with the particular DNA molecule used as the substrate and they proposed a reaction scheme which accommodates both mechanisms of DNA cleavage. Differences between DNA substrates could be due to interactions of the enzyme with DNA sequences outside the recognition site. The effects of such interactions have been observed in the reactions of the *EcoRI* enzyme towards individual recognition sites on bacteriophage λ (Halford *et al.*, 1980).

We describe here further studies on the mechanism by which the *SalGI* restriction endonuclease cleaves duplex DNA at its recognition site, using enzyme purified to homogeneity. A preliminary account of this work has been presented previously (Maxwell & Halford, 1981).

Materials and methods

Supercoiled DNA (^3H -labelled) of plasmid pMB9 was prepared as described previously (Halford & Johnson, 1981). To prepare the open-circle form of plasmid pMB9 cleaved in one strand at the *SalGI* recognition site, the supercoiled DNA (18 nm) was incubated with *SalGI* restriction endonuclease (62.5 nm) in 50 mM-Pipes (1,4-piperazinediethanesulphonic acid) / 10 mM-MgCl₂ / 125 mM-NaCl / 10 mM- β -mercaptoethanol / 0.5 mg·ml⁻¹ bovine

serum albumin, pH 5.7 at 22°C in a total volume of 0.8 ml (see Fig. 5 below). After 100 min, 0.2 ml of 0.2 M-EDTA was added and the mixture was incubated at 67°C for 30 min. Open circle and linear forms of the DNA were separated from the supercoiled substrate by centrifugation to equilibrium in a CsCl gradient as described by Halford & Johnson (1981). After centrifugation, the DNA was localized by u.v. illumination, the upper band removed, extracted several times with CsCl-saturated propan-2-ol and dialysed against 10 mM-Tris/0.1 mM-EDTA, pH 7.5. Analysis by agarose-gel electrophoresis showed that this DNA was 94% open-circle form and 6% linear form of plasmid pMB9. The open-circle form of plasmid pMB9 cleaved in one strand at random locations was prepared by incubating the supercoiled form (4.9 nm) in 50 mM-Tris/10 mM-MgCl₂/125 mM-NaCl/10 mM- β -mercaptoethanol, pH 8.0, with 0.1 mg of untreated bovine serum albumin, at 22°C in a total volume of 0.2 ml for 16 h. Analysis by agarose-gel electrophoresis showed that 95% of the DNA was the open-circle form of plasmid pMB9. [This method for the preparation of open-circle DNA, cleaved in one strand at random locations, relies on commercial preparations of bovine serum albumin (Sigma, lot number 83C-0340) being contaminated with an endonuclease which converts supercoiled DNA of plasmid pMB9 into its open-circle form. In all other experiments, solutions containing bovine serum albumin were previously incubated for 2 h at 67°C, which inactivated this contaminating activity.] The M_r of plasmid pMB9 is 3.5×10^6 (Rodriguez *et al.*, 1976). The molarities of solutions of plasmid pMB9 are equal to the concentrations of *Sal*GI recognition sites, if it is assumed that a recognition site comprises both strands on the DNA.

Restriction endonuclease *Sal*GI was prepared to at least 80% homogeneity as described previously (Maxwell & Halford, 1982a); molarities of enzyme solutions are expressed in terms of a dimeric protein of M_r 58 000. (We suggest below that the active form of this enzyme is a dimer.) All of the reactions described here, in which the DNA of plasmid pMB9 is cleaved, were shown to be due to the *Sal*GI restriction endonuclease acting at its recognition site on the DNA. Experiments using the supercoiled form of plasmid pMB8, a DNA molecule that lacks a *Sal*GI recognition site, were carried out in parallel to those reported here with plasmid pMB9, except that the reaction mixtures with pMB8 contained 10-fold higher enzyme concentrations. Under the range of MgCl₂ concentrations and pH values tested, the supercoiled form of plasmid pMB8 was not cleaved to either its open-circle or linear forms. Thus the possibilities that the observed reactions were due to a contaminating nuclease, or were occurring outside the *Sal*GI recognition site, may be dis-

counted. The *Sal*GI enzyme was found to remain catalytically active for at least 1 h under all conditions used here.

The cleavage of DNA by the *Sal*GI restriction endonuclease was monitored by removing samples (15–20 μ l) at timed intervals from 0.2 ml reaction mixtures containing [³H]DNA of plasmid pMB9 and *Sal*GI enzyme at 22°C in various buffers. Samples were immediately added to 10 μ l of stop mix [40% (w/v) sucrose/0.1 M-Tris/0.1 M-EDTA/0.05% Bromophenol Blue, pH 8.0] and analysed by electrophoresis through 1% (w/v) agarose gels. Conditions for electrophoresis, localization of DNA bands and measurement of radioactivity in each band were as described previously (Halford *et al.*, 1979). In addition to the stated constituents, all DNA-containing solutions also contained 5 mM-Tris/0.05 mM-EDTA; the concentrations of MgCl₂ given below are the total rather than the free Mg²⁺ concentrations since they have not been corrected for chelation by EDTA or other components of the reaction mixtures. All other materials and methods have been described (Maxwell & Halford, 1982a).

Results and discussion

Analysis of DNA cleavage mechanisms

The cleavage of DNA by the *Sal*GI restriction endonuclease has been studied using the supercoiled DNA of plasmid pMB9 as a substrate. This DNA molecule possesses a single *Sal*GI recognition sequence (Rodriguez *et al.*, 1976). If the enzyme were initially to cleave this DNA in only one strand, an open-circle form would be produced. This nicked DNA, now in a relaxed configuration, would subsequently be cleaved in the second strand to yield the linear product. However, if both strands of the duplex were cleaved simultaneously, then only the linear form would be produced. The supercoiled, open-circle and linear forms of plasmid pMB9 may be separated by electrophoresis through agarose (Johnson & Grossman, 1977). If the DNA is labelled with [³H]thymidine, the proportions of each of the three forms of the DNA at various times during a reaction can be evaluated (for example, Fig. 2). Preliminary studies with impure *Sal*GI enzyme (Halford *et al.*, 1979) suggested that this enzyme may cleave both strands of plasmid pMB9 in a concerted reaction, the supercoiled substrate being converted directly to linear product without the formation of an open-circle intermediate. Plasmid pMB9 also contains a single recognition site for the *Eco*RI restriction endonuclease (Rodriguez *et al.*, 1976), but this enzyme appears to cleave the DNA by two independent reactions, each on one strand of the duplex (Halford *et al.*, 1979). Hence, it appeared that the *Sal*GI and *Eco*RI restriction endo-

nucleases, although catalysing very similar reactions, may interact differently with their DNA substrates.

The optimal reaction conditions for the cleavage DNA by the *Sal*GI restriction endonuclease are 50 mM-Tris/10 mM-MgCl₂/125 mM-NaCl/10 mM- β -mercaptoethanol/0.5 mg·ml⁻¹ bovine serum albumin, pH 8.0 (Maxwell & Halford, 1982a). Under these conditions, the cleavage of supercoiled DNA of plasmid pMB9 by the *Sal*GI enzyme produces no open-circle form of the DNA (Fig. 1), in agreement with the observations of Halford *et al.* (1979). Thus the enzyme appears to cleave both strands of the DNA in a concerted reaction to yield a linear product. The decline in the concentration of the supercoiled substrate and concomitant increase in the concentration of the linear product cannot be fitted to exponential curves. This is contrary to the *Eco*RI enzyme where, under similar conditions, the reactions could be described by apparent first-order rate constants (Halford *et al.*, 1979). Instead, the concentrations of supercoiled pMB9 during cleavage by the *Sal*GI enzyme were found to decline linearly over at least 50% of the reaction (Fig. 1), indicating a zero-order process. The initial rate of the reaction was found to vary linearly over the range of enzyme concentrations employed here; thus, the cleavage of pMB9 DNA by the *Sal*GI endonuclease follows the kinetics expected for a steady-state reaction. However, to satisfy steady-state conditions, the concentration of substrate must exceed that of the enzyme; but in our experiments enzyme and substrate concentrations are similar (for example, Fig. 1). In the following paper, Maxwell & Halford (1982b) show that the zero-order nature of these reactions is due partly to the low K_m of this enzyme for its substrate and also to enzyme binding to DNA sequences outside the *Sal*GI recognition site, such that the free enzyme concentration is less than that stated. [In the studies of the *Eco*RI restriction endonuclease, the reactions could be described by apparent first-order rate constants, because the initial substrate concentrations were below the K_m value for the *Eco*RI enzyme (Halford *et al.*, 1979; Halford & Johnson, 1981).] In the present work, initial rates were measured from the slope of the linear portion of the plots of substrate or product concentration against time (for example, Fig. 1) and are quoted as nM substrate consumed per min per nM enzyme dimer. Initial rates of DNA cleavage in the range of plasmid pMB9 concentrations, 2.5–20 nM were independent of substrate concentration (results not shown).

Variation in MgCl₂ concentration and pH

Lowering the MgCl₂ concentration (below 10 mM) reduces the rate at which the *Sal*GI restriction endonuclease cleaved both strands of the DNA from

Fig. 1. Reaction of restriction endonuclease *Sal*GI at 10 mM-MgCl₂.

(a) Samples (15 μ l) were withdrawn at timed intervals from a 0.2 ml reaction mixture containing supercoiled DNA (³H-labelled) of plasmid pMB9 (4.9 nM) and *Sal*GI restriction endonuclease (2.5 nM) in 50 mM-Tris/10 mM-MgCl₂/125 mM-NaCl/10 mM- β -mercaptoethanol/0.5 mg·ml⁻¹ bovine serum albumin, pH 8.0, at 22°C. Each sample was immediately added to 10 μ l of stop mix and, after heating for 10 min at 67°C followed by quenching on ice, was analysed by electrophoresis on a 1% (w/v) agarose slab gel. The time (in min) of withdrawal of each sample is noted by each track of the gel; the zero-time point was taken before the addition of enzyme. Under the conditions employed for electrophoresis, the three forms of the DNA migrate towards the anode at the bottom of the gel in the order: supercoiled DNA > linear DNA > open-circle DNA. The slots marked (M) contain a mixture of open-circle, linear and supercoiled forms of plasmid pMB9. (b) Determinations of the percentages of supercoiled (●), open-circle (○) and linear (■) forms of plasmid pMB9 in samples from the reaction described in (a). Slices from the above gel were analysed by scintillation counting to determine the proportions of each of the three forms of the DNA in each timed sample.

a derivative of bacteriophage λ (Maxwell & Halford, 1982a). With supercoiled DNA of plasmid pMB9 as substrate, we observed a similar reduction

in the overall rate of DNA cleavage at low Mg^{2+} concentrations (Table 1) but also the appearance of products cleaved in one strand only. For example, at a $MgCl_2$ concentration of 0.3 mM (Fig. 2), a high level of open-circle DNA was produced (60%), this being subsequently converted to the linear form. In this reaction, the rate of formation of linear DNA was biphasic, being much faster in the first phase (0–15 min) than in the second (20–60 min). At a $MgCl_2$ concentration of 2 mM (Fig. 3), a low level of open-circle DNA was produced (14%). Hence, the initial phase of these reactions, when supercoiled DNA is being converted to linear and open-circle forms, require three zero-order rate constants for their description. Values for these rate constants at a range of $MgCl_2$ concentrations are given in Table 1.

Fig. 2. Reaction of restriction endonuclease *SalGI* at 0.3 mM- $MgCl_2$.

(a) Samples, withdrawn from a reaction mixture identical with that described in the legend to Fig. 1(a) except that the *SalGI* enzyme concentration was 20 nM and the $MgCl_2$ concentration was 0.3 mM, were analysed by electrophoresis as above. (b) Determinations of the percentages of supercoiled (●), open-circle (○) and linear (■) forms of plasmid pMB9 DNA in samples from the reaction in (a).

Halford *et al.* (1979) have shown that the *EcoRI* restriction endonuclease first cleaves plasmid pMB9 in one strand to yield the open-circle form; linear DNA was only produced after a lag phase. Thus, the open-circle DNA is an obligatory intermediate between the supercoiled and linear forms and a sequential mechanism of DNA cleavage by the *EcoRI* enzyme has been proposed:

where A represents the intact DNA duplex, B a DNA molecule in which one of the two strands has been cleaved at the recognition site and C a DNA molecule in which both strands have been cleaved at this site. In the reactions of the *SalGI* enzyme at $MgCl_2$ concentrations of 2 mM and below, open-circle forms of plasmid pMB9 were observed, which raises the possibility that, under these conditions, this enzyme cleaves DNA by a similar mechanism to that of the *EcoRI* enzyme. However, examination of individual reaction records shows that this is not the case. Firstly, at 2 mM- $MgCl_2$ (Fig. 3), the initial rate of formation of open-circle DNA was slower than that of linear DNA. Secondly, at 0.3 mM- $MgCl_2$ (Fig. 2), when a high level of open-circle DNA was produced, a lag phase in the production of linear DNA was not observed. Hence, the open-circle form of plasmid pMB9, produced by the cleavage of the supercoiled DNA by the *SalGI* enzyme, cannot be an obligatory intermediate between substrate and product, as in eqn. (1). However, the reaction may be described by a scheme in which products cleaved in one or both strands are produced simultaneously rather than in a sequential fashion:

where, A, B and C represent the same forms of the DNA as in eqn. (1) and the initial rates of formation of both B and C are zero-order processes. In the first phase of this reaction, the partitioning between the two products (B and C) is affected by the $MgCl_2$ concentration, with the result that the maximum level of open-circle DNA produced is a direct consequence of the relative rates of these two simultaneous reactions (Table 1). For example, at 0.3 mM- $MgCl_2$ (Fig. 2), the initial rate of formation of open-circle DNA ($A \rightarrow B$) was faster than that of linear DNA ($A \rightarrow C$), resulting in a high level of open-circle DNA. Conversely, at 2 mM- $MgCl_2$, the relative magnitude of these two rates was reversed such that a low level of open-circle DNA was formed.

In the second phase of this reaction, the open-circle form will be subsequently cleaved in the

Table 1. *MgCl₂ dependence of endonuclease SalGI*

Samples (15 μ l) were withdrawn at timed intervals from 0.2 ml reaction mixtures at 22°C containing supercoiled DNA (³H-labelled) of plasmid pMB9 (4.9 nm) and varying amounts of *SalGI* enzyme (2.5–20 nm) in 50 mM-Tris/125 mM-NaCl/10 mM- β -mercaptoethanol/0.5 mg·ml⁻¹ bovine serum albumin, pH 8.0, at the *MgCl₂* concentrations stated. Samples were analysed by electrophoresis and scintillation counting as described in the legend to Fig. 1. The maximum level of open-circle DNA produced at each *MgCl₂* concentration was noted and rates were determined from the gradient of the initial straight portion (starting at zero time) of the plots of substrate or product concentration against time.

[<i>MgCl₂</i>] (mM)	Maximum level of open-circle DNA production (%)	10 ⁻² × Initial rates (nm pMB9 DNA/min per nm <i>SalGI</i> enzyme)		
		Disappearance of supercoiled DNA	Appearance of open-circle DNA	Appearance of linear DNA
10	0	8.7	0	8.7
7	0	6.9	0	6.9
5	0	5.8	0	5.5
2	14	5.4	0.3	4.9
1	30	4.3	0.8	3.3
0.5	50	2.7	1.1	1.6
0.3	60	1.1	0.7	0.4

Fig. 3. Reaction of restriction endonuclease *SalGI* at 2 mM-*MgCl₂*

Determination of the percentages of supercoiled (●), open-circle (○) and linear (■) forms of plasmid pMB9 in samples from a reaction mixture as described in the legend to Fig. 1, except that the *SalGI* enzyme concentration was 5 nm and the *MgCl₂* concentration was 2 mM.

second strand to yield linear product (B→C). To examine this portion of the reaction, plasmid pMB9 that had been specifically cleaved in one strand at the *SalGI* recognition site was prepared and used as a substrate for the *SalGI* enzyme; the intact strand of this open-circle form was cleaved to yield the linear form (Fig. 4a). The kinetics of this reaction were studied over a range of *Mg*²⁺ concentrations (1–20 mM) and, in all cases, this substrate was cleaved at a slower rate than the intact supercoiled form. Thus, the second phase of the *SalGI* reaction

(B→C) is slower than the first phase (either A→B or A→C). Moreover, unlike the cleavage of the supercoiled form of the DNA, which is a zero-order reaction, the cleavage of the open-circle form appears to be a first-order process: the decline in substrate concentration with time follows an exponential curve. One possibility, discussed further by Maxwell & Halford (1982b), is that the *K_m* of the enzyme for the nicked substrate is higher than for the intact form.

In the above comparison, the DNA molecule in which both strands were intact at the *SalGI* recognition site was negatively supercoiled, whereas the molecule in which one strand had been cleaved at this site was in a relaxed conformation. Hence, it is possible that the different cleavage rates were a consequence of their different topological states. Thus, an open-circle form of plasmid pMB9, cleaved in one strand at random locations, was prepared; on this form of the DNA, both strands of the duplex will, in all probability, be intact at the *SalGI* recognition site. This substrate was cleaved by the *SalGI* enzyme to yield a linear product at a rate identical with that observed for the supercoiled form (Fig. 4b). Hence, the activity of the *SalGI* restriction endonuclease at its recognition site on plasmid pMB9 is unaltered by changing the conformation of the DNA from the negatively supercoiled to the relaxed state, but is altered when the DNA is nicked at the *SalGI* recognition site. The *SalGI* enzyme also has the same activity towards its recognition site on plasmid pMB9 with both negatively and positively supercoiled substrates and with a linear form of plasmid pMB9 obtained after digestion with the *EcoRI* enzyme (Halford *et al.*, 1979; Halford & Johnson, 1981). In principle, the reaction of an enzyme on a negatively supercoiled DNA molecule

Fig. 4. Cleavage of supercoiled and open-circle DNAs by the *SalGI* enzyme

Two 0.2 ml reaction mixtures contained supercoiled DNA (^3H -labelled) of plasmid pMB9 (2.45 μm) and *SalGI* restriction endonuclease (5 nM) in 50 mM-Tris/10 mM-MgCl₂/125 mM-NaCl/10 mM- β -mercaptoethanol/0.5 mg·ml⁻¹ bovine serum albumin, pH 8.0, at 22°C. Reaction mixture (a) also contained open-circle DNA (^3H -labelled) of plasmid pMB9, cleaved in one strand at the *SalGI* recognition site (2.45 μm) and reaction mixture (b) also contained open-circle DNA (^3H -labelled) of plasmid pMB9, cleaved in one strand at random locations (2.45 μm). Samples (15 μl) were withdrawn at timed intervals and analysed by electrophoresis and scintillation counting as described in the legend to Fig. 1. The percentages of the substrates in each sample were determined: (a) (●) supercoiled DNA, (○) open-circle DNA nicked at the *SalGI* recognition site; (b) (■) supercoiled DNA, (□) open-circle DNA nicked at random locations.

could differ from that on a relaxed molecule (Bauer, 1978), in that the hydrolysis of a phosphodiester bond on negatively supercoiled DNA liberates more free energy and that negatively supercoiled molecules are more easily unwound. But neither of these possibilities affects the interaction of the *SalGI* restriction endonuclease with DNA.

The reactions of the *SalGI* restriction enzyme on

the supercoiled form of plasmid pMB9 were also studied over a range of pH values from 8.0 to 5.7. In this series, the MgCl₂ concentration (10 mM) and other conditions were as in Fig. 1, except that Tris, pH 8.0, was replaced by either Tris or Pipes at the requisite pH (at pH 6.8, identical results were obtained with the two buffers). These experiments yielded data equivalent to Table 1 in that the decrease in pH not only reduced the overall rate of the reaction but also altered the partitioning between the two products (eqn. 2); increasing levels of open-circle DNA were formed at decreasing pH values. During the cleavage of the supercoiled DNA by the *SalGI* enzyme at pH 5.7, the initial rate of formation of open-circle DNA was ten times faster than the initial rate of linear DNA production, so that a very high level of open-circle DNA was obtained (Fig. 5); the subsequent conversion of this open-circle DNA to its linear form was too slow to detect.

For certain applications of restriction endonucleases in molecular biology, such as mutagenesis *in vitro* (Muller *et al.*, 1978), the enzymes are required to cleave only one strand of their recognition sites on duplex DNA. The reaction of the *SalGI* enzyme at pH 5.7 (Fig. 5) produced the highest level of nicked DNA molecules that we have observed and would therefore be the most suitable for applications of this nature. We have used this reaction to prepare the open-circle form of plasmid pMB9 cleaved in one strand at the *SalGI* recognition site, with minimal contamination from either

Fig. 5. Reaction of restriction endonuclease *SalGI* at pH 5.7

Determination of the percentages of supercoiled (●), open-circle (○) and linear (■) forms of plasmid pMB9 DNA in samples from a reaction mixture as described in the legend to Fig. 1, except that the *SalGI* enzyme concentration was 45 nM and Tris was replaced by Pipes, pH 5.7.

supercoiled or linear forms (see the Materials and methods section).

Mechanism of DNA cleavage

Under optimal reaction conditions, the *Sal*GI restriction endonuclease cleaves both strands of the supercoiled DNA of plasmid pMB9 in a concerted reaction (Fig. 1). Halford *et al.* (1979) suggested that the enzyme either cleaves both strands of the DNA within a single enzyme-substrate complex or cleaves each strand in an independent event, where the cleavage of the second strand is fast compared with the first. But under sub-optimal conditions of either low pH or Mg^{2+} concentration, open-circle DNA is produced upon the cleavage of the supercoiled DNA of plasmid pMB9 by the *Sal*GI enzyme. Therefore a mechanism with concomitant cleavage of both strands of the DNA cannot account for the *Sal*GI reaction under all conditions. However, the open-circle DNA produced in these reactions is not an obligatory intermediate between the substrate and the linear product, as would be required in a sequential mechanism (eqn. 1). Moreover, in order to account for the lack of open-circle DNA under optimal conditions, a sequential mechanism would demand that open-circle DNA, cleaved in one strand at the *Sal*GI site, be cleaved faster than intact supercoiled DNA. Instead we observe this open-circle form to be cleaved at a slower rate (Fig. 4a). Hence a mechanism involving the cleavage of each strand of the DNA in independent events is also incompatible with the above data. Therefore the *Sal*GI restriction endonuclease must cleave DNA by a mechanism which incorporates both the cleavage of each strand in separate events and also the cleavage of both strands without dissociation of the enzyme from the DNA, such as:

where E is *Sal*GI enzyme, S is supercoiled DNA of plasmid pMB9, Oc is open-circle DNA cleaved in one strand at the *Sal*GI site and L is linear DNA cleaved in both strands at the *Sal*GI site. Thus, the binding of the *Sal*GI enzyme at its recognition site on the DNA leads to the cleavage of one strand of the duplex and an intermediate with enzyme bound to the nicked DNA, $E \cdot Oc$. Whether this intermediate dissociates to give free open-circle DNA, or

proceeds to the cleavage of the second strand of the duplex, depends upon the reaction conditions. Under optimal reaction conditions (10 mM- $MgCl_2$, pH 8.0), no open-circle DNA was formed and thus both single strand scissions must occur within the same enzyme-DNA complex, i.e. $k_4 \gg k_3$. However, at either low $MgCl_2$ concentrations (0.3 mM) or low pH (5.7), high levels of open-circle DNA were produced, suggesting that the majority of complexes between enzyme and open-circle DNA dissociate, i.e. $k_3 > k_4$ under these conditions. Eqn. (3) above does not constitute a reaction mechanism, as Mg^{2+} has not been included.

In the absence of DNA, the *Sal*GI restriction endonuclease behaved principally as a monomer during gel filtration studies, though aggregated forms were observed under certain conditions (Maxwell & Halford, 1982a). Since the enzyme can cleave both strands of the DNA within a single enzyme-substrate complex, we suggest that the active form of the enzyme is a dimer. The two subunits of this protein could then interact symmetrically with the palindromic DNA sequence of the recognition site [as suggested by Kelly & Smith (1970)], each subunit catalysing the cleavage of one strand of the DNA. One hypothesis for why concerted cleavage of DNA only occurs at high $MgCl_2$ concentrations is that both subunits of the enzyme dimer must bind Mg^{2+} in order to effect catalysis. Low $MgCl_2$ concentrations would lead to enzyme dimers in which only one subunit had bound Mg^{2+} , thus cleaving the DNA in only one strand. Although direct experimental evidence to support the suggestion that the *Sal*GI enzyme is active as a dimer has not been obtained, the alternative of an active monomer carries the unattractive implication that the monomer contains two active sites. If only the dimer is active, the linear relationship between rates of DNA cleavage and enzyme concentration demands a uniform aggregation state of the protein over the range tested.

The reaction scheme in eqn. (3) has been previously proposed to account for the cleavage of the substrates, plasmid Col EI and the DNA from SV40, by the *Eco*RI restriction endonuclease (Rubin & Modrich, 1978). The reactions of the *Eco*RI enzyme on the DNA from either plasmid pMB9 or bacteriophage λ can also be accommodated by eqn. (3) provided that, for these substrates, $k_3 \gg k_4$ (Halford *et al.*, 1979, 1980). In these instances, each turnover of the enzyme liberates the nicked intermediate and eqn. (3) then simplifies to two separate but consecutive reactions. Thus, the cleavage of duplex DNA by the *Sal*GI and *Eco*RI restriction endonucleases may both be incorporated into the same general reaction scheme. It is likely that other type II restriction endonucleases cleave DNA by this pathway.

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