The *Sal*GI restriction endonuclease

Enzyme specificity

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(Received 9 September 1981/Accepted 8 December 1981)

We have analysed the kinetics of DNA cleavage in the reaction between the *Sal*GI restriction endonuclease and plasmid pMB9. This reaction is subject to competitive inhibition by DNA sequences outside the *Sal*GI recognition site; we have determined the K_m and V_{max} for the reaction of this enzyme at its recognition site and the K_i for its interaction at other DNA sequences. We conclude that the specificity of DNA cleavage by the enzyme is only partly determined by the discrimination it shows for binding at its recognition sequence compared with binding to other DNA sequences.

Type II restriction endonucleases are enzymes that specifically recognize short nucleotide sequences in double stranded DNA and cleave the DNA at fixed locations relative to their recognition sites. The wide applicability of these enzymes in molecular biology derives from the specificity of their cleavage reactions (Nathans & Smith, 1975). However, it is not yet clear how this specificity is achieved; the specificity could be primarily exerted either upon DNA binding or during catalysis. In the first case, the enzyme would show a much greater affinity for its recognition sequence compared with other DNA sequences but it would be able to catalyse the hydrolysis of any phosphodiester bond on the DNA. In the second case, the enzyme would show a similar affinity for all DNA sequences but catalysis would be much faster at the recognition sequence. These alternatives may be distinguished by determining the relative affinities of the restriction endonuclease for its recognition sequence compared with other DNA sequences. It has been shown by several different techniques that the *Eco*RI restriction endonuclease binds to DNA sequences that do not contain the *Eco*RI recognition site (Langowski *et al.*, 1980; Woodhead & Malcolm, 1980; Halford & Johnson, 1980). The equilibrium dissociation constant for the *Eco*RI enzyme binding at its recognition sequence is smaller than that for its binding to other DNA sequences; however, the ratio of these dissociation constants is less than the ratio between the rate at which this enzyme cleaves DNA at its recognition sequence compared with the rate at other DNA sequences (Halford & Johnson, 1980). Thus it appears that, with *Eco*RI enzyme, the specificity of the cleavage reaction does not derive solely from its binding specificity.

We have purified the type II restriction endonuclease *Sal*GI to virtual homogeneity and have studied its mechanism of DNA cleavage (Maxwell & Halford, 1982*a,b*). We now report on the specificity of this enzyme as analysed by the steady state kinetics of its reaction with plasmid pMB9. The observed rates of DNA cleavage were inhibited by DNA sequences outside the *Sal*GI recognition site on this substrate and we demonstrate that the specificity of this enzyme cannot arise solely from binding to its recognition sequence in preference to other DNA sequences.

Materials and methods

Supercoiled DNA (^3H -labelled) from plasmid pMB9 was prepared as described previously (Halford & Johnson, 1981). The linear form of this DNA was prepared by incubating 76 μg of the supercoiled form with *Sal*GI restriction endonuclease (8.3 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, in a total volume of 1.2 ml at 37°C for 2 h. After extracting twice with redistilled phenol and twice with chloroform, the DNA solution was made 0.3 M in sodium acetate and the DNA was precipitated with 2.5 vol. of ethanol at -20°C. The precipitate was redissolved in 10 mM-Tris/0.1 mM-EDTA, pH 7.5. (All reaction mixtures containing DNA also contained 5 mM-Tris/0.05 mM-EDTA in addition to the stated constituents.) The M_r values of plasmids pMB8 and pMB9 are 1.76×10^6 and 3.5×10^6 respectively (Rodriguez *et al.*, 1976). DNA from the bacteriophage λ 395(0) was prepared as described by Halford *et al.* (1980).

All kinetic experiments on the cleavage of DNA

by the *Sal*GI restriction endonuclease described here were performed by withdrawing samples at timed intervals from reaction mixtures containing 50 mM-Tris/10 mM-MgCl₂/125 mM-NaCl/10 mM- β -mercaptoethanol/0.5 mg·ml⁻¹ bovine serum albumin, pH 8.0. Samples were added immediately to stop mix [40% (w/v) sucrose/0.1 M-Tris/0.1 M-EDTA/0.5 mg·ml⁻¹ Bromophenol Blue] and analysed as described by Halford *et al.* (1979). The concentrations of substrate and product in each sample were plotted against time (see Fig. 1 below) and the rates of the reactions were evaluated from the initial portions of these plots. In each instance, the initial linear portion was defined by at least five samples withdrawn from the reaction mixture at different times. DNA concentrations are quoted as $\mu\text{g}\cdot\text{ml}^{-1}$ and are also expressed in terms of both the concentration of substrate (nM *Sal*GI recognition sites) and the concentration of inhibitor (μM base pairs). In reaction mixtures containing DNA from both plasmids pMB8 and pMB9, the inhibitor concentration is expressed as the total concentration of base pairs from both pMB8 and pMB9. The concentration of *Sal*GI restriction endonuclease are given in terms of a dimeric protein of M_r 58000. All other materials and methods were described previously (Maxwell & Halford, 1982a,b).

Results and discussion

Inhibition of *Sal*GI

The DNA of plasmid pMB9 has approx. 5300 base pairs and contains one *Sal*GI recognition sequence (Rodriguez *et al.*, 1976). Therefore the *Sal*GI restriction endonuclease, apart from binding at its recognition site, could in principle bind to 5300 alternative hexanucleotide sequences on this DNA. If this non-specific binding was significant compared with the binding at the recognition site, the reaction of the enzyme with plasmid pMB9 would be inhibited. To investigate this possibility, it would be necessary to measure the rates of cleavage of plasmid pMB9 by the *Sal*GI enzyme in the presence of varied concentrations of DNA sequences from outside the recognition site. However, with plasmid pMB9 alone, the concentrations of *Sal*GI recognition sequences and of DNA sequences outside the recognition site cannot be varied independently. Plasmid pMB8 is a molecule that constitutes approximately half of the DNA sequence of pMB9 but does not contain the *Sal*GI recognition site (Rodriguez *et al.*, 1976). Thus, the inclusion of plasmid pMB8 in reaction mixtures containing pMB9 and *Sal*GI enzyme increases the total DNA concentration whilst keeping the concentration of *Sal*GI recognition sites constant. Under the reaction conditions used here (10 mM-MgCl₂, pH 8.0), the enzyme appears to cleave both strands of the

DNA duplex in a concerted reaction, supercoiled DNA being converted directly to its linear form (Maxwell & Halford, 1982b). Initial rates of these zero-order reactions can be evaluated from both the disappearance of the supercoiled DNA and the appearance of the linear DNA; in practice, an average rate was determined from these two and expressed as nM substrate (*Sal*GI recognition sites)/min per nM *Sal*GI enzyme. Hence the V_{max} values given below are equivalent to catalytic-centre activities.

The addition of the supercoiled form of plasmid pMB8 reduced the rate at which the *Sal*GI restriction endonuclease cleaves the supercoiled form of plasmid pMB9 (Fig. 1). Thus, the cleavage of DNA

Fig. 1. *Inhibition of the SalGI reaction*
Samples (15 μl) were withdrawn at timed intervals from two 0.2 ml reaction mixtures (a and b), both containing 1.7 μg of supercoiled DNA (³H-labelled) of plasmid pMB9 and *Sal*GI restriction enzyme (1 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 (b) also contained 1.4 μg of supercoiled DNA of plasmid pMB8. [In reaction mixture (a), [S] = 2.4 nM, [I] = 13 μM ; in reaction mixture (b), [S] = 2.4 nM, [I] = 23 μM .] Each sample was immediately added to 10 μl of stop mix and analysed by electrophoresis and scintillation counting as described by Halford *et al.* (1979). The Figure presents the percentages of DNA in the supercoiled and linear forms at timed intervals in each reaction: under these reaction conditions, the cleavage of the supercoiled DNA of plasmid pMB9 by the *Sal*GI restriction enzyme yields none of the open-circle form (Maxwell & Halford, 1982b). The straight lines drawn are those used in the evaluations of the zero-order reaction rates (v , nM substrate or product/min per nM enzyme). Reaction (a), in the absence of plasmid pMB8: (○) supercoiled plasmid pMB9, (■) linear plasmid pMB9. Reaction (b), in the presence of plasmid pMB8: (○) supercoiled plasmid pMB9, (□) linear plasmid pMB9.

by the *SalGI* enzyme is inhibited by DNA sequences which do not contain the *SalGI* recognition site. Similar experiments were carried out using the linear form of plasmid pMB9 that had been cleaved previously at the *SalGI* site; this DNA also inhibited the rate at which the *SalGI* enzyme cleaved the supercoiled form of plasmid pMB9. The same concentration of base pairs from either the linear form of plasmid pMB9 or the supercoiled form of plasmid pMB8 resulted in identical reductions in reaction rate. (In this paper, we describe only the data obtained with plasmid pMB8 as the inhibitor.) This indicates that, with respect to non-specific binding to DNA, the enzyme does not discriminate between supercoiled and relaxed DNA molecules. We have previously observed that the *SalGI* enzyme shows the same reactivity towards its recognition sequence on DNA molecules that are linear, supercoiled or randomly nicked open-circles (Halford *et al.*, 1979; Maxwell & Halford, 1982b).

In contrast to the *SalGI* restriction enzyme, the rate at which the *EcoRI* restriction endonuclease cleaves plasmid pMB9 at its single recognition site was found, by experiments similar to Fig. 1, not to be measurably inhibited by the other DNA sequences on this substrate. Under the conditions 50 mM-Tes (2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulphonic acid)/50 mM-NaCl/10 mM-MgCl₂/100 µg·ml⁻¹ gelatin, pH 7.0, the same rate was observed for the cleavage of 10 µg·ml⁻¹ supercoiled DNA of plasmid pMB9 by 0.3 nM-*EcoRI* enzyme in either the absence or presence of 10 µg·ml⁻¹ DNA of bacteriophage λ395(0); the DNA from this derivative of bacteriophage λ lacks *EcoRI* recognition sites (Murray & Murray, 1974). Langowski *et al.* (1980) have demonstrated that DNA lacking the *EcoRI* recognition sequence could inhibit this enzyme; however, the minimum concentration of the alternative DNA required to inhibit significantly was an order of magnitude higher than those used in our experiments.

Determination of kinetic parameters

The reaction of the *SalGI* restriction endonuclease is inhibited by DNA sequences which do not contain the *SalGI* recognition site. If this inhibition is competitive, then the reaction velocity will be given by:

$$v = \frac{V_{\max} [S]}{K_m^{\text{app}} + [S]} \quad (1)$$

where

$$K_m^{\text{app}} = K_m (1 + [I]/K_i) \quad (2)$$

In our studies, [S] represents the concentration of *SalGI* recognition sites and [I] the total concentration of base pairs on the DNA. (Since this value of [I] determines K_i , it assumes that all

alternative sequences are inhibitors.) However, in order to determine K_m^{app} by varying [S], the concentration of plasmid pMB9 in the reaction mixture has to be varied; this will produce a variation in the total base pair concentration and hence alter [I]. Thus, four series of experiments were carried out; in each series, the rates of cleavage of supercoiled plasmid pMB9 by the *SalGI* enzyme were measured over a range of plasmid pMB9 concentrations, but the total DNA concentration (in base pairs) within each series was kept constant by compensatory adjustments to the concentration of plasmid pMB8. Examples of these data are shown in Fig. 2, from which values of $V_{\max}$ and K_m^{app} were determined by non-linear regression. All four series yielded similar $V_{\max}$ values in the range $0.12 \pm 0.02 \text{ min}^{-1}$, but the values of K_m^{app} were found to increase with increasing [I] (Fig. 3).

Hence the inhibition of the *SalGI* restriction enzyme by DNA sequences which do not contain its recognition site is competitive; non-competitive and uncompetitive inhibition both alter $V_{\max}$ and non-competitive inhibition does not affect K_m . From eqn. (2), a plot of K_m^{app} versus the inhibitor concentration permits the determination of K_m and of the ratio of K_m/K_i (Fig. 3). The value of K_m from the ordinate intercept was found to be 0.5 nM. However, as this intercept falls close to zero, this value cannot be considered a unique solution; the experimental data would also be consistent with much lower values of K_m . The ratio, K_m/K_i , was evaluated from the gradient of the plot in Fig. 3 as 8×10^{-5} ; but values for this parameter substantially different from that given would not remain consistent with the experimental data. The value of K_i , calculated from the above values of K_m and K_m/K_i , was 6 µM; this carries the same margin of error as the K_m .

The catalytic centre activities of most enzymes lie in the range $10\text{--}10^3 \text{ s}^{-1}$ (Fersht, 1977), whereas those determined for some restriction endonucleases are much lower than this: e.g. *EcoRI*, 7.5 min^{-1} (Halford & Johnson, 1981). We have found the catalytic-centre activity ($V_{\max}$) for the *SalGI* enzyme to be about 0.12 min^{-1} , considerably lower than that for the *EcoRI* enzyme. The low catalytic-centre activity for the *SalGI* enzyme may be partly due to the presence of inactive enzyme in our preparations, but until kinetic data for other restriction endonucleases are available, it is not possible to say whether the *SalGI* enzyme is atypical in this respect. [The catalytic-centre activities noted above were evaluated by extrapolating the measured rates of DNA cleavage to an infinite concentration of DNA substrate, but each is an apparent value, referring to a particular concentration of Mg²⁺; the catalytic-centre activity of the *EcoRI* enzyme varies with the concentration of MgCl₂ (Halford & Johnson, 1981).] The similarity between the specific

Fig. 2. Determination of $V_{\max}$ and K_m^{app} .

Initial velocities, v , of the rates of cleavage of plasmid pMB9 by the *Sal*GI restriction enzyme were measured over a range of pMB9 concentrations ($[S] = 0.6\text{--}12\text{ nM}$ in *Sal*GI recognition sites) in series of experiments. In each series, the total DNA concentration in the reaction mixtures ($[I]$ in base pairs) was kept constant by the requisite amounts of DNA from plasmid pMB8: (a) $[I]$ constant at $75.5\text{ }\mu\text{M}$; (b) $[I]$ constant at $20.5\text{ }\mu\text{M}$. The composition of the reaction mixtures and the evaluation of the initial velocities were as described in the legend to Fig. 1. The figures show plots of v against $[S]$ from these data. The lines drawn are theoretical lines for Michaelis-Menten kinetics fitted by non-linear regression: (a) $K_m^{\text{app}} = 7.6\text{ nM}$, $V_{\max} = 0.13\text{ min}^{-1}$; (b) $K_m^{\text{app}} = 1.6\text{ nM}$, $V_{\max} = 0.10\text{ min}^{-1}$.

activities of the *Sal*GI and the *Eco*RI restriction enzymes (Maxwell & Halford, 1982a) is consistent with the dissimilarity between their catalytic-centre activities. In the assay used by Maxwell & Halford (1982a) to define one unit of the *Sal*GI enzyme, the concentration of DNA recognition sites is above the K_m for this enzyme, whereas the assay used by Halford *et al.* (1979) to define one unit of the *Eco*RI enzyme contains a DNA concentration below its K_m .

Fig. 3. Determination of K_m and K_I .

Values of K_m^{app} were determined for four values of $[I]$ (the total base pair concentration) for reactions of the *Sal*GI restriction enzyme on plasmid pMB9 in the presence of plasmid pMB8, as described in the legend for Fig. 2. The graph is a plot of K_m^{app} versus $[I]$. The line drawn, fitted by linear regression (correlation coefficient 0.96), yields values for the ordinate intercept (K_m) of 0.5 nM and for the gradient (K_m/K_I) of 8×10^{-5} .

Specificity of *Sal*GI

The kinetic parameters determined above now permit the binding specificity of this enzyme to be compared with the specificity of its cleavage reaction. Maxwell & Halford (1982a) showed that the concentration of purified *Sal*GI restriction enzyme required to cleave plasmid pMB8 (which has no *Sal*GI recognition sites) is more than 2000 times greater than that required to cleave plasmid pMB9 (which has a single *Sal*GI recognition site). However, whereas the reaction on pMB9 DNA occurs at the *Sal*GI recognition site, the reaction on pMB8 DNA could occur at any phosphodiester bond on this DNA molecule. Since plasmid pMB8 contains over 5000 phosphodiester bonds, and if we assume that all sequences differing from the *Sal*GI recognition sequence would be cleaved at the same rate, then the reactivity of the *Sal*GI restriction enzyme towards its recognition site is at least a factor of 1×10^7 greater than that towards other sequences. This factor for the specificity of DNA cleavage by the *Sal*GI enzyme is a lower limit and the true value may be much greater; Maxwell & Halford (1982a) could not detect any cleavage of plasmid pMB8 at the highest enzyme concentration tested. The value of K_m/K_I determined above shows that the *Sal*GI restriction enzyme will be more frequently bound to

its recognition sequence than to other DNA sequences by a factor of about 1.2×10^4 . Hence the binding specificity of the *SalGI* enzyme accounts for only a small part of the observed specificity of its cleavage reaction: i.e., events occurring after the formation of the initial enzyme–DNA complex must also contribute to the specificity of the enzyme reaction.

The discrimination shown by the *EcoRI* enzyme between binding at its recognition sequence compared with other DNA sequences (Halford & Johnson, 1980) is about 30 times greater than that shown by the *SalGI* enzyme; this accounts for the observation that whereas the *SalGI* enzyme is significantly inhibited by DNA sequences outside its recognition site on plasmid pMB9, the *EcoRI* enzyme is not. From the difference between the equilibrium dissociation constant of the *EcoRI* enzyme from its recognition site on DNA and that from other DNA sequences (Halford & Johnson, 1980), it can be calculated that, when the *EcoRI* enzyme is present at a lower concentration than plasmid pMB9, 99% of the enzyme bound to the DNA in the absence of MgCl_2 would be located at the *EcoRI* recognition site and only 1% at other sequences on this DNA molecule. In contrast, the value of K_m/K_i for the *SalGI* restriction enzyme demonstrates that, during a steady state reaction with plasmid pMB9 at 10mM- MgCl_2 , 70% of the *SalGI* enzyme bound to the DNA would be located at its recognition site and 30% at other sequences. However, it must be noted that the binding specificity of the *EcoRI* enzyme was determined by equilibrium binding studies in the absence of Mg^{2+} , whereas that for the *SalGI* enzyme was evaluated from steady state kinetic experiments in the presence of Mg^{2+} ; hence the above comparison may not be strictly valid.

One question that remains unanswered is why the *SalGI* restriction endonuclease fails to cleave DNA sequences outside the *SalGI* recognition site, even when it is bound to these sequences.

Zero-order reactions

It is now possible to examine whether the non-specific binding of the *SalGI* restriction endonuclease to DNA accounts for the zero-order nature of the *SalGI* reactions. Re-examination of eqn. (1) shows that, under conditions where $[S] > K_m$ (as in our experiments), we may write:

$$v \simeq \frac{V_{\max.}}{1 + \frac{n \cdot K_m}{K_i}} \quad (3)$$

where

$$n = \frac{[I]}{[S]}$$

Thus, in a reaction with plasmid pMB9 alone, the initial rate will depend only upon n ; at zero time, n will be equal to the number of base pairs in pMB9. This accounts for the same initial rate of cleavage of plasmid pMB9 by the *SalGI* enzyme being obtained irrespective of the DNA concentration in the range 2.5–20 nM (Maxwell & Halford, 1982b). Using eqn. (3) and the values of $V_{\max.}$ and K_m/K_i determined above, a value of 0.084 min^{-1} may be calculated for v in the reaction of the *SalGI* enzyme with plasmid pMB9 alone; this agrees closely with the experimental value (Maxwell & Halford, 1982b). During the time course of a reaction, the concentration of the substrate, $[S]$, will decrease whereas the total base pair concentration, $[I]$, will remain constant: hence the value of n in eqn. (3) will increase. Calculation of v when, for example, 30% of the substrate has been consumed gives a value of 0.077 min^{-1} . This change in rate during the reaction would be too small to measure from the plot of substrate concentration against time; thus the rate will appear to be invariant over the initial part of the reaction.

Maxwell & Halford (1982b) reported that when an open-circle form of plasmid pMB9, that had been cleaved in one strand at the *SalGI* recognition site, was used as a substrate for the *SalGI* restriction enzyme, the concentration of this substrate declined exponentially during the reaction. From eqn. (1), an exponential decline in the substrate concentration requires that $K_m^{\text{app}} \gg [S]$. But at the same total DNA concentration, both the values of $[I]$ and of K_i must be the same in this exponential reaction as in the zero-order reaction on the supercoiled form of plasmid pMB9. Since K_m^{app} is determined only by K_i , $[I]$ and K_m (eqn. 2), the *SalGI* enzyme must have a higher K_m for the DNA molecule in which one strand is cleaved at the *SalGI* site than for the DNA molecule with both strands intact at this site.

We thank Mrs. N. P. Johnson for her assistance with these experiments and the Science Research Council for financial support.

References

- Fersht, A. (1977) *Enzyme Structure and Mechanism*, W. H. Freeman, Reading
- Halford, S. E. & Johnson, N. P. (1980) *Biochem. J.* **191**, 593–604
- Halford, S. E. & Johnson, N. P. (1981) *Biochem. J.* **199**, 767–777
- Halford, S. E., Johnson, N. P. & Grinstead, J. (1979) *Biochem. J.* **179**, 353–365
- Halford, S. E., Johnson, N. P. & Grinstead, J. (1980) *Biochem. J.* **191**, 581–592
- Langowski, J., Pingoud, A., Goppelt, M. & Maass, G. (1980) *Nucleic Acids Res.* **8**, 4727–4737

- Maxwell, A. & Halford, S. E. (1982a) *Biochem. J.* **203**, 77–84
- Maxwell, A. & Halford, S. E. (1982b) *Biochem. J.* **203**, 85–92
- Murray, K. & Murray, N. E. (1974) *Nature (London)* **251**, 476–481
- Nathans, D. & Smith, H. O. (1975) *Annu. Rev. Biochem.* **44**, 273–293
- Rodriguez, R. L., Bolivar, F., Goodman, H. M., Boyer, H. W. & Betlach, M. (1976) in *Molecular Mechanisms in the Control of Gene Expression* (Nierlich, D. P., Rutter, W. J. & Fox, C., eds.), pp. 471–477, Academic Press, New York
- Woodhead, J. L. & Malcolm, A. D. (1980) *Nucleic Acids Res.* **8**, 389–403