

# Asn<sup>141</sup> is essential for DNA recognition by *EcoRI* restriction endonuclease

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**Abstract** The amino acid residue Asn<sup>141</sup> of the restriction endonuclease *EcoRI* was proposed to make three hydrogen bonds to both adenine residues within the recognition sequence -GAATTC-. We have mutated Asn<sup>141</sup> to alanine, aspartate, serine, and tyrosine. Only the serine mutant is active under normal buffer conditions although 1000-fold less than wild-type *EcoRI*. The alanine and aspartate mutants can be activated by Mn<sup>2+</sup>. At acidic pH the latter mutant becomes even more active than the wild-type enzyme in the presence of Mn<sup>2+</sup>. We conclude that Asn<sup>141</sup> is essential for DNA recognition and that serine can partly substitute it.

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**Key words:** DNA-protein interaction; Type II restriction enzyme; Sequence specificity; Site-directed mutagenesis; Restriction modification; Hydrogen bond

## 1. Introduction

The restriction endonuclease *EcoRI* binds and, in the presence of Mg<sup>2+</sup>, cleaves within the palindromic double-stranded DNA sequence -GAATTC- with very high specificity. Sequences deviating by one base pair are cleaved at least five orders of magnitude more slowly [1,2]. Under various 'star buffer conditions' the cleavage of these sequences is markedly enhanced and canonical cleavage slightly reduced, resulting in comparable velocities [3]. These conditions are (i) high pH together with low ionic strength [4], (ii) addition of organic solvents such as glycerol or dimethyl sulphoxide [5], or (iii) substitution of Mg<sup>2+</sup> by Mn<sup>2+</sup> [6]. The phenomenon of star activity is common to many restriction enzymes (for review see [7,8]) although the primary protein sequences show very little or no homology. However, in the four co-crystal structures of class II enzymes solved to date (*EcoRI*, *EcoRV*, *BamHI*, *PvuII*) a central  $\beta$ -sheet, two  $\alpha$ -helices packed against it [9] and the catalytic centres built by two strands of this  $\beta$ -sheet are clearly homologous (reviewed in [8,10]) and they may function similarly [11,12]. This can be extended even to the catalytic domain of the class II enzyme *FokI* [13].

Sequence recognition, on the other hand, is completely different in the four class II enzymes. Although they all contact bases in the major groove of the DNA the main body of the enzyme may be positioned on either the major or minor groove side and the contacting amino acid residues may originate from loops (*EcoRV* [14]; *BamHI* [15]),  $\beta$ -sheets (*PvuII*

[16]), or an extended chain (*EcoRI* [17]). The importance of the 'extended chain motif' of *EcoRI* for DNA recognition was confirmed recently by showing that a dodecapeptide comprising this sequence binds specifically to -GAATTC-, albeit with a low binding constant [18]. The amino acid residue Asn<sup>141</sup> is part of the extended chain and, as deduced from X-ray structure analysis, receives two hydrogen bonds from the exocyclic amino groups of both adenines of the recognition sequence and donates one hydrogen bond to the N7 of the outer adenine (Fig. 1) [19]. These contacts represent a substantial portion of the direct contacts between the dimeric enzyme and its palindromic substrate. The two Asn<sup>141</sup> residues are involved in six out of the total 16 hydrogen bonds. In addition to these hydrogen bonds, van der Waals contacts to the bases, as well as ionic interactions to the phosphates of the DNA, take part in specific recognition. Therefore, the interactions of the *EcoRI* endonuclease with the DNA are clearly redundant.

In order to investigate the contribution of Asn<sup>141</sup> to this interaction network we have mutated this amino acid residue to alanine, aspartate, serine and tyrosine. The results show the importance of Asn<sup>141</sup>, but the redundancy of the recognition prevents a change of specificity.

## 2. Materials and methods

### 2.1. Bacterial strains and plasmid vectors

The following *Escherichia coli* strains were used: WK6mutS( $\lambda$ ) for transformation of DNA after mutagenesis; LK111( $\lambda$ ) for propagation of DNA; and TGE900 for the expression of *EcoRI* and the mutant enzymes. Prior to transformation with the pRIF309+ plasmid harbouring the *EcoRI* gene [20], all strains were transformed with pEcoR4 carrying the *EcoRI* methyltransferase gene [21].

### 2.2. Mutant enzymes

Site-directed mutagenesis by means of the gapped duplex method was performed as described [22], using mismatch primers which were prepared on a Cyclone DNA synthesiser using  $\beta$ -cyanoethyl phosphoramidites (MWG). Positive clones were screened by restriction enzyme analysis and verified by sequencing of the entire gene. Mutant enzymes were purified as described [22]. Protein concentration was determined spectrophotometrically using the molar absorption coefficient for wild-type *EcoRI*:  $\epsilon_{278} = 5.2 \times 10^4 \text{ l mol}^{-1} \text{ cm}^{-1}$  [23].

### 2.3. Circular dichroism

Circular dichroism (CD) spectra and CD melting curves of the mutants were recorded and analysed as described [24].

### 2.4. Cleavage of bacteriophage $\lambda$ -DNA

A concentration of 1  $\mu\text{g}/60 \mu\text{l}$  bacteriophage  $\lambda$ -DNA (Boehringer-Mannheim) was used to determine the specific activity of the mutant enzymes under varying buffer conditions. The buffer contained 20 mM Tris-HCl with varying pH (6.8, 7.5 or 8.8) as indicated, 50 mM NaCl, 10 mM MgCl<sub>2</sub> or 0.75 mM MnCl<sub>2</sub>. Only the N141S mutant was tested in low salt buffers containing 20 mM Tris-HCl with varying pH (6.8, 7.5, or 8.8), 1 mM MgCl<sub>2</sub>. The enzyme concentration was adjusted to the activity of the mutant tested. Samples were

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**Abbreviations:** CD, circular dichroism

incubated at 37°C, aliquots were taken at varying time intervals and analysed by agarose gel electrophoresis. The shortest time interval at which complete cleavage was detected was used for estimation of enzyme units. 1 unit is defined as the amount of enzyme which cleaves 1 µg λ-DNA per hour in 60 µl reaction volume at 37°C.

### 2.5. DNA binding experiments

Binding constants were determined by nitrocellulose filter binding as described [25]. The double-stranded oligodeoxynucleotide TAC GAATTC TGCTG with 5'-overhanging T residues in both single strands was used as the binding substrate. It was radiolabeled using T4 polynucleotide kinase and [ $\gamma$ - $^{32}$ P]ATP (Amersham).

## 3. Results

Asn<sup>141</sup> was mutated by the gapped duplex method using the degenerate mutagenesis primer 1: 5'-GAT TTA ATG GCT GCT GGT (G or T)(A or C)T GCT ATC GAA AGG TCT CAT AAG AAT ATA TCA G-3' (mismatched positions for amino acid mutation in the 5' half and for silent mutation at a *Bgl*II site in the 3' half are underlined). This primer should lead to all four mutations: Asn<sup>141</sup> → Asp (GAT), Asn<sup>141</sup> → Tyr (TAT), Asn<sup>141</sup> → Ala (GCT), and Asn<sup>141</sup> → Ser (TCT). Only the first two of these mutants were found in four independent experiments. Presumably, annealing of those primers with only one mismatch in the first codon position and the canonical A in the second codon position was very much preferred. Therefore, we repeated the mutagenesis experiment with a second primer 2: 5'-GAT TTA ATG GCT GCT GGT (G or T)CT GCT ATC GAA AGG TCT CAT AAG AAT ATA TCA G-3' (mismatched positions underlined). This experiment resulted in the last two desired mutants. All mutants were verified by sequencing of the whole gene.

To get a first impression of the activity of the mutant enzymes, the viability of cells after induction of overexpression was tested. Cells are effectively killed by the nucleolytic activity of wild-type *Eco*RI. Methylation of the recognition sequence by *Eco*RI methylase only partly protects the cells [26]. This is reflected by the viability factors for the wild-type enzyme shown in Fig. 2. Only the N141S mutant is toxic. This toxicity is completely blocked by the *Eco*RI methylase. Overexpression of all other mutants is not toxic for the cells.

In order to correlate these results with in vitro activity of the mutants, we tested crude cell lysates for specific cleavage of bacteriophage λ-DNA. In normal buffer, only the N141S mutant showed the *Eco*RI-specific cleavage pattern in accordance with the viability assay. Using different star buffers, the N141A and N141D mutants could be activated to cleave λ-DNA to shorter fragments while the N141Y mutant showed no cleavage at all (data not shown).

The mutants were purified according to a procedure developed for wild-type *Eco*RI, consisting of DEAE-cellulose and

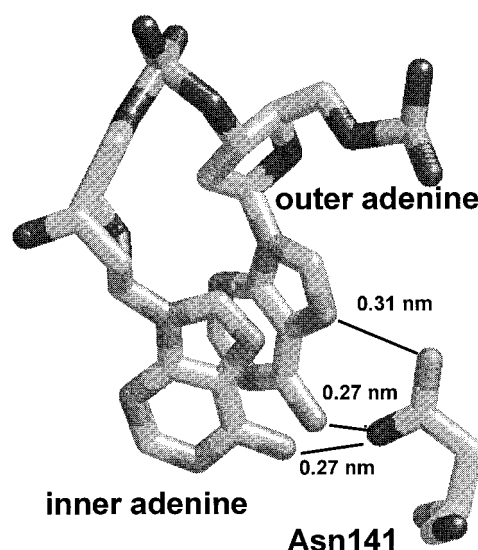


Fig. 1. Structural representation of the contacts between Asn<sup>141</sup> and the two adenine bases of the recognition sequence using the coordinates given in 1ERI in the Brookhaven Data Bank and the program Rasmol. Distances between the heteroatoms believed to be engaged in hydrogen bonds are indicated.

hydroxyapatite chromatography [22]. On a denaturing polyacrylamide gel, the purified proteins migrated as single bands corresponding to more than 95% of the stained material. The identity of the band was verified by a Western blot with an anti-*Eco*RI antibody (data not shown). CD spectroscopy showed no significant differences to wild-type *Eco*RI nor does CD melting for the N141S and N141D mutants. These enzymes all denature around 45°C. This denaturation is concentration-dependent and irreversible because of an aggregation and precipitation of the denatured protein. The N141A mutant at 43°C lost about 70% of the structure while 30% were more stable and melted in a second transition at about 58°C (Fig. 3).

Under normal buffer conditions (20 mM Tris pH 7.5, 50 mM NaCl, 10 mM MgCl<sub>2</sub>), only the N141S mutant cleaves -GAATTC- sites, though its specific activity is three orders of magnitude less than that of wild-type *Eco*RI (Table 1). In contrast to the wild-type enzyme, this mutant is activated 10-fold by increasing the pH to 8.8, while no cleavage is detectable at pH 6.8. Lowering the ionic strength (20 mM Tris pH 8.8, 1 mM MgCl<sub>2</sub>), which results in the star buffer described by Polisky et al. [4], activates this mutant an additional five-fold compared to the wild-type enzyme (data not shown), but only results in cleavage of canonical sites. The other mutants show no DNA cleavage at any pH in the nor-

Table 1  
Specific activities (U/mg) of wild-type and mutant enzymes under various buffer conditions

| Divalent cation          | pH  | wt <i>Eco</i> RI    | N141A               | N141D               | N141S               | N141Y |
|--------------------------|-----|---------------------|---------------------|---------------------|---------------------|-------|
| 10 mM Mg <sup>2+</sup>   | 6.8 | 6 × 10 <sup>6</sup> | nd                  | nd                  | nd                  | nd    |
|                          | 7.5 | 1 × 10 <sup>7</sup> | nd                  | nd                  | 1 × 10 <sup>4</sup> | nd    |
|                          | 8.8 | 8 × 10 <sup>6</sup> | nd                  | nd                  | 1 × 10 <sup>5</sup> | nd    |
| 0.75 mM Mn <sup>2+</sup> | 6.8 | *                   | < 10 <sup>2</sup>   | 4 × 10 <sup>4</sup> | 7 × 10 <sup>3</sup> | nd    |
|                          | 7.5 | *                   | 2 × 10 <sup>2</sup> | 7 × 10 <sup>3</sup> | 1 × 10 <sup>4</sup> | nd    |
|                          | 8.8 | *                   | < 10 <sup>2</sup>   | < 10 <sup>2</sup>   | *                   | nd    |

\*: cleavage of star sites, i.e. DNA sequences deviating in one base pair from the recognition sequence; nd: after 24 h even a start of DNA cleavage was not detectable; < 10<sup>2</sup>: cleavage activity starts but does not become complete within 24 h.

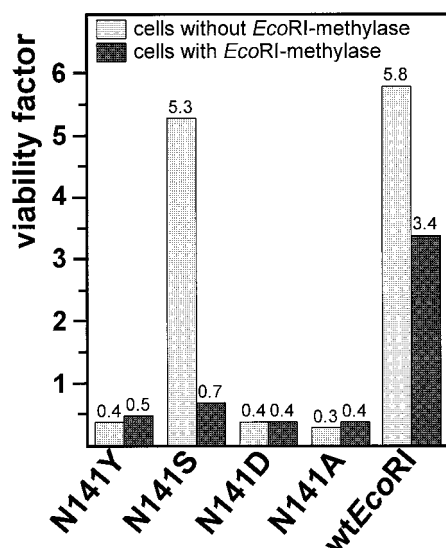


Fig. 2. In vivo toxicity of wild-type *EcoRI* and its mutants. The viability factor is defined as the negative decade logarithm of the cell count of induced cells (42°C) divided by the cell count of non-induced cells (30°C) [26].

mal buffer. But, in contrast to the N141Y mutant, the N141A and N141D mutants can be activated to a low specific activity by changing 10 mM  $Mg^{2+}$  to 0.75 mM  $Mn^{2+}$ . The N141S mutant shows the same activity with both divalent cations. A comparison with wild-type *EcoRI* under these conditions is difficult, because it cleaves non-canonical star sites nearly as fast as the canonical sites and a clear canonical band pattern is never observed. However, a rough estimation from initial velocities gives an approximately 50-fold less specific activity at the canonical sites in star buffers for the wild-type enzyme. Therefore, the N141D and N141S mutants are respectively only 30-fold and 20-fold less active than wild-type *EcoRI* while the N141A mutant is still 1000-fold less active. The N141S mutant, as seen in the  $Mg^{2+}$  buffer, is activated by alkaline pH to such an extent that it starts star cleavage at non-canonical sites.

Interestingly, N141D is the only mutant which can cleave DNA at pH 6.8 in the  $Mn^{2+}$  buffer. In order to characterise the pH dependence of this cleavage in more detail, we used a short PCR fragment with only one canonical and no star sites as a substrate. It is cleaved only once by the mutant and the

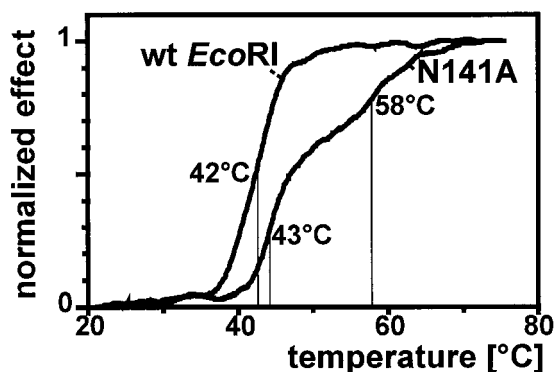


Fig. 3. CD melting of wild-type *EcoRI* and the N141A mutant. The N141S and N141D mutants behave like wild-type *EcoRI*.

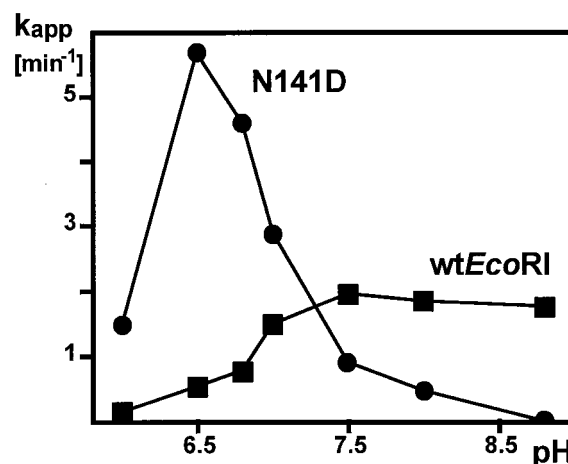


Fig. 4. pH dependence of the cleavage rates of wild-type *EcoRI* and the N141D mutant in the  $Mn^{2+}$  star buffer. A 173 bp long PCR fragment of pBR322 [11] was cleaved with varying concentrations of the two enzymes in 20 mM Tris-HCl with various pH values (6.0, 6.5, 6.8, 7.0, 7.5, 8.0, 8.8), 50 mM NaCl, 0.75 mM  $MnCl_2$  at 37°C.

wild-type enzyme in  $Mn^{2+}$  star buffer. While the cleavage rate of wild-type *EcoRI* decreases at pH values below 7.5 the N141D mutant is activated. Thus at pH 6.5 the mutant is about 10 times more active than the wild-type enzyme (Fig. 4).

Only the N141S mutant shows significant DNA-binding (Fig. 5). The binding constant of  $8 \times 10^7 M^{-1}$  is about 10-fold lower compared to the wild-type enzyme. For the N141A mutant, a binding constant could not be calculated because, even at maximal concentration of this enzyme, only a faint binding was detectable which did not reach saturation. The same behaviour was seen in experiments in which the mutant competed with single turnover cleavages of the same oligodeoxynucleotide substrate by the wild-type enzyme under normal buffer conditions (data not shown). The situation was even worse for the N141Y and N141D mutants. Although the latter cleaves DNA faster than the N141A mutant, specific binding was not detectable. Neither a change of the pH to

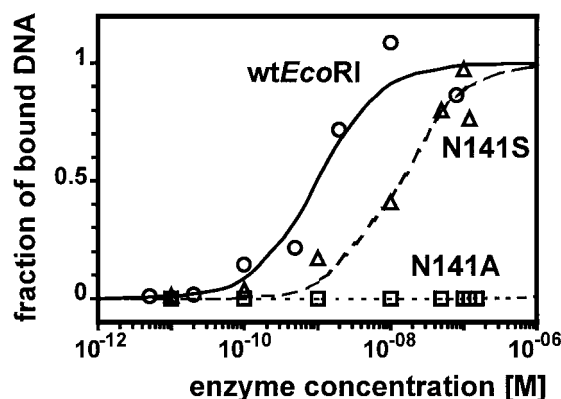


Fig. 5. Specific DNA binding by wild-type *EcoRI* and the N141S and N141A mutants. The experiments shown represent complexes formed by 0.1 nM radiolabelled ds(TAC GAATTC TGCTG) and widely varying concentrations of the enzymes quantified by nitrocellulose filter binding. The lines represent fits with binding constants of  $1 \times 10^9 M^{-1}$  (solid line),  $8 \times 10^7 M^{-1}$  (broken line) and  $1 \times 10^5 M^{-1}$  (dotted line), respectively.

6.5 nor the electrophoretic mobility shift assay at both pH values led to a detection of DNA binding by this mutant, although the wild-type enzyme showed binding under all these conditions (data not shown).

#### 4. Discussion

As deduced from the X-ray structure, Asn<sup>141</sup> plays an important role in the specific interaction of the *EcoRI* restriction endonuclease with its recognition sequence -GAATTC- [17]. It was suggested to be engaged in three hydrogen bonds with the two adenines of this sequence [19]. To test this suggestion we mutated Asn<sup>141</sup> to Ala, Asp, Tyr and Ser.

The expression of the N141A, N141D and N141Y enzymes does not impair the viability of *E. coli* cells while the N141S mutant is almost as toxic as the wild-type enzyme. But, in cells expressing the *EcoRI* methylase, the N141S mutant is barely toxic in contrast to wild-type *EcoRI* which at high enzyme concentration cleaves at star sequences not protected by methylation. Obviously, the N141S mutant under cellular conditions is more accurate.

While the single point mutations did not reduce secondary structure composition and stability of the mutants, the N141A mutant is partly stabilised against thermal denaturation. Such a biphasic melting behaviour has also been detected for the Q115A and Q115D mutants and was explained by an uncoupling of the melting of the arm domain from the main body of the enzyme due to the loss of intramolecular hydrogen bonds [24]. However, in the co-crystal structure Asn<sup>141</sup> is oriented to the bases of the DNA and the stabilisation of its structure may rather be caused by a retardation of aggregation and precipitation, which make the melting of wild-type *EcoRI* a concerted event.

We have extensively characterised the cleavage activity of the purified mutants. As was seen in crude cell extracts, N141S is the only mutant which cleaves DNA under normal buffer conditions. In contrast to the wild-type enzyme it can be activated by alkaline pH. The help of preformed OH<sup>−</sup> in catalysis may indicate difficulties in activation of the attacking water molecule as was seen for the cleavage of H-phosphonate substituted substrates by wild-type *EcoRI* [27].

The N141A and N141D mutants need a star buffer (for example Mn<sup>2+</sup> in Table 1) to show cleavage activity at canonical cleavage sites. This was also seen for other mutants of base contacting amino acid residues in *EcoRI* [11,24], *BamHI* [28] and *EcoRV* [29–31]. The main influence of star buffers seems to be that coupling of recognition and catalysis is not as strict as in normal buffers. This causes cleavage (i) by mutants which have an interaction surface disturbed by a mutation of a contacting amino acid residue or (ii) by the wild-type enzyme at star sequences which offer a disturbed interaction surface [8]. Therefore, the cleavage behaviours of the N141A and N141D mutants show that their DNA recognition process is strongly disturbed and needs relaxation of coupling to become capable of trigger cleavage.

Besides the N141Y mutant, whose tyrosine may be too large for an accommodation in the enzyme-DNA interface, the N141A mutant has the lowest activity. This is in accordance with the structure of an alanine residue which has no hydrogen bonding capacity and is short enough not to interfere with other DNA contacts. One may wonder whether the loss of these hydrogen bonds would lead to a relaxation of

sequence specificity. However, the methyl groups of both thymines are in a hydrophobic cluster with the three residues Gln<sup>115</sup>, Gly<sup>140</sup> and Ala<sup>142</sup> and, furthermore, both adenines are additionally hydrogen bonded to Arg<sup>145</sup> and the inner thymine is hydrogen bonded to the main chain at position 142. Therefore, the redundant recognition prevents any relaxation of sequence specificity.

The completely changed pH dependence of the N141D mutant compared to wild-type *EcoRI* may result from the necessity of protonation of the aspartate residues for DNA interaction as seen for the Q115E mutant [24]. The protonated forms are able to establish all DNA contacts similarly to asparagine residues. But this should only lead to an activity comparable to the wild-type enzyme. The activation to higher cleavage rates may be due to the star conditions. It has to be pointed out that we have no indication of an asymmetry in the reaction due to protonation of only one of the two aspartate residues. This should result in a preferential nicking of the DNA which we have never observed in cleavage reactions with plasmid substrates (data not shown). Therefore, it is reasonable that the enzyme is inactive as long as one of the two aspartates is deprotonated. That may in part also explain the sharp activation by lowering the pH.

Given that the wild-type enzyme cleaves canonical sites 50-fold more slowly when 10 mM Mg<sup>2+</sup> is changed to 0.75 mM Mn<sup>2+</sup>, the same activity at both conditions for the N141S mutant resembles an activation by star conditions. At pH 8.8 this mutant even starts to cleave non-canonical sites, which again confirms the strong activation of this mutant by alkaline pH. Although serine is shorter than asparagine, it has the capacity to give and accept hydrogen bonds. Even if larger rearrangements are not tolerated by neighbouring DNA contacts, it seems plausible that at least one hydrogen bond is made by this residue. Both adenines are equally well oriented for an interaction between their exocyclic amino groups and the hydroxyl group of serine. Although an interaction with the carbonyl group of guanine would also be possible, the redundancy of contacts to the AT base pairs already described rules out such a change in specificity for this mutant.

For the N141S and N141A mutants, binding resembles their cleavage behaviour. It is more than 10-fold reduced for the still rather active N141S mutant and hardly detectable for the inactive N141A mutant. The situation is much more complicated for the N141D mutant. Given its fairly high activity in a Mn<sup>2+</sup> buffer at acidic pH one may expect specific binding at least at a pH of 6.5. Although we tried very hard we could not detect any binding. It is reasonable to expect that the aspartates have to be protonated to allow binding and cleavage. But there is no reason for a strong pK shift of these residues. If so, even at lower pH, only a small fraction of enzyme molecules would be protonated simultaneously at both aspartates, and then the thermodynamic binding experiment could not detect the interaction with the DNA, although the kinetic cleavage experiment would trap the low fraction of bound species.

The behaviour of the four mutants at position 141 is fully compatible with the contacts to the adenine bases deduced from the X-ray structure. We cannot decide whether really three or only two hydrogen bonds are made by Asn<sup>141</sup>. But, the differential effect in DNA cleavage and binding going from asparagine to serine to alanine speaks for the progressive loss of at least two contacts.

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