Ability of DNA and Spermidine To Affect the Activity of Restriction Endonucleases from Several Bacterial Species†

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ABSTRACT: Previous work has described the novel ability to modulate in vitro the activity of restriction endonuclease *Nae*I from *Nocardia aerocoligenes* by using cleavable DNA and spermidine [Conrad & Topal (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9707–9711]. In this paper we report the results of a study of 49 type II restriction enzymes from a variety of bacterial species. On the basis of the rates of cleavage observed, we found that in addition to expected *cleavable* sites a number of enzymes had *slow* and *resistant* cognate recognition sites. Resistant sites were identified for *Bsp*MI, *Nae*I, and *Nar*I; slow sites were identified for *Hpa*II, *Nae*I, and *Sac*II. Cleavage of these sites was found to be significantly enhanced by the addition of cleavable DNA or spermidine. We demonstrate that for *Bsp*MI, as for *Nae*I, activator DNAs increased V_{max} without altering K_m, whereas for *Hpa*II, *Nar*I, and *Sac*II activator DNAs decreased K_m without changing V_{max}. Comparison among the K_ms for *Nae*I cleavage of several different substrates demonstrated that distant DNA sequences can affect DNA recognition by the activated enzyme. Our observations extend DNA activation of the *Nocardia Nae*I endonuclease to restriction endonucleases from *Nocardia argentinensis* (*Nar*I), *Bacillus species M* (*Bsp*MI), *Haemophilus parainfluenza* (*Hpa*II), and *Streptomyces achromogenes* (*Sac*II). In addition, activation has now been found to affect slow as well as resistant recognition sites.

Previous work from our laboratory (Conrad & Topal, 1989) reported the ability to control in vitro the activity of the type II restriction endonuclease *Nae*I isolated from *Nocardia aerocolonigenes*; this enzyme cleaves within the DNA sequence GCC/GGC. Both cleavable and resistant *Nae*I recognition sequences were found. In the presence of cleavable sites, either cis (intramolecular) or trans (intermolecular), resistance was overcome in a reaction that followed Michaelis–Menten kinetics. Activation affected the maximum velocity of the cleavage reaction (V_{max}) rather than the apparent affinity of substrate for the enzyme (K_m). Saturation studies indicated that *Nae*I endonuclease contains independent binding sites for substrate and activator.

Spermidine also stimulated cleavage of *Nae*I resistant sites. Surprisingly, in the presence of activating levels of spermidine, the addition of cleavable sites had an inhibitory effect on *Nae*I cleavage of resistant sites (Conrad & Topal, 1989). Spermidine is a polyamine known to stimulate restriction enzymes (Pingoud et al., 1984), other DNA-binding enzymes such as topoisomerases (Pommier et al., 1989), and RNA polymerases (Blair, 1986; Jain & Tyaqi, 1987). Spermidine cellular concentration is also tightly correlated with cell cycle (Tabor & Tabor, 1984).

The ability to control the activity of the type II restriction enzyme *Nae*I in vitro and reports that *Eco*RII, another type II restriction enzyme, can be activated by exogenous DNAs (Hattman et al., 1979; Kruger et al., 1988) raised the question of whether this possible regulatory mechanism is more widely found among bacterial species. To look for this activation mechanism in other bacterial species, we surveyed 49 enzymes (including *Nae*I) for the presence of resistant sites whose cleavage could be activated by cleavable DNA and spermidine.

The kinetics of cleavage for these enzymes were measured; resistant sites were identified for *Bsp*MI, *Nae*I, and *Nar*I. In addition, sites that were cleavable, but with much slower kinetics, were identified for *Hpa*II, *Nae*I, and *Sac*II. Cleavage of resistant and slowly cleaved sites was significantly enhanced by the addition of cleavable DNA or spermidine. Thus, although these restriction enzymes were isolated from different microorganisms, we demonstrate that they share an activating mechanism similar to the one previously described for *Nae*I endonuclease.

Materials and Methods

DNA Substrates. pBR322 and φX174 DNAs were purchased from Promega (Madison, WI); M13mp18 and pSP64 DNA were purchased from Boehringer Mannheim (Indianapolis, IN); SV40 DNA was purchased from Bethesda Research Laboratories (Gaithersburg, MD), and pUC-f1 DNA was purchased from Pharmacia (Piscataway, NJ). Plasmid pMB3 (DHFR-pUC18) was a gift from Dr. Jane Azizkhan, Lineberger Comprehensive Cancer Center, University of North Carolina.

Restriction Endonucleases. The following enzymes were purchased from New England Biolabs (Beverly, MA): *Aat*II, *Afl*III, *Ase*I, *Ban*I, *Bgl*I, *Bsp*MI, *Bss*HI, *Bst*BI, *Dra*III, *Eag*I, *Fsp*I, *Msc*I, *Nae*I, *Nci*I, *Nru*I, *Sca*I, *Sma*I, and *Ssp*I. The following enzymes were purchased from Bethesda Research Laboratories (Gaithersburg, MD): *Ava*I, *Ava*II, *Bcl*I, *Bgl*II, *Cla*I, *Hae*II, *Hpa*II, *Hin*dIII, *Msp*I, *Nae*I, *Nar*I, *Nde*I, *Pst*I, *Pvu*I, *Pvu*II, *Sal*I, *Xho*I, and *Xma*III. The enzymes *Acc*I, *Apa*I, *Bam*HI, *Eco*RV, *Kpn*I, *Nhe*I, *Sac*II, *Sfi*I, *Sph*I, *Stu*I, *Taq*I, and *Xba*I were purchased from Promega (Madison, WI). *Eco*RI and *Crf*1OI were purchased from U.S. Biochemicals (Cleveland, OH).

Reaction Conditions. Reactions were typically performed in the presence of 100–200 ng of substrate DNA in a 10-μL volume. The manufacturer's definition of a unit of restriction enzyme activity is that amount of enzyme needed to cleave 1 μg of DNA to completion, after a 60-min incubation at 37 °C, in a 50-μL volume. This standard DNA is usually from bacteriophage λ or adenovirus 2 (Ad2), and the number of

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Table I: Restriction Enzymes with Cleavable Sites

enzyme	site[a]	DNA substrate	enzyme	site	DNA substrate
*Aat*II	GACGT/C	pBR322	*Hae*II	UGCGC/Y	SV40
		ϕX174[b]	*Hin*dIII	A/AGCTT	M13mp18
*Acc*I	GT/XMAC	M13m18			pBR322
		SV40	*Kpn*I	GGTAC/C	M13mp18
*Afl*II	C/TTAAG	SV40			SV40
*Apa*I	GGGCC/C	SV40	*Msc*I	TGG/CCA	M13mp18
		λ	*Msp*I	C/CGG	SV40
*Ase*I	AT/TAAT	pBR322	*Nci*I	CC/WGG	ϕX174
*Ava*I	C/YCGUG	pBR322	*Nde*I	CA/TATG	M13mp18
		ϕX174			pBR322
*Ava*II	G/GZCC	M13mp18	*Nhe*I	G/CTAGC	pBR322
		ϕX174			λ
*Bam*HI	G/GATCC	M13mp18	*Nru*I	TCG/CGA	pBR322
		pBR322	*Pst*I	CTGCA/G	pBR322
		SV40			ϕX174
*Ban*I	G/GYUCC	SV40			SV40
*Bcl*I	T/GATCA	SV40	*Pvu*I	CGAT/CG	M13mp18
*Bgl*I	$GCCN_5GGC$	M13mp18			pBR322
		SV40	*Pvu*II	CAG/CTG	pBR322
*Bgl*II	A/GATCT	M13mp18	*Sal*I	G/TCGAC	M13mp18
*Bss*HII	G/CGCGC	ϕX174			pBR322
*Bst*BI	TT/CGAA	Ad2	*Sca*I	AGT/ACT	pBR322
*Cla*I	AT/CGAT	pBR322	*Sfi*I	$GGCCN_5GGCC$	SV40
*Cfr*10I	U/CCGGY	M13mp18	*Sma*I	CCC/GGG	M13mp18
		SV40	*Sph*I	GCATG/C	M13mp18
*Dra*III	$CACN_3/GTG$	M13mp18			pBR322
		ϕX174	*Ssp*I	AAT/ATT	pBR322
*Eag*I	C/GGCCG	pBR322			ϕX174
		pMC1neo	*Stu*I	AGG/CCT	ϕX174
*Eco*RI	G/AATTC	M13mp18	*Taq*I	T/CGA	SV40
		pBR322	*Xba*I	T/CTAGA	M13mp18
		SV40			λ
*Eco*RV	GAT/ATC	pBR322	*Xho*I	C/TCGAG	ϕX174
		SV40			λ
*Fsp*I	TGC/GCA	M13mp18	*Xma*III	C/GGCCG	pBR322
		ϕX174			

[a] 5′ to 3′ DNA sequence of the restriction enzyme recognition site from supplier catalogs. /, cleavage position; X, A or C; M, G or T; W, G or C; Z, A or T. [b] Considerable nicking.

restriction sites varies with the enzyme. To correct for the different number of recognition sequences in these DNAs for each of the different enzymes, we corrected the manufacturer's unit value for the different enzymes taking into account (1) the micrograms of DNA used in the reaction, (2) the molecular weight of the substrate, and (3) the number of restriction sites in the standard DNA and in the substrate DNA. The activity of restriction enzymes was tested at 1–30× equivalent units. Each enzyme was tested in the buffer suggested by the manufacturer. Cleavage reactions were carried out for 60 min at 37 °C unless otherwise indicated.

Kinetics. K_m measurements were done by varying the concentration of substrate from 1 to up to 60 nM with incubation times between 15 and 40 min; the amount of cleavage was kept at less than 10% of the starting material. All kinetic studies were done on supercoiled DNA substrates. The electrophoresis of DNA samples was performed in 1% agarose gels in 1× TAE buffer (40.0 mM Tris–acetate, pH 8.0, and 2.0 mM EDTA); the gels were stained with 1 μg/mL ethidium bromide and photographed with a UV-light transilluminator and Polaroid instant film, type 665. Negatives from photographs of the gels were scanned on a Bio-Rad Model 1650 densitometer (Hoefer). Scans were analyzed by using a Maxima chromatography workstation from Dynamic Solutions Corp. (Ventura, CA).

Results

Presence of Cleavable, Slow, and Resistant Sites. On the basis of the *Nae*I cleavage data (Conrad & Topal, 1989), the presence of a cognate recognition site in the DNA does not guarantee cleavage. Therefore, we examined the cleavage of DNA substrates having only one recognition site for each enzyme. Digestion of the DNAs with appropriate restriction enzymes showed the presence of sites that are cleaved at widely different rates. For the purposes of this discussion, we define cleavable, slow, and resistant sites for our reaction conditions. A *cleavable* site is defined as a site where 90% or more of the DNA is cleaved within 1 h with a 1–5-fold excess of enzyme predicted from its unit activity to be necessary for complete cutting of the single-site substrate. A *slow* site is defined as a site where between 5% and 90% cleavage is achieved with a 5-fold excess of enzyme; the addition of a 10- or 30-fold excess of enzyme increased cleavage at slow sites. A *resistant* site is defined as a site at which less than 5% cleavage can be achieved with a 5-fold excess of enzyme; the addition of a 10–30-fold excess of enzyme did not increase cleavage at resistant sites. For example, Figure 1 shows the digestion of pBR322 DNA by two enzymes, *Eco*RI and *Bsp*MI; both of these enzymes have a unique site in this DNA. Plasmid pBR322 DNA was found to have a *cleavable Eco*RI site, but a *resistant Bsp*MI site.

The 49 enzymes surveyed were catagorized in terms of their ability to cleave their respective recognition sequence. Table I lists the restriction enzymes that had cleavable sites and the substrate DNA used. Table II lists the restriction enzymes that had *slow* or *resistant* sites. Five of 49 restriction enzymes surveyed showed sites that were slow or resistant. Three of these enzymes, *Hpa*II, *Nae*I, and *Sac*II, had substrates that were *slow*. In addition to *Nae*I, the restriction enzymes *Bsp*MI and *Nar*I had substrates that were *resistant*.

Cleavage at Resistant Sites. Table II lists the resistant substrates found for *Bsp*MI, *Nae*I, and *Nar*I. All resistant

Table II: Slow and Resistant Restriction Enzyme Sites

resistant sites				with activator		
enzyme	site	DNA substrate	class[a]	K_m (nM)	V_{max} (nM/min)	activation by DNA
*Bsp*MI	ACCTGCN$_4$/	pUC-f1	V	7.0	*d*	yes by ϕX174
						yes by M13mp18
		pBR322		3.5	*d*	yes by ϕX174
						yes by M13mp18
*Nae*I	GCC/GGC	M13mp18	V	2	0.06[c,d]	yes by oligo[f]
						yes by pBR322
						yes by SV40
		pUC-f1		42	*d*	yes by oligo
		DHFR-pUC18		24.5	*d*	yes by oligo
		pMC1neo		6.6	*d*	yes by oligo
		pSP64		23.7	*d*	yes by oligo
		λ		nd[b]		no by pBR322
						no by oligo
*Nar*I	GG/CGCC	M13mp18	K	1.1[c,d]	0.004	yes by oligo
						yes by pBR322
						yes by ϕX174
						no by λ
		λ		nd		yes by pBR322
						yes by ϕX174
						no by M13mp18

slow sites			without activator			
enzyme	site	DNA substrate	class	K_m (nM)	V_{max} (nM/min)	activation by DNA
*Hpa*II	C/CGG	SV40	K	10[c,d,e]	0.162	yes by pBR322
						yes by oligo
*Nae*I		SV40	V	30	0.01	yes by oligo
						yes by pBR322
*Sac*II	CCGC/GG	pMB3	K	*e*	*e*	yes by ad2
						no by λ
						no by ϕX174
		ϕX174		32	0.15	no by ad2
						no by λ
						no by pMB3

[a] Class refers to whether the enzyme is part of a positive allosteric V- or K-system. [b] Not determined. [c] Value measured at saturating concentration of activator. [d] Varied with the amount of activator. [e] Sigmoidal kinetics without activator. [f] Double-strand DNA containing unique cleavable *Nae*I, *Nar*I, and *Hpa*II restriction sites.

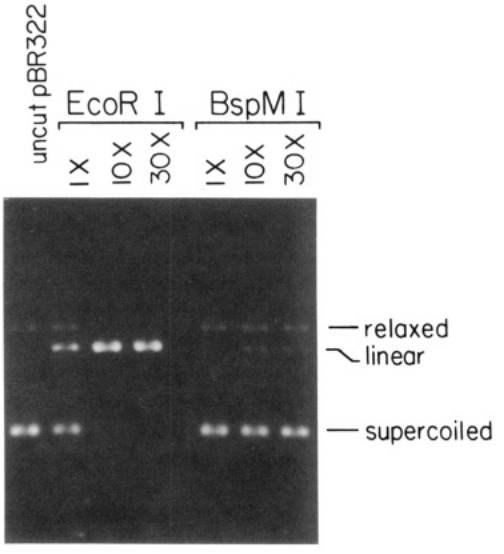

FIGURE 1: Cleavage of pBR322 DNA by *Eco*RI and *Bsp*MI endonucleases. The enzymes were added at 1-, 10-, and 30-fold excess as described under Materials and Methods. The substrate, pBR322 DNA, was present at 3.5 nM in a 10-μL reaction volume. The reactions were incubated for 1 h at 37 °C.

recognition sites showed activation of cleavage by addition of cleavable or slow DNA sites to the reaction. To determine whether the activator DNA affected the V_{max} or the K_m of the reaction, these parameters were measured in the presence of various concentrations of activator. The V_{max} for *Nae*I cleavage of the resistant M13mp18 DNA site increases with increasing concentration of activator, whereas the K_m remains constant (Conrad & Topal, 1989). Similarly, *Bsp*MI showed an increase in V_{max} for cleavage of the resistant site in pUC-f1 DNA with increasing concentrations of activating ϕX174 DNA, whereas K_m remained constant (Figure 2a).

For *Nar*I, however, the V_{max} for cleavage of M13mp18 double-stranded DNA remained constant, whereas the K_m decreased with increasing concentrations of activator; activator was an oligonucleotide duplex DNA containing a *Nar*I site (Table II; Conrad & Topal, 1989).

In general, resistant cognate recognition sites were unable to activate cleavage of other resistant sites (Table II). The only exception was the inability of the activating oligonucleotide duplex to stimulate cleavage of the resistant *Nae*I site in λ DNA for reasons that we do not understand.

Cleavage at Cleavable Sites. For comparison with enzymes showing slow and resistant sites, the K_m and V_{max} for cleavage of pBR322 DNA by *Eco*RI were determined to be 12 nM and 0.3 nM/min, respectively (not shown). The measured K_m is similar to the value of 8 nM for cleavage of ColE1 DNA (from which pBR322 DNA was derived) by *Eco*RI under similar conditions (Modrich & Zabel, 1976).

Cleavage at Slow Sites. For *Hpa*II (Figure 2b), the K_m for cleavage of its single SV40 DNA recognition site decreased with increasing concentrations of pBR322 activator DNA. The shape of the velocity/saturation curve for *Hpa*II cleavage of SV40 DNA without activator present was sigmoidal; the shape of the curve changed to hyperbolic in the presence of activator (Figure 2b). The assignment of the SV40 DNA *Hpa*II site as slow was somewhat arbitrary; the sigmoidal shape of the velocity/saturation curve means that the ability to cleave this site was highly dependent on the concentration

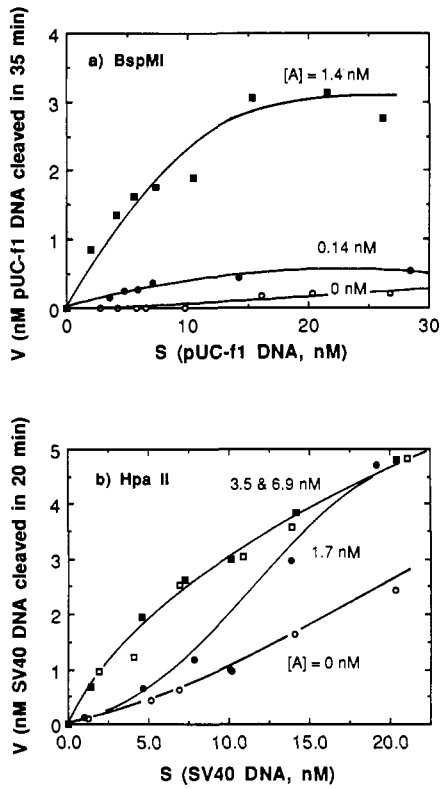

FIGURE 2: Allosteric activation of resistant and slow sites. (a) V versus S plot for the *Bsp*MI resistant site in pUC-f1 DNA. The activator DNA [A] was ϕX174. The enzyme concentration was 0.8 unit of *Bsp*MI/reaction. (b) V versus S plot for the *Hpa*II slow site in SV40 DNA. The activator DNA [A] was pBR322. The enzyme concentration was 0.12 unit/reaction.

of the DNA substrate.

The resistance to cleavage of the *Hpa*II site in SV40 DNA was not due to methylation. Methylation of this site with *Hpa*II methylase completely blocked cleavage at that site, and no activation by pBR322 DNA was observed (results not shown). Similar results were obtained upon methylation of the *Nae*I site in M13mp18 DNA.

In contrast to the slow cleavage of *Hpa*II and *Nae*I sites, the slow cleavage of a *Msc*I site in pBR322 is probably due to methylation. That slow site overlaps a *dcm* methylase site, CC(A or T)GG. Cleavage of pBR322 DNA with *Eae*I (Py/GGCCPu), which overlaps the *Msc*I (TGG/CCA) site and is inhibited by 5-meC [methylation inhibition tabulated by Nelson and McClelland (1987)], demonstrated that three of the four *Eae*I sites in pBR322 DNA were cleaved; the uncleaved site was the unique *Msc*I/*Eae*I recognition site that overlaps the *dcm* site (not shown). Methylation can explain why this was the only slow or resistant site found that could not be activated by cleavable DNA.

Figure 3 shows two examples of V (nanomoles of substrate cleaved per liter per minute) versus S (nanomolar) plots for cleavage of two different *Sac*II slow sites. Cleavage of the *Sac*II slow site in ϕX174 DNA showed the rectangular hyperbola characteristic of Michaelis–Menten kinetics (Figure 3a). By contrast, *Sac*II cleavage of the pMB3 DNA slow site showed a sigmoidal dependence on substrate concentration (Figure 3b). A Hill coefficient of 1.6 was determined (Figure 3c) for the binding of pMB3 DNA; if we assume that two substrate sites are present on the enzyme, the Hill coefficient indicates 80% cooperativity for substrate binding.

*Nae*I and *Sac*II could be activated by cleavable DNA to cleave their *slow* sites at a faster rate (not shown); the effect was not as strong for *Sac*II as for *Nae*I. This is understandable, since the activated and nonactivated saturation curves for *Sac*II are not that different in terms of rates of cleavage (panel a vs b of Figure 3). This small difference could explain the inability to observe activation of *Sac*II cleavage of pMB3 DNA by λ DNA. The general inability to activate *Sac*II cleavage of ϕX174 DNA is probably related to the already activated shape of the saturation curve for cleavage of this site; the more active form of the enzyme binds this substrate. In general, we observed that cleavable sites at high molar ratio to the slow sites were required to activate cleavage of slow sites; this is in contrast to the lower molar ratios required to activate cleavage of resistant sites.

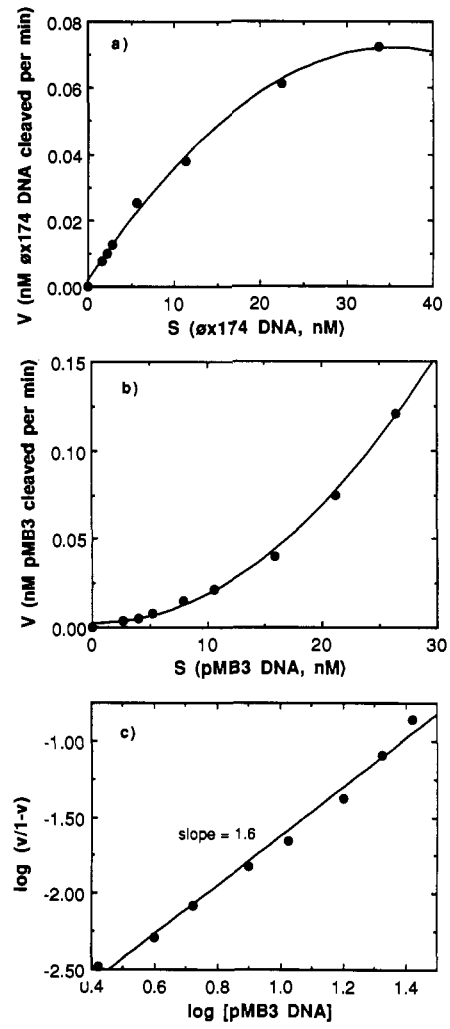

FIGURE 3: Kinetics of cleavage of *Sac*II at two different slow sites. (a) V versus S plot for the *Sac*II slow site in ϕX174 DNA. Variable amounts of substrate were incubated for 40 min (37 °C) with 1.0 unit of enzyme in a 10-μL reaction volume. (b) V versus S plot for the *Sac*II slow site in pMB3 DNA. Variable amounts of substrate were incubated for 40 min (37 °C) with 1.6 units of *Sac*II in a 10-μL reaction volume. (c) Hill plot for the *Sac*II slow site in pMB3 DNA. The slope of log $(v/1 - v)$ versus log S gives the Hill coefficient, a measure of cooperativity.

Spermidine Effects. Spermidine was able to activate the cleavage of resistant sites and to increase the rate of cleavage of slow sites. The activation of cleavage of resistant sites by spermidine exhibited activation maxima at concentrations of spermidine between 0.5 and 10 mM (tested at the manufacturer's recommended salt conditions for each enzyme). The effect of spermidine was dependent on the concentration of Mg^{2+} as previously shown (Conrad & Topal, 1989). Figure 4 shows *Nae*I endonuclease digests of the SV40 slow site in the absence and presence of spermidine. Under identical

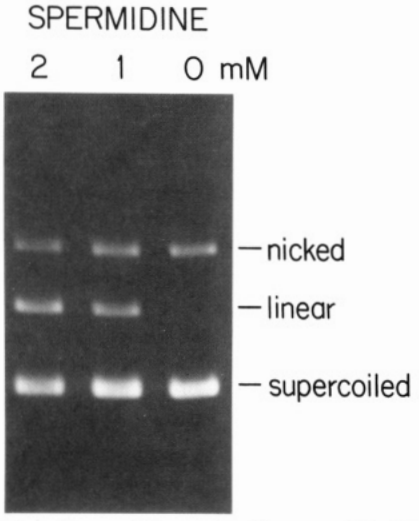

FIGURE 4: Effect of spermidine on the cleavage of the *Nae*I slow site in SV40 DNA. Reaction conditions: spermidine as indicated, SV40 DNA (2.9 nM) in a 10-μL reaction, 1.1 units of *Nae*I, 10 mM $MgCl_2$, 20 mM NaCl, 10 mM Tris-HCl (pH 8.0), 5 mM β-mercaptoethanol, and 100 μg of bovine serum albumin/mL. Reactions were incubated for 1 h at 37 °C.

conditions of DNA and enzyme concentration, the addition of 2 mM spermidine increased cleavage 40-fold from 0.4% to 17%.

We previously reported that the addition of an activating concentration of spermidine to an *Nae*I cleavage reaction containing activating DNA inhibited cleavage. The experiments were done in the presence of low concentrations of NaCl and $MgCl_2$. We have repeated those experiments at 50 mM NaCl and 10 mM $MgCl_2$. The results remain the same, but the spermidine concentration at which the switch occurs is at 8 mM instead of the 1 mM at low salt concentration (not shown). This agrees with the dependence of the activating effect of spermidine on salt concentration (Conrad & Topal, 1989). The effect of spermidine in the presence of activator for *Bsp*MI, *Hpa*II, *Nar*I, or *Sac*II has not been examined. The effects of spermidine on the kinetics of activation for these enzymes are currently being examined.

Discussion

In this study, 49 restriction enzymes were screened by using standard restriction enzyme assays. In addition to *Nae*I endonuclease, we found that *Bsp*MI and *Nar*I also have recognition sites resistant to cleavage. Furthermore, we found that *Hpa*II, *Nae*I, and *Sac*II endonucleases have slow sites. Cleavage of resistant and slow sites by these enzymes was enhanced by the addition of either cleavable sites in trans or spermidine.

Commercially available DNA substrates with one recognition site per molecular were used to test the activity of restriction enzymes; a substrate with more than one site could obscure the presence of a resistant site because of cis activation (Conrad & Topal, 1989). The use of readily available DNAs with unique restriction sites enabled us to screen a large number of enzymes. It is possible, however, that some of the 44 enzymes for which only cleavable sites were detected may have other untested substrates with resistant sites. Our observations may, therefore, underestimate the presence of both resistant/slow sites and restriction enzymes whose activity is subject to modulation by DNA.

Resistant and Slow Sites. Kinetic analysis of the cleavage of either resistant or slow substrates by some enzymes indicated two different mechanisms of activation. The enzyme *Bsp*MI, as with *Nae*I (Conrad & Topal, 1989), gave hyperbolic substrate saturation curves with varying V_{max} and constant K_m; the curves were hyperbolic at all activator concentrations studied. In the absence of activator, very little cleavage of resistant sites could be detected, and cleavage was not significantly increased by increasing substrate concentrations substantially above K_m. These results indicate that *Bsp*MI, as with *Nae*I, is an allosteric positive V-system enzyme according to the classic allosteric protein model (Monod et al., 1965), in which the protein contains independent binding sites for activator and substrate and has at least two conformations; the substrate has the same affinity for both conformations of the protein, but the two protein conformations differ in their catalytic activity. Activator DNA must have maximum affinity for the more active state of the protein.

The kinetics for the cleavage of substrate by the enzyme *Hpa*II, on the other hand, were sigmoidal with respect to substrate concentration, indicating that substrate binding at the active site was cooperative. With increasing concentrations of DNA activator, the kinetics for cleavage changed from a sigmoidal dependence on substrate concentration to Michaelian hyperbolic; K_m decreased with increasing activator concentration, whereas V_{max} remained constant. Measurements of K_m for *Nar*I at different activator concentrations showed similar kinetics to *Hpa*II. These results indicate that *Hpa*II and *Nar*I are allosteric positive K-system enzymes; these proteins have independent binding sites for substrate and activator and at least two conformations. In contrast to the V-system enzymes, however, both activator and substrate have differential affinities toward the two conformations of protein. The presence of activator increases the affinity of protein for substrate at the active site (Monod et al., 1965).

The choice of designating sites for K-system enzymes as either resistant or slow was based on the amount of cleavage at the defined concentration of substrate used to screen all of the enzymes (see Materials and Methods). This choice turned out to be somewhat arbitrary; higher substrate concentrations of originally resistant sites give the appearance of slow sites, and very high concentrations may appear to be cleavable by K-system enzymes.

The slow cleavage sites found for *Sac*II in ϕX174 DNA and pMB3 DNA gave differing kinetics; cleavage of pMB3 DNA by *Sac*II endonuclease was sigmoidal with respect to substrate concentration, whereas cleavage of ϕX174 DNA was hyperbolic with respect to substrate concentration. Similar differences in substrate binding were seen for various triphosphonucleosides acting as phosphoryl donors in the deoxythymidine kinase reaction (Okazaki & Kornberg, 1964). With ATP, for example, the rate/concentration curve is strongly cooperative (sigmoidal), whereas with dATP the curves are hyperbolic. Furthermore, CDP converts the ATP sigmoidal curve to hyperbolic, indicating allosteric activation (Okazaki & Kornberg, 1964). Monod et al. (1965) argue that the deoxythymidine kinase observations support their model so that "when the binding of two analogous ligands depends very much on steric factors it may be expected that the ratios of the affinities of each ligand toward the two states of the protein will be different. If so, the two ligands might bind to the same sites with widely different interaction coefficients."

*Sac*II also appears to support this model; homotropic allosteric effects are apparent for *Sac*II. The *Sac*II DNA substrates ϕX174 DNA and pMB3 DNA apparently have very different relative affinities for the two states of the protein. Activation of cleavage of pMB3 DNA was obtained by the addition of other DNA containing cleavable *Sac*II sites. This

activation indicates activating heterotropic allosteric effects in addition to the homotropic allosteric effects of substrate.

Relative Ability to Activate Cleavage. Determination of the rank order of sites, cleavable, slow, or resistant, required to activate cleavage of any other type of site (Table II; Conrad & Topal, 1989) shows, from our population, that (a) each site susceptible to activation required a more cleavable site to activate its cleavage and (b) the amount of activating site required for activation of cleavage of substrate appeared to be proportional to the cleavability of that substrate. One model that can explain these characteristics assumes that resistant and slow sites, and perhaps cleavable sites as well, differ only in their relative ability to bind to the activator site of their cognate enzymes. According to this model, a higher concentration of cleavable sites is necessary to activate cleavage of a slow site than is necessary to activate cleavage of a resistant site because the cleavable site must compete with the slow site for binding to the activator site of the enzyme.

According to the model, resistant sites do not bind the enzyme activator site so they cannot activate cleavage of any of the sites. Slow sites can potentially activate another slow site or cleavable site; however, the high concentrations required would competitively inhibit substrate cleavage. Cleavable sites theoretically are already fully activated either because they bind to the activation site as well as to the substrate site or because they bind the active conformation of the protein in a manner analogous to the *Sac*II/ϕX174 system discussed above. Thus, it is possible that the class of restriction enzymes that contain an activator site as well as a substrate site is much larger than the five enzymes we have characterized so far.

An exception to the above model is the behavior of λ DNA with several of the restriction enzymes. The reason for its inability to be cleaved by *Nae*I even in the presence of potentially activating DNAs and its inability to activate resistant and slow sites for enzymes that cleave λ (Table II) is not known.

Flanking Sequence Effects. Slow and resistant sites must exist because of the effect of sequences outside the recognition sequence. These sequences can interact directly with enzyme. For example, site-directed mutagenesis, chemical protection experiments, and X-ray crystallography show that, in addition to bases within the recognition site, bases outside the site are also contacted by *Eco*RI (Lu et al., 1981; McClarin et al., 1986). This could be the reason that *Eco*RI varies up to 10-fold in its ability to cleave the five recognition sites in λ DNA (Thomas & Davis, 1974) and the five recognition sites in adenovirus DNA (Forsblom et al., 1976).

Our results provide evidence for interactions of the restriction enzymes with distant DNA sequences. Comparison of the sequences immediately flanking the several *Nae*I recognition sequences used as substrates for *Nae*I endonuclease (Table II) showed no obvious correlation of sequence with K_m differences. In fact, two of the most disparate K_ms are for the single *Nae*I sites whose flanking sequences are almost identical with each other for 126 base pairs downstream and 384 base pairs upstream from the recognition sequence.

This situation arises for the bacteriophage f1 intergenic region (514 base pairs) engineered into pUC18 to give the vector pUC-f1. The f1 intergenic region contains the *Nae*I site and is almost identical with its homologue in M13; single base differences occur 86 and 212 base pairs upstream from the *Nae*I recognition site (Hill & Peterson, 1982). This region in the context of M13 gives a K_m of 2 nM, whereas in the context of pUC-f1 it gives a K_m of 42 nM. Thus, in this instance distant sequences affect the relative affinity of the protein for its binding site by 20-fold. We do not know the basis for this interesting effect of distant sequence on K_m; we are studying the basis for the resistance to cleavage of the *Nae*I site in M13 DNA (Frediani, Rossi, and Topal, unpublished results) to understand the basis for these apparently long-range effects.

Other Regulated Restriction Enzymes. A survey of the literature indicated two other type II restriction enzymes for which resistant sites have been reported. Resistant *Eco*RII restriction sites exist (Hattman et al., 1979) in ϕX174 DNA that can be cleaved in the presence of an uncharacterized, heterologous "stimulator DNA." Resistant *Eco*RII sites have also been reported (Kruger et al., 1988; Pein et al., 1989) in phage T3 and T7 DNAs. These were cleaved in the presence of a high density of cleavable *Eco*RII DNA sites, so the authors suggest that at least two bound recognition sites are needed for cleavage (Kruger et al., 1988). No kinetics were measured, however, and the basis for the activation was not pursued. The authors reported that spermidine does not affect the *Eco*RII cleavage of the resistant sites. However, since the concentration range over which spermidine activates cleavage of slow and resistant sites is narrow, the effect could easily have been missed; the concentrations of spermidine used in the study (Kruger et al., 1988) were not reported. Therefore, the relation between *Eco*RII cleavage of resistant sites and the modulation of *Bsp*MI, *Hpa*II, *Nae*I, *Nar*I, and *Sac*II activity by DNA and spermidine is not clear.

Also, *Pae*R7, a type II restriction enzyme from *Pseudomonas aeruginosa*, shows a resistant site in Ad2 DNA in the presence of other susceptible Ad2 DNA sites (Gingeras et al., 1983); resistance was overcome by replacement of upstream sequences with sequence from a different source. When the gene for *Pae*R7 was expressed in *Escherichia coli*, the bacteria were unable to restrict the growth of incoming phage even though cell extracts displayed the expected restriction activity on the phage DNA (Gingeras et al., 1983); the authors speculate that this may indicate the presence of a control element.

Other Enzymes Regulated by DNA. We wonder whether DNA/polyamine modulation of endonucleolytic cleavage DNA plays a role in bacteria to increase the accuracy of restriction/modification systems. The requirement for more than one restriction site to enable cleavage would reduce the possibility of cleaving the host genome by mistake. It is also interesting to note that the ability of *Nae*I to juxtapose and cleave distant recognition sequences (Topal et al., 1991) is reminiscent of some aspects of site-specific recombination and transposition [see Alberts et al. (1989) for a general discussion of these processes]. During the study of the mechanism of transposition of insertion sequence IS50, it was observed that methylation at one end of the sequence could influence interaction of the transposition complex with the other end (Tomcsanyi & Berg, 1989).

Conclusions. We have shown that the ability of DNA and spermidine to activate *Nae*I endonuclease from *Nocardia aerocolonigenes* is not unique to this enzyme or to this species. *Bsp*MI, *Hpa*II, *Nar*I, and *Sac*II share a similar mechanism. The activating mechanism was found to belong to two different classes; one, for *Bsp*MI and *Nae*I, in which the catalytic activity (k_{cat}) of the enzyme is increased by the activator (V-system), and the other, for *Hpa*II, *Nar*I, and *Sac*II, in which the affinity of binding of the resistant substrate is increased by the activator (K-system).

These enzymes have been isolated from four different species of bacteria, *Bacillus*, *Haemophilus*, *Nocardia*, and *Strepto-*

myces, suggesting a broader distribution of this regulatory mechanism among bacterial species. In addition, activation has now been extended to slow as well as resistant sites.

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Stimulation of the ATPase Activity of Rat Brain Protein Kinase C by Phospho Acceptor Substrates of the Enzyme†

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ABSTRACT: We recently reported that autophosphorylated rat brain protein kinase C (PKC) catalyzes a Ca^{2+}- and phosphatidylserine- (PS-) dependent ATPase reaction. The Ca^{2+}- and PS-dependent ATPase and histone kinase reactions of PKC each had a $K_{m\,app}$(ATP) of 6 μM. Remarkably, the catalytic fragment of PKC lacked detectable ATPase activity. In this paper, we show that subsaturating concentrations of protein substrates accelerate the ATPase reaction catalyzed by PKC and that protein and peptide substrates of PKC induce ATPase catalysis by the catalytic fragment. At subsaturating concentrations, histone III-S and protamine sulfate each accelerated the ATPase activity of PKC in the presence of Ca^{2+} and PS by as much as 1.5-fold. At saturating concentrations, the protein substrates were inhibitory. Poly(L-lysine) failed to accelerate the ATPase activity, indicating that the acceleration observed with histone III-S and protamine sulfate was not simply a result of their gross physical properties. Furthermore, histone III-S induced the ATPase activity of the catalytic fragment of PKC, at both subsaturating and saturating histone concentrations. The induction of ATPase activity was also elicited by the peptide substrate Arg-Arg-Lys-Ala-Ser-Gly-Pro-Pro-Val, when the peptide was present at concentrations near its $K_{m\,app}$. The induction of the ATPase activity by the nonapeptide provides strong evidence that the binding of phospho acceptor substrates to the active site of PKC can stimulate ATP hydrolysis. Taken together, our results indicate that PKC-catalyzed protein phosphorylation is inefficient, since it is accompanied by P_i production. Our results also provide support for a model of PKC catalysis in which the binding of the phospho acceptor substrate to the active site of PKC enhances the rate of phospho donor substrate hydrolysis.

Protein kinase C (PKC)[1] consists of a family of closely related Ca^{2+}- and phosphatidylserine- (PS-) dependent protein kinase isozymes that are activated in vivo by the second messenger diacylglycerol (Kikkawa et al., 1989; O'Brian & Ward, 1989a). We recently reported that purified, autophosphorylated rat brain PKC has an intrinsic Ca^{2+}- and PS-dependent ATPase activity. The Ca^{2+}- and PS-dependent histone kinase and ATPase activities of PKC each had a

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[1] Abbreviations: BSA, bovine serum albumin; cAMP, adenosine cyclic 3′,5′-phosphate; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PMSF, phenylmethanesulfonyl fluoride; PS, phosphatidylserine; RRKASGPPV, Arg-Arg-Lys-Ala-Ser-Gly-Pro-Pro-Val; TCA, trichloroacetic acid; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

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