

# Subunit assembly modulates the activities of the Type III restriction–modification enzyme PstII *in vitro*

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## ABSTRACT

**We demonstrate that, like other Type III restriction endonuclease, PstII does not turnover such that a DNA substrate is only fully cleaved at a Res<sub>2</sub>Mod<sub>2</sub>-to-site ratio of ~1:1. However, unlike other Type III enzymes, the cleavage rate profiles varied with protein concentration: using 5 nM DNA and 25 nM PstII, approximately half of the DNA was cut at a fast rate while the remainder was cut 24 times more slowly; in comparison, with 100 nM PstII cleavage occurs at a single fast rate. The inclusion of the methyl donor S-adenosyl methionine does not alter the rates with 100 nM PstII but with 25 nM PstII the reaction stopped after completion of the initial fast cleavage phase owing to methylation. Concentration-dependent rates were also observed in methylation assays: at 100 nM PstII, a single slow rate was measured while at lower PstII concentrations both fast and slow rates were measured. We propose a model in which the intact Res<sub>2</sub>Mod<sub>2</sub> complex favoured at high PstII concentrations is a fast endonuclease/slow methyltransferase while the various subassemblies which coexist at lower concentrations are fast methyltransferases. A potential role for disassembly in control of restriction activity *in vivo* is discussed.**

## INTRODUCTION

To function as an efficient restriction–modification (RM) system, the newly characterized Type III RM enzyme PstII (1) must be able to accurately control its methyltransferase and endonuclease activities so that double strand breaks are introduced into infecting phage DNA yet not into the host DNA. Just how accurate is this control? It is well established that some RM enzymes can be transferred to recipient cells in

which the chromosomal DNA is unmodified (2,3). It is also well established that a temporary loss of restriction proficiency, called restriction alleviation (RA), can occur in response to DNA damage (4–6). This suggests that temporal and/or spatial enzyme control is being exercised. For the heterooligomeric Type I and III enzymes, which catalyse both RM functions within the same complex (4,7), this poses two related problems: what is to prevent a multifunctional complex cutting ‘self’ DNA before modifying it, and what is to prevent the same multifunctional complex modifying ‘foreign’ DNA before cleaving it? The relatively simple answer is that modulation of the subunit composition of a Type I or III complex determines whether a DNA site is modified or cleaved (8).

How do bacterial cells regulate the protein assembly of Types I and III RM enzymes? In neither system is there any evidence of transcriptional regulation (3,9–11). For some Type I enzymes (e.g. EcoKI and EcoAI), disruption of a DNA cleavage event can be triggered by an ATP-dependent protease complex, ClpXP, in response to RA signals (4–6). The protease is targeted to endonucleases on genomic DNA which are in the process of DNA cleavage (5); proteolysis of the HsdR subunit during DNA translocation prevents the reaction from proceeding to completion. General RA independent of the protease may also occur due to the packaging and condensation of the genomic DNA, conditions that disfavour translocation (12). ClpXP does not target endonuclease complexes translocating on phage DNA—this may be because translocation is unencumbered on the naked bacteriophage genome and is too rapid for proteolysis to occur (12). There is also some evidence that Type III enzymes could be controlled by RA (13). For plasmid-borne Type I enzymes the assembly of a fully active endonuclease is limited by the affinity of the HsdR subunits for the core complex (10,14, 15). This may allow the methyltransferase activity to act in advance of the endonuclease activity (8). Although this can explain transmission of RM activity into new hosts, it can less readily explain RA phenomena. For the Type III enzymes EcoP15I and EcoPI there is evidence that the availability of the endonuclease subunit Res is controlled both at the

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translational level and by the associated Mod subunit (11). EcoP15I and EcoPI are freely transferable from cell to cell by phage infection, conjugation and transformation [(11) and references therein], and this may be due to these controls on Res. In contrast, horizontal transfer of the Type III enzyme StyLTI results in cell death (16). It, therefore, appears that different Type III enzymes may utilize different mechanisms, or levels, of RM control.

In the accompanying paper (1) we show that PstII has endonuclease activities characteristic of a Type III enzyme and that it can function *in vivo* as an RM system. We have also observed that PstII is difficult to establish in a new cell line (when a cell line is transformed using a high copy number plasmid expressing the PstII operon from natural promoters). Given that the PstII is assembled from Res and Mod subunits, does the protein composition of the complex affect the relative RM activities? From the data in this study we suggest that while the intact Res<sub>2</sub>Mod<sub>2</sub> tetramer is a fast endonuclease and slow methyltransferase, thereby favouring DNA cleavage, subassemblies of PstII in which the Res subunits have dissociated are more efficient methyltransferases. DNA cleavage by these lower molecular weight species may only occur if sufficient HsdR associates to form a Res<sub>2</sub>Mod<sub>2</sub> tetramer before methylation occurs. This dynamic association of Res and Mod might play a key role in the control of PstII activity *in vivo*.

## MATERIALS AND METHODS

### DNA and proteins

The construction and preparation of the DNA substrates and the purification of PstII are described in the accompanying paper (1).

### Analytical gel filtration

Gel filtration was carried out using a 24 ml Superose 6 column plumbed into an AKTA FPLC (Amersham Biociences). The system was pre-equilibrated at 0.4 ml/min with either NEB 4 (20 mM Tris-acetate, pH 7.9, 10 mM Mg acetate, 50 mM potassium acetate and 1 mM DTT) or NEB 4 plus 0.02% (v/v) Triton X-100. A 100 µl loop was filled with protein sample in storage buffer [19 mM Tris-HCl, pH 8.0, 0.4 mM EDTA, 150 mM NaCl, 0.4 mM DTT and 50% (v/v) glycerol] supplemented where necessary with 0.02% (v/v) Triton X-100. Protein was eluted with the equilibration buffer at 0.4 ml/min and was monitored by measuring the A<sub>280</sub> using an averaging time (sensitivity) setting of 1.3 s. Fractions (333 µl) were collected and aliquots analysed by SDS-PAGE. Gels were stained using Bio-Safe Coomassie (Bio-Rad), destained in water overnight and digital images captured on a Kodak Image Station 440CF without editing and using a linear intensity scale. The percentage of Res and Mod was calculated from the relative band intensities using ImageQuant software (Molecular Dynamics) and assuming a linear relationship between protein size and dye intensity. The Superose column was calibrated using at least five molecular weight standards.

### Cleavage reactions

Cleavage reactions contained 5 nM <sup>3</sup>H-labelled pLJP11b and 4 mM ATP in NEB 4 supplemented with 100 µM AdoMet

and/or 0.02% (v/v) Triton X-100 where indicated. Cleavage was initiated by addition of PstII (concentrations are given in the figure legends/text). Reactions were carried out at 37°C and either left for 1 h or aliquots removed at the indicated time points. Cleavage was stopped by the addition of 0.5 vol of STEB buffer [0.1 M Tris-HCl, pH 7.5, 0.2 M EDTA, 40% (w/v) sucrose and 0.4 mg/ml bromophenol blue]. The DNA substrate and product fragments were separated by agarose gel electrophoresis and the percentage of DNA in each band was evaluated by scintillation counting as in Sears *et al.* (1).

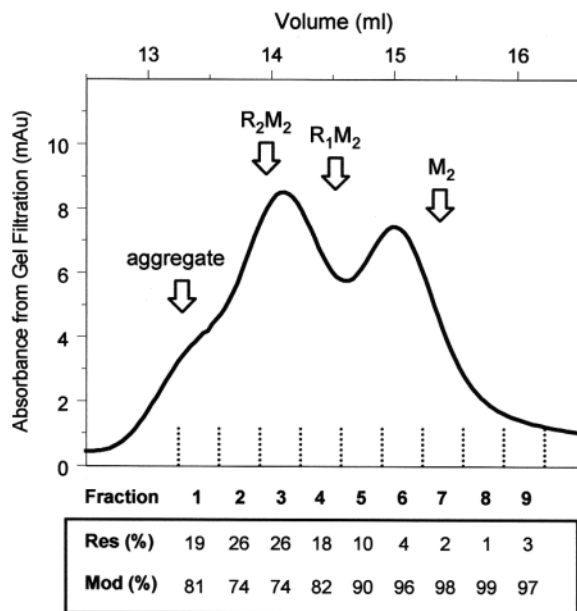
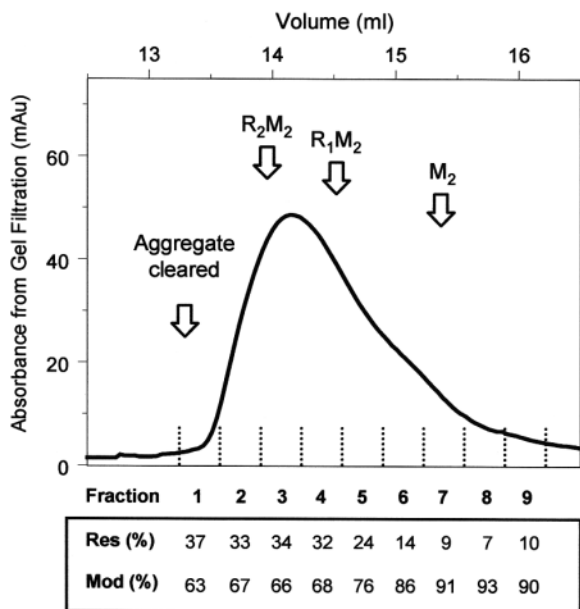
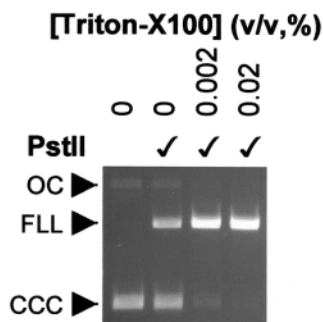
### Apparent methylation rates from inhibition of cleavage

<sup>3</sup>H-labelled pLJP11b (5 nM) was pre-incubated with varying concentrations of PstII in NEB 4 plus 0.02% (v/v) Triton X-100 supplemented with 100 µM AdoMet. Methylation was allowed to proceed at 37°C. At the time points indicated 20 µl aliquots were removed from the master reaction and mixed with ATP and the appropriate amount of PstII so that the final reaction conditions were 129 nM PstII mix and 4 mM ATP. These reactions were then incubated for a further hour at 37°C and cleavage stopped by the addition of STEB as above. The DNA substrate and product fragments were separated by agarose gel electrophoresis and the percentage of DNA in each band evaluated by scintillation counting.

## RESULTS

### The subunit stoichiometry of PstII and the effect of Triton X-100

In the accompanying paper we describe the purification of PstII, a new Type III enzyme from *Providencia stuartii* (1). The ratio of Res to Mod subunits in the purified PstII samples was estimated from densitometry of SDS-PAGE gels as ~1:3 (Materials and Methods). All protein concentrations are quoted in terms of this ratio, although this is not considered an active species but as a 'PstII mix'. Previous analysis of EcoPI and EcoP15I demonstrated that for each enzyme the Res and Mod subunits were in equal proportion and that the complexes assembled into stable tetramers with the subunit stoichiometry Res<sub>2</sub>Mod<sub>2</sub> (17). No evidence for intermediates in the assembly pathway (e.g. Res<sub>1</sub>Mod<sub>2</sub>) was obtained. Maximum endonuclease activity only arises when each site of a pair of indirectly repeated sites is bound by a Res<sub>2</sub>Mod<sub>2</sub> tetramer (18). The most probable reason for the under-representation of Res in our PstII samples is that during anion exchange chromatography on DEAE-Sepharose (1), the Res subunit eluted across a broader range of Cl<sup>-</sup> ion concentrations than Mod (data not shown). The fractions that were pooled for further purification were those which contained both Res and Mod; the samples containing Res alone were contaminated with significant levels of co-eluted proteins and were discarded. Therefore, the purification protocol depleted the preparation of Res subunits. Depletion of an RM complex during purification has also been observed with the Type I enzyme EcoR124I (14). When purified from cells transformed with the complete EcoR124I operon, the resulting purified protein had only one HsdR subunit per methyltransferase complex. Since two HsdR subunits are needed for cleavage, full endonuclease activity was only observed with an ~7-fold molar excess of

**(A) Buffer NEB 4 alone****(B) Buffer NEB 4 + Triton-X100****(C)**

EcoR124I over DNA sites, where additional HsdR subunits are provided from the excess of unbound complexes in solution (14,15,19).

To investigate further the subunit composition of our PstII preparations, we analysed samples using analytical gel filtration under one set of volumetric and flow conditions. The most rigorous method for determining subunit assembly is sedimentation equilibrium analysis (17). However, for technical reasons we were unable to use this technique with our PstII preparations. There are a number of limitations in the interpretation of gel filtration data, particularly in relating the species observed during chromatography to those present under reactions conditions. However, our goal with these experiments was to observe the changes in subunit assembly as a function of conditions and to try to relate the observations in general terms to changes in activity in solution; i.e. does activity change as the subunit assembly changes?

Scouting experiments indicated that the endonuclease activity of PstII required  $Mg^{2+}$  ions and ATP, CTP or GTP (1) but was not affected by the composition of monovalent ions in the buffer (data not shown). We used a standard  $K^{+}$  ion-based buffer (NEB 4; Materials and Methods) for all subsequent experiments described here and in the accompanying paper (1). An aliquot of 100  $\mu$ l of a 1.2  $\mu$ M PstII mix sample was loaded onto a 24 ml Superose 6 column (Amersham Biosciences) pre-equilibrated in NEB 4 and the sample eluted at 0.4 ml/min. Elution was monitored by continuous measurement at  $A_{280}$  and by SDS-PAGE of 333  $\mu$ l fractions (Figure 1A). The percentage of Res and Mod in each fraction was estimated by gel densitometry (Materials and Methods and figure legend). The PstII sample eluted across a broad range of apparent molecular weights ( $\sim$ 600 to  $\sim$ 80 kDa) with two distinct peaks at  $\sim$ 330 and  $\sim$ 165 kDa (Figure 1A). The  $K_{av}$  of the main peak (at  $\sim$ 14 ml) coincides almost exactly with that calculated for a  $Res_2Mod_2$  complex (343 999 Da). The fractions corresponding to this peak (e.g. 2 and 3) had a Res/Mod ratio of  $\sim$ 1:3. It may be difficult to capture a clear-cut  $Res_2Mod_2$  complex under these conditions owing to mass transport effects (dissociation of Res may occur during elution).

The apparent molecular weight of the second peak lies halfway between the calculated values for a  $Res_1Mod_2$  complex (235 722 Da) and a  $Mod_2$  methyltransferase complex (127 446 Da). However, the fractions corresponding to this peak (e.g. 6) had significantly more Mod than Res (10- to 20-fold), which suggests that the second peak may represent the  $Mod_2$  complex eluting with an anomalous  $K_{av}$ . The Mod subunits of both the EcoPI and EcoP15I enzymes have been shown to assemble

**Figure 1.** Subunit assembly of PstII and the effect of Triton X-100. Gel filtration of PstII was carried out in (A) NEB 4 or (B) NEB 4 plus 0.02% Triton X-100, as described in the text and in Materials and Methods. Elution profiles are shown from 12.5 to 16.5 ml following PstII injection. Fractions 1–9 (333  $\mu$ l) were collected and aliquots analysed by gel densitometry following SDS-PAGE assuming a linear relationship between staining intensity and polypeptide size. The percentage of Res and Mod in each fraction is indicated. With the exception of the aggregate, the open arrows indicate ideal  $K_{av}$  values calculated from the theoretical MW of each species ( $M = Mod$ ,  $R = Res$ ) and using the calibration curve specific to the individual Superose 6 column. (C) The effect of different concentrations of Triton X-100 on DNA cleavage by PstII: 5 nM pLJPI1b (10 nM sites) were incubated for 1 h in NEB 4 with 15 nM PstII mix as indicated and with Triton X-100 at the concentrations shown.

as active dimers (20,21). A smaller peak was also observed eluting across a broad range of high molecular weights centred at ~520 kDa (Figure 1A). The composition of the fractions around this peak are over-represented in Mod (5-fold excess over Res). We suggest that this is an inactive aggregated state, possibly driven by self-association of the excess methyltransferase subunits.

It would appear from our gel filtration analysis of PstII in NEB 4 that there are more than two enzyme species present; a methyltransferase (Mod<sub>2</sub>) followed by several higher order assemblies with Res. Given the similarity of PstII in enzymatic terms to EcoPI and EcoP15I, we fully expect that the active PstII species on DNA is a Res<sub>2</sub>Mod<sub>2</sub> complex. We propose that under the conditions here, the endonuclease and methyltransferase forms are in dynamic equilibrium as:



Equation 1 assumes that the Res subunits assemble as monomers onto a dimeric methyltransferase. This model is based on similar models suggested for the assembly of Type I restriction enzymes (8). Type III endonuclease activity can be reinstated by mixing separate Mod and Res fractions *in vitro* (22), which suggests that assembly does not rely on protein folding and/or chaperone activity. A discrete PstII peak corresponding to the Res<sub>1</sub>Mod<sub>2</sub> intermediate was not observed (Figure 1A) although its presence may have been masked. From the relative proportions of Res and Mod in Fractions 3–5, it is perfectly possible that this species is present.

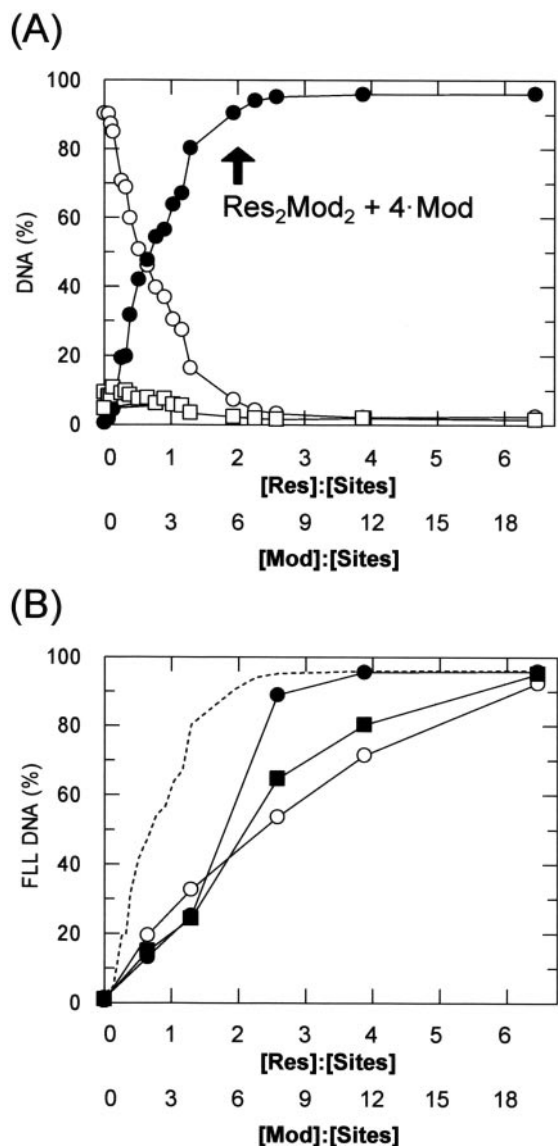
Our initial attempts to characterize the endonuclease activity of PstII using NEB 4 alone were hampered by a low apparent specific activity (data not shown). Given the gel filtration data, this is most probably due to both partial subunit assembly and protein aggregation (albeit at different concentrations of total protein). We, therefore, investigated a number of changes to our buffer conditions (e.g. adding glycerol, spermidine, detergents) to try to improve the DNA cleavage activity (data not shown). The most useful additive—that which recovered the endonuclease activity to expected levels—was Triton X-100 at a concentration of  $\geq 0.02\%$  (v/v) (Figure 1C). This detergent concentration (~0.5 mM) is well below the critical micelle concentration of Triton X-100 (~25 mM). Triton X-100 is a common additive to commercial restriction enzyme buffers and can improve endonuclease activity and specificity (23), most probably through a disruption of aggregated states (24). The effect of the detergent on the assembly of PstII was analysed using analytical gel filtration as above. A clear difference in the elution profile was observed (Figure 1B). The distribution of eluted species is narrower than seen with NEB 4 buffer alone and is dominated by a single peak with a  $K_{av}$  corresponding to a Res<sub>2</sub>Mod<sub>2</sub> tetramer. However, the relative proportions of Res and Mod in Fractions 1–4 are still not equal as would be expected for a Res<sub>2</sub>Mod<sub>2</sub> tetramer. This may represent inaccuracy in the densitometry and/or equilibrium redistribution during chromatography. There are still lower molecular weight species present, most probably corresponding to the Res<sub>1</sub>Mod<sub>2</sub> and Mod<sub>2</sub> forms (Fractions 5–9), but these are less prevalent than in Figure 1A. Addition of Triton X-100 has also disrupted the putative aggregate observed in Figure 1A.

Given a scheme such as Equation 1 in which there is a dynamic equilibrium between four protein species (Res, Mod<sub>2</sub>, Res<sub>1</sub>Mod<sub>2</sub> and Res<sub>2</sub>Mod<sub>2</sub>), the analysis of gel filtration profiles is made more difficult by associations/dissociations of the components in the gel matrix during elution. Nonetheless, adding Triton X-100 has two clear effects on PstII assembly: in stabilizing higher order assemblies that correspond, in molecular weight terms at least, with a Res<sub>2</sub>Mod<sub>2</sub> tetramer and in preventing the appearance of high molecular weight species (most probably inactive aggregates). We suggest that the improvement in DNA cleavage activity observed in Figure 1C as a function of Triton X-100 may also correspond to similar changes in the subunit interactions at the lower enzyme concentrations used in the cleavage assays. For PstII, conditions which favour the higher order structures also favour DNA cleavage. We will investigate this in more detail below. Attempts to purify PstII using buffers supplemented with Triton X-100 proved unsuccessful (data not shown). Consequently, PstII was purified in normal buffers and then analysed in NEB 4 supplemented with 0.02% (v/v) Triton X-100. Given the Res/Mod ratio of ~1:3 in the purified samples, we suggest that the equilibrium as indicated by Equation 1 will lie more to the left. Therefore, DNA cleavage will require an apparent excess of PstII to supply the requisite number of subunits per site; in same manner, assembly of active EcoR124I complexes on DNA can be driven by the association of endonuclease subunits from solution (14,15,19).

### Efficient DNA cleavage by PstII is dependent on subunit assembly

Cleavage of 5 nM pLJP11b [10 nM sites, (1)] at a range of PstII concentrations was analysed after incubation for 1 h in buffer NEB 4 plus Triton X-100 (Figure 2A). This DNA substrate contains two indirectly repeated sites and is thus a substrate for the endonuclease (1). All components except PstII were pre-incubated at 37°C. After 2 min, enzyme was added, DNA cleavage was allowed to proceed for 1 h, the reactions were stopped by the addition of EDTA and the products were analysed by gel electrophoresis (Materials and Methods).

Cleavage of pLJP11b followed a roughly linear dependence on enzyme concentration until maximum activity was achieved at a Res/site ratio of ~2:1 and a Mod/site ratio of ~6:1. Only one of the two sites was ever cleaved and the addition of further enzyme did not result in cleavage of the remaining site. No endonuclease turnover following cleavage was observed (data not shown) although ATPase activity continued regardless (1). If DNA cleavage requires the long-range interaction of two sites by separate enzyme molecules, one bound at each site, and the conditions are such that DNA–protein binding is not limiting, then the cleavage profile should saturate at an enzyme/site ratio of 1:1 (25). Addition of further enzyme should not, *per se*, inhibit or enhance the reaction. This is what we observed in a previous study using the heterotetrameric form of EcoPI (17). In contrast, if DNA cleavage requires the long-range interaction of two DNA sites by a single enzyme molecule then cleavage will saturate at an enzyme/site ratio of 1:2 (25). Addition of excess enzyme will then inhibit the reaction. This is what we observed in a previous study using the tetrameric Type II restriction enzyme SfiI (26). We have assumed above that our PstII preparations



**Figure 2.** Stoichiometry of pLJPI1b cleavage by PstII as a function of Triton X-100 and AdoMet: 5 nM DNA (10 nM sites) was incubated for 1 h with 0–129 nM PstII mix in NEB 4. Where indicated Triton X-100 was included at 0.02% (v/v) and AdoMet was included at 100  $\mu$ M. (A) Full cleavage profiles in NEB 4 plus Triton X-100. The covalently closed circular DNA substrate (CCC, open circles) was separated from the nicked intermediate (OC, open squares) and full length linear product cut at one site (FLL, closed circles) by agarose gel electrophoresis (data not shown). Fragments were excised and the percentages of the <sup>3</sup>H-labelled DNA fragments were quantified by scintillation counting. (B) Changes in the reaction profile with buffer conditions. As above, the percentage of FLL was determined in NEB 4 alone (closed circles), NEB 4 plus AdoMet (closed squares) and NEB 4 plus AdoMet plus Triton-X100 (open circles). The FLL profile from (A) is shown for comparison as a dashed line.

do not represent a single species but are a continuum of assembled states as described by Equation 1. From the relative proportions of Res and Mod (1:3), a Res<sub>1</sub>Mod<sub>2</sub> complex could readily form on DNA but to form the tetrameric species would require further PstII to be added. It would be difficult to accommodate the PstII data with a model in which a single Res<sub>2</sub>Mod<sub>2</sub> complex interacted with two sites simultaneously. Furthermore, we do not believe that the DNA is bound by a

Res<sub>2</sub>Mod<sub>6</sub> complex. The more plausible explanation of the PstII data in Figure 2A, and one which would coincide with the EcoPI data (1,17), is that separate Res<sub>2</sub>Mod<sub>2</sub> complexes must bind at each site before an interaction occurs but this will only occur under conditions where there will also be an excess of non-interacting Mod subunits. We do not believe that these subunits play any direct role in the reaction.

In Figure 1A we showed that in NEB 4 buffer alone, PstII disassembles. The efficiency of DNA cleavage is also reduced under these conditions (Figure 1C). Nonetheless, if Res subunits can be obtained from unbound PstII complexes in solution then elevating the concentration of PstII should eventually restore full activity by supporting the assembly of DNA-bound tetramers. To examine this suggestion we repeated our titration experiments using NEB 4 alone (Figure 2B). Cleavage at low enzyme concentrations was less efficient than in equivalent conditions with Triton X-100 and the data were noticeably sigmoidal in shape suggesting that a threshold is reached before active species are formed. However, by adding a sufficient excess of PstII, full DNA cleavage was restored.

The experiments in Figure 2A and B report on cleavage after 1 h of incubation but the kinetics of PstII assembly cannot be inferred from the gel filtration data in Figure 1. If assembly/disassembly were rapid then the Res<sub>2</sub>Mod<sub>2</sub> state may be visited numerous times even if it was under-occupied on average. Therefore, although 100% cleavage can be achieved at a given apparent PstII concentration, the steady-state concentration of the Res<sub>2</sub>Mod<sub>2</sub> species may actually be significantly lower. This point is addressed in the next section on the rates of DNA cleavage.

In previous assays of EcoPI, the cofactor AdoMet was shown to play three roles in a Type III reaction: as a methyl donor during methylation, as an allosteric activator of DNA-protein interactions and as a specificity factor preventing cleavage of inappropriate DNA substrates (18). However under relaxed reaction conditions (K<sup>+</sup> ion-based buffers) and using a DNA substrate with a pair of head-to-head sites, AdoMet had no effect on the cleavage profiles; maximal cleavage activity was obtained at a tetramer/site ratio of 1:1 both in the presence and absence of the cofactor (18). In contrast, the addition of AdoMet to PstII titrations altered the reaction profiles compared with those described above (Figure 2A). In NEB 4 supplemented with 0.02% (v/v) Triton X-100 and 100  $\mu$ M AdoMet, the titration was markedly linear with maximal DNA cleavage only observed upon the addition of a large excess of enzyme (Figure 2B). A similar reaction profile was observed if Triton X-100 was omitted from the buffer (Figure 2B). These results contrast with the results in the absence of AdoMet where Triton X-100 enhances the reaction (Figures 1C, 2A and 2B).

It is well established that AdoMet can inhibit Type III cleavage reactions owing to competition between methylation and cleavage activities. This is distinct from the Type I enzymes where the rate of methylation of unmodified DNA is significantly slower than the rate of DNA cleavage (4,8). For EcoPI this is not a significant problem as the stable Res<sub>2</sub>Mod<sub>2</sub> complex cuts the majority of the DNA before significant co-methylation can occur [assuming that the enzyme is added last to the *in vitro* assays (18), as also done here]. However, given that PstII most probably separates into different species, even in the presence of Triton X-100, the

possibility of co-methylation may be increased due to the presence of species with methyltransferase activities greater than their endonuclease activities. Although Triton X-100 appears to facilitate DNA cleavage by PstII, the inhibitory effect of AdoMet appears to be greater. Regardless of the detergent, as the concentration of PstII is increased then the amount of inhibition decreases. This suggests that increasing the concentration of PstII favours a form of the enzyme which cuts the DNA rather than methylating it.

### Relative cleavage/methylation rates dictates the extent of restriction

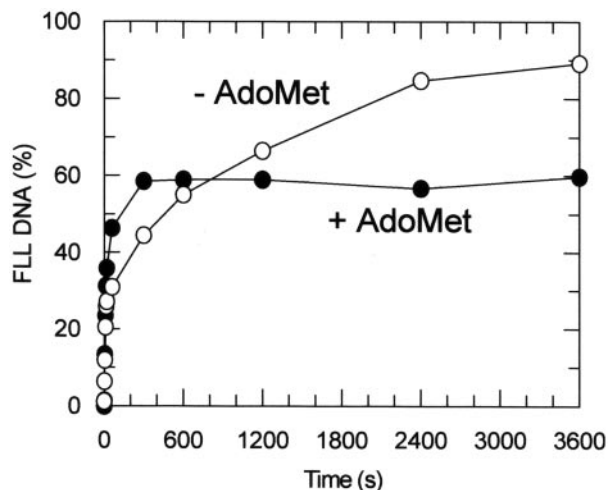
In Figure 2A we showed that at a concentration of  $\sim 1$  Res<sub>2</sub>Mod<sub>2</sub> complex per site, pLJP11b is completely cleaved after 1 h of incubation with PstII. However, if PstII exists as a mixture of different complexes then the kinetics of cleavage may reflect the relative activities of each species. To investigate this we examined the rate of cleavage of 5 nM pLJP11b (10 nM sites) using 33 nM PstII mix (Figure 3), conditions at which maximum DNA cleavage is observed after 1 h. In the absence of AdoMet, the cleavage profile as judged by the appearance of the FLL product DNA is characterized by two distinct rates with roughly equal amplitudes; an initial fast rate followed by an  $\sim 30$ -fold slower rate. Although 50% of the DNA is cut within 60 s, cleavage of the remainder of the DNA is only completed after  $>60$  min. In the presence of AdoMet, the profile becomes monophasic as the reaction is inhibited by co-methylation after completion of the initial fast phase. In additional reactions where we either raised or lowered the concentration of the PstII mix, the amplitude of the fast cleavage phase was also raised or lowered, respectively (data not shown). In the accompanying paper (1) we show that by elevating the PstII concentration to 129 nM, cleavage proceeds at a single fast rate to 100% completion independent of AdoMet. These results suggest that high PstII concentrations favour a form of the enzyme which can cleave DNA quickly

before methylation can occur. At lower enzyme concentrations however, while some complexes can also cleave the DNA at a rate substantially faster than the methylation rate, the reaction outcome for the remaining complexes is dependent upon the reaction conditions; in the presence of AdoMet, methylation and cleavage will be in competition. The slower cleavage rate observed in the absence of AdoMet in Figure 3 may reflect an intrinsically slower rate or a slow protein rearrangement/assembly step (Discussion).

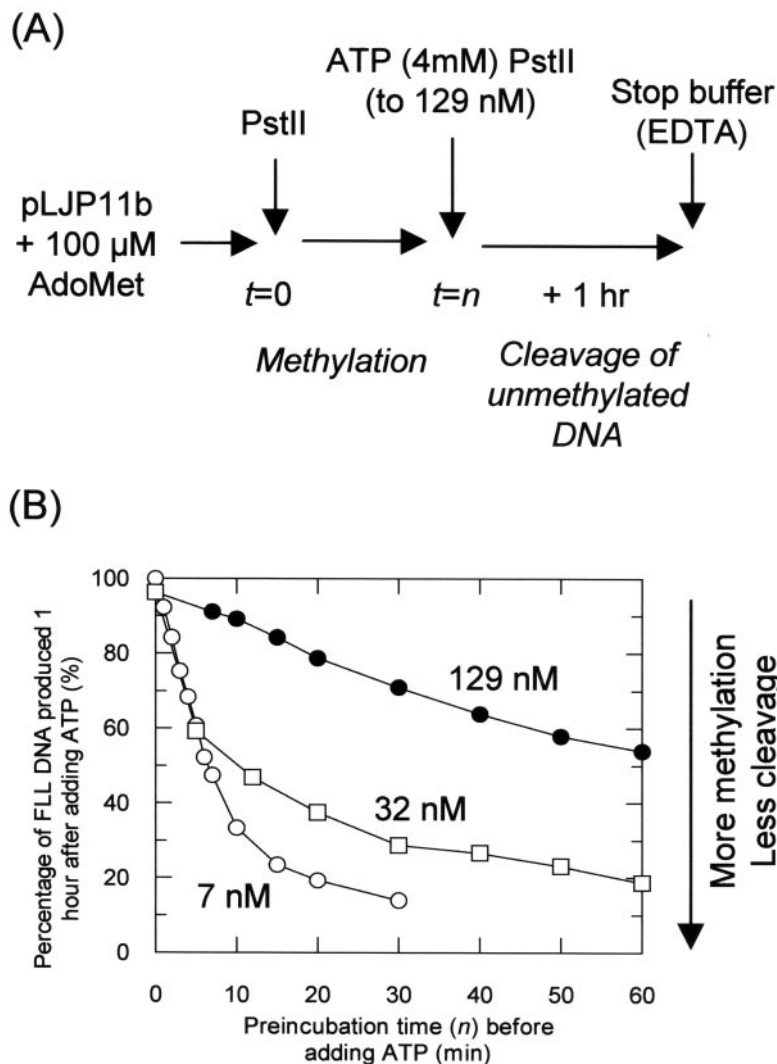
### PstII species with fast cleavage rates have slow methylation rates and vice versa

The results described above suggest that the initial rate of DNA cleavage can be modulated by changing the concentration of PstII. We, therefore, investigated the rate of DNA methylation using an indirect approach (Figure 4A). Fixed concentrations of PstII were pre-incubated with 5 nM pLJP11b for different lengths of time in the absence of ATP but presence of AdoMet. Under these first conditions, enzyme complexes with methyltransferase activity can proceed to modify the DNA while cleavage by any species is prevented. At the end of each pre-incubation period ( $t = n$ , Figure 4A), we added ATP (and extra PstII if necessary) so that the final mixtures contained  $\sim 5$  nM DNA (a mixture of methylated and unmethylated molecules), 129 nM PstII mix, 4 mM ATP and 100  $\mu$ M cofactor (a mixture of AdoMet and the cofactor product *S*-adenosyl homocysteine). The reactions were then allowed to proceed for a further hour. Under these second conditions, namely a high concentration of PstII, substrates with two unmodified sites will be rapidly cleaved (1) whilst DNA methylated at one or other site (or both) will remain intact. The amount of methylation during the initial incubation of PstII and DNA can then be judged by the amount of FLL DNA that remains at the end of the reaction: the greater the amount of FLL DNA remaining, the greater the amount of methylation that must have occurred during pre-incubation ( $t = n$ ).

Data from reactions using initial PstII mix concentrations of 7, 32 and 129 nM are shown in Figure 4B. At 129 nM PstII the co-methylation rate appears to be slow—even after 60 min pre-incubation with AdoMet,  $<50\%$  of the DNA molecules are resistant to cleavage. At the same PstII concentrations, DNA cleavage would be completed in  $\sim 10$  s (1). This explains why AdoMet does not adversely affect the cleavage rate at high enzyme concentrations (1); cleavage will always occur in preference to methylation owing to the  $>700$ -fold difference in the relative rates. As the concentration of PstII was lowered, however, the profiles revealed two distinct phases which varied in amplitude with enzyme concentration (Figure 4B). At 32 nM PstII,  $\sim 40\%$  of the DNA is inhibited within 5 min. This indicates a fast methylation rate. The remaining 60% of the DNA is then methylated at a slow rate, comparable with the rate at 100 nM PstII. At 7 nM PstII,  $\sim 70\%$  of the DNA is inhibited within 10 min. This matches the fast rate seen at 32 nM PstII. The remaining 30% of the DNA is then methylated at a slow rate comparable to that at 32 and 129 nM PstII. The rapid methylation rate seen at low enzyme concentrations is faster than the accompanying slow cleavage rate. It is therefore unsurprising that AdoMet inhibits the PstII cleavage reactions at low enzyme concentrations.



**Figure 3.** Rate of DNA cleavage at low PstII concentrations as a function of AdoMet. pLJP11b (5 nM) was incubated in NEB 4 plus Triton X-100 with 32 nM PstII mix and 4 mM ATP for various times at 37°C (see main text for full details). Where indicated AdoMet was included at 100  $\mu$ M. The percentage of FLL product (indicated as circles) was quantified as in Figure 2.

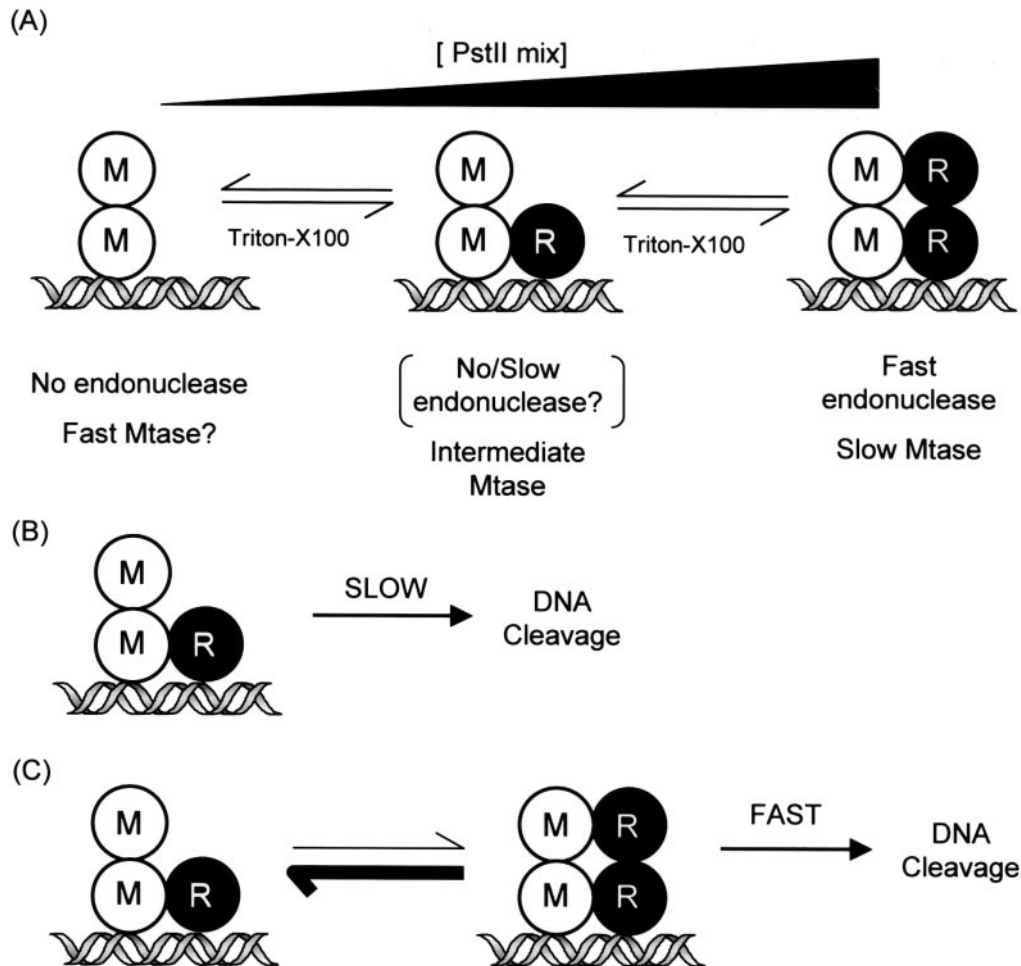


**Figure 4.** Estimating the methylation rate of PstII species. (A) Experimental flow chart. pLJP11b (5 nM) was mixed with 7, 32 or 129 nM PstII in NEB 4 plus Triton X-100 and the reactions executed as shown. (B) The percentage of FLL product was quantified as in Figure 2 for reactions pre-incubated with PstII as shown. See main text for full details.

## DISCUSSION

In many respects, the enzyme activity of PstII is very similar to other Type III enzymes [this study, (1)]. However, our analysis indicates that unlike EcoPI and EcoP15I, the association of the Mod and Res subunits of PstII is dynamic. This is reflected in the concentration dependence of the relative rates of restriction and modification. In Figure 5A we illustrate a model (based on Equation 1) which offers a possible explanation of our results. We envisage an equilibrium on DNA between the methyltransferase ( $\text{Mod}_2$ ) and the Res subunits which leads to the stepwise assembly of  $\text{Res}_1\text{Mod}_2$  and  $\text{Res}_2\text{Mod}_2$  species. With a fixed concentration of DNA (e.g. in this study 5 nM plasmid DNA and 10 nM sites), the assembly of the higher order species can be driven by simply increasing the PstII concentration. This will increase the pool of available Res subunits which can associate with the specific DNA-bound species. This is the only way a  $\text{Res}_2\text{Mod}_2$  complex can form because the Res subunit is under-represented in the PstII preparations. In a similar manner, some partially assembled, and

thus inactive, Type I restriction endonucleases, such as EcoR124I and EcoAI, can be rescued by increasing the pool of free HsdR subunits (14,15,19). In Figure 5A we propose that the effect of Triton X-100, in addition to preventing protein aggregation, is to stabilize the formation of the higher order species. Each species is then allocated kinetic properties as a function of the relative proportions of Res and Mod. The  $\text{Mod}_2$  species does not have any endonuclease motifs and thus can only act as an efficient methyltransferase. We have preliminary evidence that this is the case (A. Sears and M. D. Szczelkun, unpublished data). From the cleavage rate data in the accompanying paper [Figure 5 in Ref. (1)], the  $\text{Res}_2\text{Mod}_2$  species favoured at high PstII concentrations is an efficient endonuclease. In Figure 4 we demonstrate that this species also has an accompanying methyltransferase activity. However, the relative rate of the modification reaction is too slow to effect the cleavage reaction. The question then is which species confer(s) the slow endonuclease/intermediate methyltransferase rates seen at lower PstII concentrations. The different methylation rates observed could be explained if



**Figure 5.** Proposed model for PstII assembly and its effect on enzyme activity. Mod (M) and Res (R) are shown as open and closed circles, respectively. (A) The effect of increasing protein concentration and Triton X-100 on the equilibrium between different PstII species. The Mod<sub>2</sub> species cannot cut DNA and has a methyltransferase activity in excess of that seen for any Res-bound species (A. Sears and M. D. Szczelkun, unpublished data). (B and C) Alternative models to explain the slow cleavage rate seen at low PstII concentrations. See main text for full details.

binding of Res to the methyltransferase slowed the intrinsic modification rate in a dose-dependent manner: binding of one Res partially inhibiting the modification rate and binding of a second Res inhibiting the rate further still. There are then two possible explanations for the different cleavage rates. In the first model, illustrated in Figure 5B, the Res<sub>1</sub>Mod<sub>2</sub> complex can still cut DNA but does so at a slower rate. In the presence of AdoMet this slow rate could be exceeded by the methylation rate and the inhibition of cleavage would occur. In the alternative model, illustrated in Figure 5C, the Res<sub>1</sub>Mod<sub>2</sub> complex cannot cleave the DNA. Instead, it must first assemble into a Res<sub>2</sub>Mod<sub>2</sub> complex by the association of a Res subunit. If the occupancy of the tetramer species were limited kinetically (by slow Res binding and/or by fast Res dissociation), then the observed cleavage rate would be slower than the intrinsic rate. As above, in the presence of AdoMet there would be a competition between endonuclease and methyltransferase functions resulting in inhibition of cleavage at low PstII concentrations.

It has been suggested that a simple control of RM activity can be obtained on the basis of the affinities of the protein

subunits (8). An equilibrium between different PstII species with different relative enzyme activities may therefore be important to the control of its RM activity *in vivo*. Freely dissociating Type I enzymes may have evolved where elements of the complex are genetically mobile (4,10,15). In these cases new specificities can be generated by the free exchange of genes between bacteria. The new system must first establish its methylation pattern and a dynamic complex assembly may allow this to occur. However, PstII is chromosomally expressed so genetic exchange may not play such an important role. In fact, we have observed that PstII appears to be difficult to establish in a new cell line (1). This is in contrast to the EcoPI and EcoP15I enzymes, which are freely transferable *in vivo* [(11) and references therein] and which form stable tetramer complexes *in vitro* (17). One caveat to our observations is that expression from a high copy number plasmid (pMB1 origin plus *rop*) may result in an excess of PstII Res protein such that Res<sub>2</sub>Mod<sub>2</sub> complexes are formed before all sites are methylated. Nonetheless, it would appear that at least one Type III enzyme has evolved to allow modulation of its RM activities through subunit assembly.



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