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Immobilization of the Restriction Endonucleases *Pvu*II and *Hin*dIII

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ABSTRACT

The effects of several chemical reagents on the activity of the restriction endonucleases *Pvu*II and *Hin*dIII were investigated. Carbodiimide, which reacts preferentially with carboxyl groups, was found to inactivate these enzymes. This specific effect could be prevented by Mg^{2+} cation. pBR322 DNA, which contains *Pvu*II and *Pvu*II* sites and *Hin*dIII and *Hin*dIII* sites, did not protect the enzymes from the carbodiimide.

On the other hand, glutaraldehyde, which reacts primarily with lysine residues, inactivates *Pvu*II and *Hin*dIII enzymes. This specific effect could not be prevented by pBR322 DNA.

Preincubation with high concentrations of *N*-ethylmaleimide, which reacts with sulfhydryl groups, caused slight inhibition of *Pvu*II activity, but had no effect on the activity of *Hin*dIII enzyme.

The effects of glutaraldehyde, carbodiimide, and *N*-ethylmaleimide on other restriction endonucleases were also investigated.

Restriction endonucleases *Pvu*II and *Hin*dIII were immobilized by covalent coupling to various insoluble carriers. Both immobilized enzymes retained partial enzyme activities, when immobilized through phenolic groups and were stable for at least two months.

Index Entries: Restriction endonucleases; endonuclease *Pvu*II; endonuclease *Hin*dIII; endonucleases *Pvu*II and *Hin*dIII, glutaraldehyde; carbodiimide; *N*-ethylmaleimide; immobilization.

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INTRODUCTION

Type II restriction endonucleases are widely used in analysis and manipulation of DNA molecules (*1,2*). These enzymes recognize and cleave DNA at a limited number of recognition sites (*3,4*). Immobilization of restriction endonucleases, which permits stabilization, removal of the enzyme from the reaction mixture in active form, and possible reuse over several times, could facilitate their utilization. Furthermore, the immobilization of the endonucleases also permits the study of the effect of insolubilization on their activity and specificity.

The methods of immobilizing enzymes are divided into five groups: adsorption, cross-linking, entrapment, microencapsulation, and covalent coupling (*5,6*). Only low molecular weight substrates can diffuse and reach cross-linked, microencapsulated, or included enzymes easily, whereas covalent linkage of enzymes to insoluble carriers offers greater accessibility of macromolecular substrates. Covalent linkage involves the formation of covalent bonds between reactive groups present on the enzyme and those at the surface of the insoluble carrier. Covalent binding must be carried out via functional groups on the enzyme, which are nonessential for the catalytic activity.

In order to determine which functional groups on the endonuclease are essential for catalytic activity, different approaches have been employed. The most widely used approach has been to study the effect of modification of functional groups by several protein-modifying reagents on the enzyme activity (*7–12*).

Few methods for the immobilization of restriction endonucleases have been described (*10,13,14*). *Eco*RI and *Bam*HI were first immobilized on cyanogen bromide-activated Sepharose-4B (*13*). *Eco*RI, *Bam*HI and *Hin*dIII also were immobilized by adsorption on tritylagarose (*14*). *Pst*I was immobilized in agarose (*10*).

In the present paper, we have investigated the effect of several protein-modifying reagents [glutaraldehyde (GA), carbodiimide, and *N*-ethylmaleimide (NEM)] on *Pvu*II, *Hin*dIII, and other endonucleases in order to determine the functional groups essential for the catalytic activity. We have also described the preparation and properties of immobilized *Pvu*II and *Hin*dIII endonucleases. Immobilized endonucleases were prepared by covalent coupling of enzymes, by three different types of functions (amine, carboxyl, and phenolic), to various insoluble carriers, such as: nylon, *p*-aminobenzoyl cellulose, gelatin membrane, and Sepharose.

MATERIALS AND METHODS

Materials

*Hin*dIII, *Pvu*II, and all other restriction endonucleases were purchased from New England Biolabs Inc. pBR322, *Hin*dIII linker, and *Col*EI

DNA were from Boehringer Mannheim. *N*-ethylmaleimide, glutaraldehyde, and carbodiimide were obtained from Sigma. *p*-amino benzoyl cellulose (*p*-ABcellulose) was obtained from Serva.

Enzyme Essays

The reactions of *Pvu*II and *Hin*dIII endonucleases were carried out in the following incubation media: 6 m*M* Tris-HCl, pH 7.4, 6 m*M* $MgCl_2$, 50 m*M* NaCl, and 6 m*M* 2-mercaptoethanol. Reaction media containing immobilized enzyme were gently stirred with a tilting agitator at 37°C.

Chemical Reagent Incubations

The studies on incubation of restriction endonucleases with GA, carbodiimide, and NEM were performed by incubating 10 U of the enzyme in 10 μL of appropriate buffer with the chemical reagent at 25°C. Aliquots (2 μL) were withdrawn at desired time intervals to test the residual activity at 37°C in standard conditions of the reaction.

Gel Electrophoresis

Electrophoresis of DNA was performed on horizontal slab gels of 1% (w/v) agarose. DNA was visualized by fluorescence in the presence of ethidium bromide and photographed.

Diazotization of p-ABcellulose

p-ABcellulose (100 mg) was suspended in 2 mL cold 2*N* HCl, and the mixture was further cooled in an ice batch. To the cold preparation, 10–20 mg of solid $NaNO_2$ were added, and the reaction mixture was stirred for 30 min in a cold room. After 30 min, the derivative (diazotizable *p*-ABcellulose) was removed on a suction filter and washed with cold water.

Coupling of Endonucleases to the Diazotizable p-ABcellulose

Diazotizable *p*-ABcellulose (20 mg) was suspended in 1 mL phosphate buffer, pH 8.5, 50% glycerol. Five-hundred units of enzyme were added, and the reaction mixture was left, gently stirred, overnight at 4°C. After washing with cold water and 1*M* KCl to remove noncovalently coupled enzyme, the remaining activated groups in the matrix were blocked by immersion of the derivative in 0.05*M* histidine, pH 8.0, for 1 h.

RESULTS

Effect of GA on PvuII and HindIII Endonucleases

In order to show if *Pvu*II and *Hin*dIII endonucleases contain reactive amino groups essential for the enzyme activities, the endonucleases

were incubated with GA at 25°C in 20 m*M* phosphate buffer, pH 6.8, containing 10% glycerol. Preincubation with 0.4% GA at 25°C for 5 min caused a total inhibition of *Pvu*II and *Hin*dIII (results not shown). At lower concentrations, the inhibitory action of GA was increased with increasing incubation time (Fig. 1). At 0.03% of GA, partial digestion of pBR322 was obtained after 5 min of incubation of *Hin*dIII with GA. At 0.01%, partially digestion fragments began to appear after 15 min of GA incubation (Fig. 1). Since GA essentially reacts with amino groups of lysine residues (*15–17*), the specific inactivation of the endonucleases, *Pvu*II and *Hin*dIII could be from modification of lysine residues essential for catalytic activities. Methyl acetimidate, which reacts specifically with lysine residues, was also found to inactivate the endonucleases *Pvu*II and *Hin*dIII (results not shown).

The endonucleases *Ava*I, *Ava*II, *Pst*I, *Eco*RI, *Bst*I, *Hin*cII, *Bgl*I, and *Sal*I, treated in the same manner, but with 0.4% GA, were completely

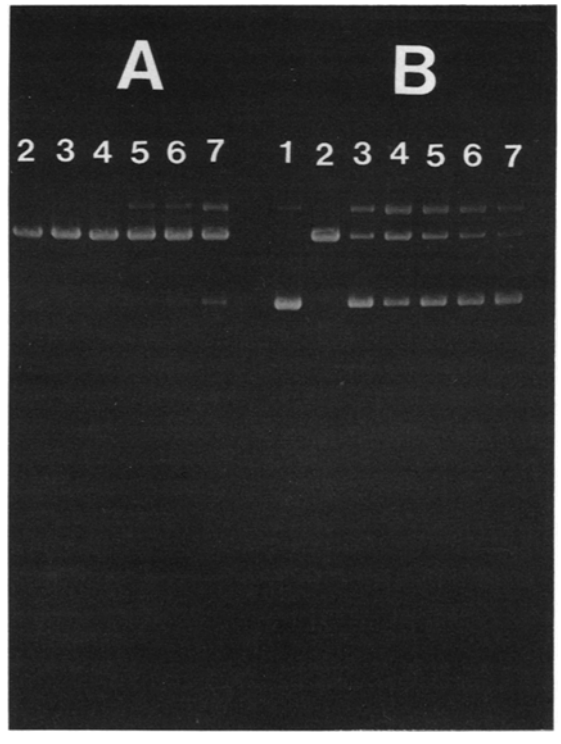

Fig. 1. Effect of glutaraldehyde on the activity of *Hin*dIII endonuclease. Ten units of *Hin*dIII endonuclease were incubated in 10 μL (20 m*M* phosphate buffer, pH 6.8, 10% glycerol) containing 0.01% (A) or 0.03% GA (B) at 25°C. Samples (2 μL) were removed from the incubation mixtures at different intervals, and the residual activities were tested in 20 μL standard buffer containing 0.5 μg pBR322 for 1 h at 37°C. GA incubations were: (2) 0; (3) 5; (4) 10; (5) 15; (6) 20; and (7) 30 min; and (1) pBR322 DNA standard.

inactive after 15 min. The effect of GA on *Bam*HI, *Hin*dIII, *Eco*RI, and *Tth*111I were also studied by Olszewski and Wasserman (*12*). They showed that the four endonucleases exhibited differential sensitivity to inactivation.

Experiments were also carried out by incubating *Pvu*II and *Hin*dIII with GA in the presence of pBR322 DNA. pBR322 DNA contains 1 *Pvu*II standard site (CAGCTG), 16 different *Pvu*II* sites (*18*), 1 *Hin*dIII standard site (AAGCTT), and more than 8 *Hin*dIII* different sites (*19*). We found that DNA concentrations ranging from 0.05 to 0.5 μg/μL do not protect the *Pvu*II and the *Hin*dIII endonucleases from inactivation by high concentrations of GA.

Effect of Carbodiimide on PvuII *and* HindIII *Endonucleases*

When preincubated at 25°C, the restriction endonucleases *Pvu*II and *Hin*dIII were affected very little by the low carbodiimide concentrations (results not shown). However, at higher concentrations, carbodiimide caused a significant inhibition of *Pvu*II and *Hin*dIII endonucleases. Figure 2 shows that the activities of both enzymes are decreased with increasing time of incubation. The inhibition is more pronounced with *Pvu*II than with *Hin*dIII. Preincubation for 1 h with 100 m*M* carbodiimide caused total inhibition in *Pvu*II and partial inhibition in *Hin*dIII activity.

With *Hin*dIII enzyme, an appreciable amount of form II DNA intermediate (open circular form) was produced. The easy detection of open circular DNA in the reaction mixture shows that the rate of hydrolysis of the first phosphodiester function is higher than that of the second one located on the second strand inside the same restriction site.

Treatment of *Pst*I and *Bgl*I with 100 m*M* carbodiimide for 15 min caused total loss of enzyme activities for the cleavage of pBR322 DNA, whereas there was only partial loss of the *Bst*I activity after 1 h of carbodiimide incubation (results not shown).

Effect of NEM on PvuII *and* HindIII *Endonucleases*

The carbodiimide used is 1-cyclo-hexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene sulfonate. This reagent reacts preferentially with carboxyl groups and also with tyrosine and cysteine residues. (*20*).

In order to determine whether the inactivation of *Pvu*II and *Hin*dIII endonucleases by the carbodiimide is caused by sulfhydryl modification, the effect of NEM, which reacts with sulfhydryl groups (*21*), on these enzymes was studied. Preincubation for 1 h with 100 m*M* NEM caused slight inhibition in *Pvu*II activity, but had no effect on the activity of *Hin*dIII enzyme.

Preincubation with 100 m*M* NEM (1 h) caused a total inhibition in *Bgl*I, but had no effect on *Bst*I activity. In contrast to the results obtained by Nath (*9*), a 5-min preincubation with 100 m*M* NEM caused slight inhibition of *Pst*I activity, which became evident after 1 h of preincubation.

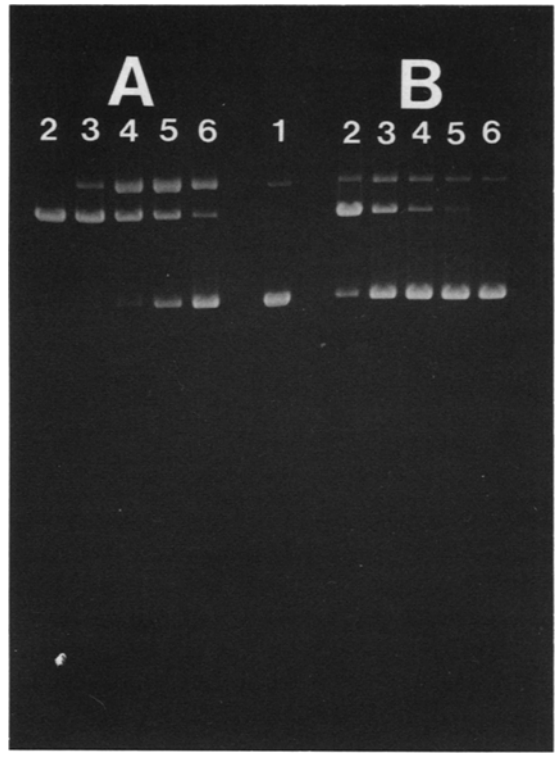

Fig. 2. Effect of carbodiimide on the activities of *Hin*dIII and *Pvu*II endonucleases. Ten units of *Hin*dIII (A) or *Pvu*II (B) were incubated in 10 μL, 60 m*M* triethanolamine pH 7, containing 100 m*M* carbodiimide. Samples (2 μL) were removed from the incubation mixtures at different intervals, and the residual activities were tested. Carbodiimide incubations were: (2) 15; (3) 30; (4) 45; (5) 60; and (6) 120 min; and (1) pBR322 DNA standard.

The above results show that the inactivation of *Hin*dIII and *Bst*I by carbodiimide is not caused by sulfhydryl modification.

Protection of HindIII *and* PvuII *from Carbodiimide Inactivation*

Experiments were also carried out in order to determine the effect of carbodiimide on *Pvu*II and *Hin*dIII activities in the presence of Mg^{2+} or DNA (pBR322, *Col*EI, *Hin*dIII linker). All inactivation experiments were carried out by incubating the enzymes for 60 min with 100 m*M* carbodiimide in the presence of DNA or/and Mg^{2+}. The presence of Mg^{2+} cation in the incubation mixture was found to protect the enzymes considerably from carbodiimide inactivation (Fig. 3, track 4). The inactivation of *Hin*dIII decreases with increasing Mg^{2+} concentration (results not shown).

*Pvu*II and *Hin*dIII enzymes could not be protected by the DNA against inactivation by carbodiimide. The presence of *Hin*dIII linker in the carbodiimide incubation increases the inactivation of *Hin*dIII enzyme

Fig. 3. Effect of carbodiimide on the activity of *Hin*dIII in the presence of Mg^{2+}, DNA. Ten units of *Hin*dIII endonuclease were incubated with 100 m*M* carbodiimide in 10 μL incubation mixture, containing: (2) no DNA, no divalent cation; (3) 0.1 μg/μL *Hin*dIII linker; (4) 6 m*M* Mg^{2+}; and (5) 0.1 μg/μL *Hin*dIII linker, 6 m*M* Mg^{2+}. After 1 h incubation at 25°C, aliquots were removed, and the residual activities were assayed at 37°C. (1) undigested pBR322 DNA.

(Fig. 3, track 3). The rate of inactivation increases with increasing DNA concentrations. However, protection from carbodiimide inactivation is more enhanced when both Mg^{2+} and DNA are present in the incubation mixture (Fig. 3, track 5). Similar results were obtained when pBR322 or *Col*EI DNA were used. These data suggest the presence of carboxyl groups essential for *Hin*dIII and *Pvu*II activities.

Immobilization of PvuII *and* HindIII

In order to study the activity of the immobilized restriction endonucleases, linearized pBR322 was used as substrate. *Eco*RI-linearized pBR322 was used as substrate with immobilized *Pvu*II. *Pvu*II and *Eco*RI cleave pBR322 at positions 2067 and 4282, respectively. Each enzyme converts superhelical pBR322 DNA to linear DNA. Double digestion of pBR322 with *Eco*RI and *Pvu*II gives two fragments of 2069 and 2293 base pair lengths, respectively (Fig. 4, track 3).

*Pvu*II and *Hin*dIII endonucleases were first immobilized on cyanogen bromide-activated Sepharose-4B (*13*) and on gelatin membranes

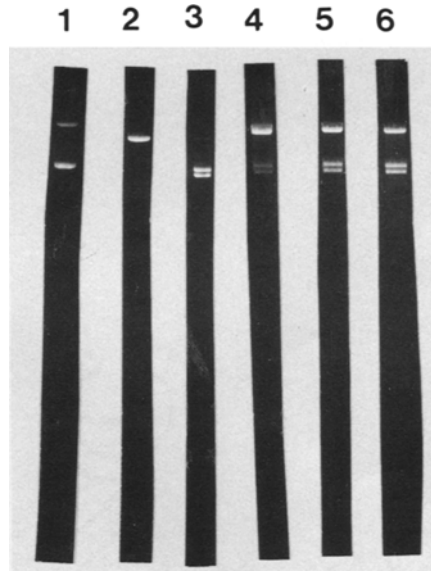

Fig. 4. Cleavage pattern of DNA with *Pvu*II immobilized on *p*-ABcellulose. The reaction mixture contained 4 μg of *Eco*RI-linearized pBR322 DNA and the immobilized *Pvu*II endonuclease (500 U) in 160 μL of incubation buffer (6 m*M* Tris-HCl, pH 7.4, 6 m*M* $MgCl_2$, 50 m*M* NaCl, and 6 m*M* 2-mercaptoethanol). Incubation was carried out at 37°C. Samples (20 μL) were removed from the incubation mixture at the following time intervals: (4) 30; (5) 120; and (6) 240 min. (1) undigested pBR322 DNA; (2) *Eco*RI-linearized pBR322 DNA; (3) pBR322 hydrolyzed by both free *Eco*RI and *Pvu*II endonucleases.

using GA, according to a previously described method (*22*). The endonucleases *Pvu*II and *Hin*dIII were also immobilized by cross-linking with serum albumin, using GA as a bifunctional reagent under various technical conditions, to produce membranes or soluble polymers (*23,24*). The above techniques involve reaction groups on solid support with amino groups of the enzyme.

*Pvu*II and *Hin*dIII were also coupled covalently with their carboxyl groups to polyisonitrile nylon, according to a previously described method (*25*), and to amino-ethyl cellulose. The immobilized enzymes did not show any detectable enzyme activity. These results and those described above indicate the presence of reactive amino and carboxyl groups essential for catalytic activity.

Finally, *Pvu*II and *Hin*dIII endonucleases were immobilized on diazotizable *p*-ABcellulose (*26*). In this technique, coupling was effected through azo bonds mainly with tyrosine and histidine residues on the enzymes. With immobilized *Pvu*II and *Hin*dIII endonucleases, ranging from 50 to 500 U, a partial cleavage of DNA was obtained. Figure 4 shows that the *Eco*RI-linearized pBR322 is not completely cleaved by immobilized *Pvu*II endonuclease (500 U) even after prolonged incubation. This is essentially because of diffusional and steric limitations, since pBR322

DNA substrate is very large. Another and more plausible possibility is that: with soluble preparation, the endonuclease binds nonspecifically to double-stranded DNA, then protein proceeds along the DNA macromolecule until it recognizes the right sequence, and the specific complex DNA-enzyme is formed (*27,28*). This linear diffusion, probably, is of great importance in localizing the recognition sites. However, with immobilized preparation, enzyme is covalently coupled to the carriers. Consequently, coupled enzyme cannot diffuse along the DNA and in most cases cannot reach the specific site.

DNA fragments produced by immobilized *Pvu*II endonuclease were identical to those produced with free enzyme. Analysis of the fragment patterns, after double digestion of *Eco*RI-linearized pBR322 DNA by immobilized *Pvu*II enzyme and other free endonucleases (*Ava*I, *Sal*I . . .), has shown that the immobilized enzyme recognizes and cleaves the standard *Pvu*II site CAGCTG.

The immobilized *Pvu*II and *Hin*dIII were found to be very stable. No decrease in activity was detected after storage for 2 mo, and these immobilized enzymes can be reused several times.

Effect of Insolubilization on PvuII* *and* HindIII* *Activities*

As described in the previous papers (*18,19*), in altered reaction environments [addition of dimethyl sulfoxide (DMSO)], soluble *Pvu*II (*18*) and *Hin*dIII (*19*) showed relaxed specificities and cleaved DNA at both the standard and a number of additional sites. Under standard conditions, *Pvu*II and *Hin*dIII recognize the sequences CAGCTG and AAGCTT, respectively. In the presence of DMSO, the enzymes recognize sites that differ by a single position on the hexanucleotide. For *Pvu*II enzyme, we found that any base can be substituted at any position of the hexanucleotide site. In a previous paper (submitted results), we have shown that with 30% DMSO, relaxed sites appeared to be cleaved at the same rates. Thus, cleavages can occur at additional sites before the DNA is completely cleaved in the standard site.

Similar experiments were done to study the effect of DMSO on the specificity of immobilized *Pvu*II and *Hin*dIII endonucleases. Both *Pvu*II and *Hin*dIII immobilized on *p*-ABcellulose do not show relaxed specificities. The immobilized enzymes were found to cleave pBR322 DNA only at standard sites. On the other hand, at higher DMSO concentrations, no activity loss is noticed when compared to the native enzyme.

DISCUSSION

Results of this study strongly show that:

(a) Glutaraldehyde, which reacts essentially with lysine residues, inactivates all restriction endonucleases used; GA is a bifunctional reagent (*29*). The specific inactivation of the

endonucleases with GA is not only related to modification of amino groups essential for catalytic activity, but is also attributed to conformational change in the enzyme structure. The direct involvement of amino groups essential for catalytic activity of *Pvu*II and *Hin*dIII endonucleases was demonstrated by the chemical modification of the endonucleases with methyl acetimidate. It seems likely, as was suggested by Helene (*30*), that essential lysine residues are involved in electrostatic interactions with the phosphate groups of the DNA.

(b) Carbodiimide, which reacts preferentially with carboxyl groups, also inactivated all restriction endonucleases used. *Pst*I and *Bgl*I are the most sensitive enzymes, whereas *Hin*dIII and *Bst*I are the most stable. This specific inactivation may be related to modification of carboxyl groups essential for catalytic activity. Mg^{2+} cation was found to protect all the endonucleases used, from the carbodiimide inactivation. These experiments provide evidence that carboxyl groups could bind the Mg^{2+} cation, as does glutamate-72 in carboxypeptidase (*31*).

(c) Experiments carried out by incubating the restriction endonucleases with NEM, which reacts with sulfhydryl groups, showed that *Pvu*II, *Pst*I, and *Bgl*I are sensitive to the action of the sulfhydryl group inhibitor, but the inhibition appeared to be partial. *Hin*dIII and *Bst*I are not sensitive to NEM action, thus, the inactivation of these enzymes with carbodiimide is not caused by sulfhydryl modification.

DNA, such as pBR322, *Col*EI, and *Hin*dIII linker, did not protect *Pvu*II and *Hin*dIII from inactivation by high concentrations of glutaraldehyde and carbodiimide. This demonstrates the presence of amine and carboxyl groups near the DNA binding site essential for catalytic activity. Strictly speaking, our results do not completely rule out the presence of essential amine and carboxyl groups in the DNA binding site.

*Pvu*II and *Hin*dIII immobilized through amine and carboxyl groups do not show any detectable activity. On the other hand, *Pvu*II and *Hin*dIII immobilized on pABcellulose through phenolic groups retain partial activity. However, since several restriction endonucleases appeared to be resistant to the action of *N*-ethylamaleimide, we propose to immobilize these enzymes with their SH-groups.

Several factors may affect the kinetics of immobilized enzymes and consequently lead to enzyme activity decrease: (a) interaction with matrix could result in a modification of the enzyme conformation (*32*); (b) steric hindrances (*33,34*), in fact, the decrease in enzyme activity observed with high-molecular-weight substrates can, in most cases, be related to steric limitations on the accessibility of substrates (*35*); (c) external and internal

diffusional limitations (*36*); and (d) partition effects between the micro- and macroenvironments (*35,37*).

According to the results obtained in this paper, the absence or decrease in immobilized enzyme activity is attributed to several factors: (a) modification of functional groups at or near the active site of endonucleases; and (b) diffusional and steric limitations, since our substrate (pBR322) is very large (*38*).

Finally, when the endonucleolytic reactions are carried out in solutions containing DMSO, alteration of the endonuclease specificity is observed with soluble *Pvu*II and *Hin*dIII enzymes, although no additional activity was observed with immobilized endonucleases. This suggests that the relaxation of the specificity is not related to an additional activity in the enzymatic preparation masked under standard conditions, but to changes in conformation of the quaternary structure of the active form.

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