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Covalent Joining of the Subunits of a Homodimeric Type II Restriction Endonuclease: Single-chain *Pvu*II Endonuclease

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The *Pvu*II restriction endonuclease has been converted from its natural homodimeric form into a single polypeptide chain by tandemly linking the two subunits through a short peptide linker. The arrangement of the single-chain *Pvu*II (sc *Pvu*II) is (2-157)-GlySerGlyGly-(2-157), where (2-157) represents the amino acid residues of the enzyme subunit and GlySerGlyGly is the peptide linker. By introducing the corresponding tandem gene into *Escherichia coli*, *Pvu*II endonuclease activity could be detected in functional *in vivo* assays. The sc enzyme was expressed at high level as a soluble protein. The purified enzyme was shown to have the molecular mass expected for the designed sc protein. Based on the DNA cleavage patterns obtained with different substrates, the cleavage specificity of the sc *Pvu*II is indistinguishable from that of the wild-type (wt) enzyme. The sc enzyme binds specifically to the cognate DNA site under non-catalytic conditions, in the presence of Ca^{2+}, with the expected 1:1 stoichiometry. Under standard catalytic conditions, the sc enzyme cleaves simultaneously the two DNA strands in a concerted manner. Steady-state kinetic parameters of DNA cleavage by the sc and wt *Pvu*II showed that the sc enzyme is a potent, but somewhat less efficient catalyst; the k_{cat}/K_M values are 1.11×10^9 and 3.50×10^9 min^{-1} M^{-1} for the sc and wt enzyme, respectively. The activity decrease is due to the lower turnover number and to the lower substrate affinity. The sc arrangement provides a facile route to obtain asymmetrically modified heterodimeric enzymes.

Keywords: single-chain protein; *Pvu*II restriction endonuclease; single-chain enzyme; DNA binding; DNA cleavage

Introduction

The *Pvu*II restriction endonuclease (*Pvu*II) is the nuclease component of one of the type II restriction-modification systems of *Proteus vulgaris*.[1,2] Like most type II endonucleases,[3–6] *Pvu*II is homodimeric and cleaves its double-stranded cognate DNA substrate in the presence of Mg^{2+} so that each subunit acts on one DNA strand in a concerted manner. The cleavage takes place in the 5′-CAGCTG-3′ sequence between the central GC residues and generates blunt ends with a 5′-phosphate group.

*Pvu*II is one of the smallest type II endonucleases. The gene for *Pvu*II codes for a protein of 157 amino acid residues[7,8] and the subunits of the mature protein contain 156 amino acid residues. *Pvu*II is structurally well characterized: X-ray structures for the apoenzyme[9] and for complexes with cognate DNA exist.[10–13] NMR techniques have been used to study the conformation and metal-binding properties of the enzyme.[14–16] The structural studies identified dimerization, catalytic and DNA recognition regions. Mutations in these regions have been obtained by site-directed mutagenesis and by selection from randomly mutated populations. Some of these mutants were characterized in functional[17,18] and structural[11] studies.

Abbreviations used: sc, single-chain; wt, wild-type; ds, double-stranded.

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The crystal structures of the homodimeric *Pvu*II show that the C terminus of one subunit is in close proximity to the N terminus of the other one. This structural feature raises the interesting possibility of linking these termini by a designed peptide linker and thereby to create a single-chain (sc) enzyme. Such a functional sc enzyme should prove to be an invaluable tool in protein engineering studies, both in basic research and in practical applications.

To initiate such studies, we constructed the prototype of the sc *Pvu*II which, to the best of our knowledge, is the first sc restriction endonuclease. A tandem gene containing two *Pvu*II coding regions joined by a sequence encoding the GlySerGlyGly peptide linker was constructed and the effect of its expression, in comparison with that of the wild-type (wt) gene, was assessed in *in vivo* tests. The sc and wt *Pvu*II were expressed, purified and their DNA binding and catalytic properties were compared. The results of the *in vivo* and the *in vitro* tests showed that although the sc enzyme is somewhat less efficient, its DNA recognition and cleavage specificities are very similar to those of the wt enzyme. A heterodimeric sc *Pvu*II mutant containing a wt and a mutant subunit was constructed and used in this study to clarify the mechanism of DNA cleavage by the sc *Pvu*II.

Results and Discussion

Design and construction of a single-chain derivative of *Pvu*II

The structure of the homodimeric *Pvu*II in complex with specific DNA[10] shows that the distance between the C-terminal Tyr157 of one subunit and the N-terminal Ser2 of the other one is approximately 12 Å. A peptide linker of four amino acid residues is long enough to span this distance without causing conformational changes in the subunits and it is expected that it would not interfere with the folding or functioning of the protein. To minimize the probability of such interference, charged and bulky amino acid residues were avoided and a linker of GlySerGlyGly sequence was designed (Figure 1). Similar sequence motifs were used successfully in substantially longer linkers of sc antibodies[19] and in several sc DNA-binding proteins.[20,21] Interruption of runs of Gly residues with Ser or Ala was shown to increase protein stability without compromising the linker flexibility.[20] Of practical considerations, the GlySer portion is advantageous, since it can be encoded by the recognition site for *Bam*HI, which was used during the sc gene construction.

Construction of the sc *Pvu*II gene containing tandem coding regions was performed by PCR modification of the wt *Pvu*II gene. Two independent amplifications were performed to obtain cloned genes for the subunits in two different arrangements (see Materials and Methods). These genes were then joined in the pRIZ′ expression vector[22]

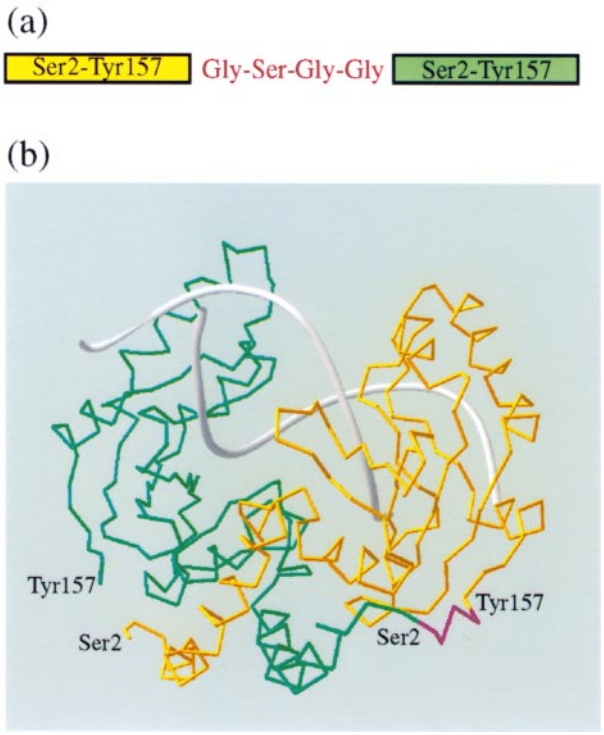

Figure 1. (a) Map of the homodimeric sc *Pvu*II. (b) Schematic view of the sc *Pvu*II-DNA complex. The model, based on a *Pvu*II-cognate DNA structure (pdb 1PVI),[10] was generated by the Swiss-Pdb-Viewer.[36] The two identical subunits (yellow and green) and the designed peptide linker (red) are shown as C^{α} traces, the DNA backbone is shown as grey wire.

so that the stop codon of the first subunit was replaced by a GlySerGlyGly coding linker containing the *Bam*HI site at the junction of the tandem genes. The gene coding for the wt *Pvu*II was also inserted into the pRIZ′ expression vector. The *Escherichia coli* hosts used during cloning, protein expression and *in vivo* tests contained the *Pvu*II methyltransferase gene on a compatible, low-copy number plasmid pLGM, which was constructed from pLG339.[23]

Testing the activity of sc *Pvu*II *in vivo*

The activity of the sc *Pvu*II was tested, in comparison with the wt *Pvu*II, in two different *in vivo* assays. The corresponding expression vectors were transformed into two isogenic *E. coli* strains that contained a plasmid either with (pLGM) or without (pLG339) the *Pvu*II methyltransferase gene and the transformants were plated to test their ability to form colonies. The results of this viability test showed that both the wt and the sc *Pvu*II-containing hosts required the presence of *Pvu*II methyltransferase for survival, indicating the expression of *Pvu*II endonuclease activity in both cases.

To compare the *in vivo* activities in a quantitative manner, λ phage restriction assays were per-

formed. By using the two-plasmid expression system as above, the *E. coli* hosts expressing either the wt or the sc *Pvu*II under uninduced conditions exhibited strong restriction, but the results were not sufficiently reproducible to make quantitative comparisons. Since the two-plasmid system was developed for protein overexpression and its regulation should be completely different from that of the native *pvuIIRCWM* gene cluster,[1,8,24] it seemed reasonable to compare the two enzymes in the background of the native arrangement. For this reason, we converted this cluster into *pvuIIR^{sc}CWM* by replacing the wt endonuclease gene (*R*) with the sc gene (*R^{sc}*), leaving the native system otherwise identical. As the *Pvu*II restriction-modification system in *P. vulgaris* is carried on a low copy number plasmid,[25] the wt and the modified systems were tested after cloning them into the low copy number vector pGB2.[26] The phage restriction assays using these plasmids were highly reproducible and the efficiency of plating values obtained were 1.5×10^{-6} and 2.0×10^{-4} in for the wt and the sc *Pvu*II, respectively. The *in vitro* data (see below) show much less difference between the two enzymes.

Expression and purification of the wt and sc *Pvu*II enzymes

In order to compare the *in vitro* catalytic and DNA-binding properties of the sc and wt *Pvu*II, both enzymes were produced by using the same expression system and purification steps. Both enzymes were expressed at high levels by using the pRIZ′ expression vector (Figure 2(a) and (b)), and were purified from the soluble cell fraction to apparent homogeneity by using two chromatographic steps (Figure 2(c)). Electrophoresis on SDS/polyacrylamide gel showed that the sc *Pvu*II had about double the molecular mass compared to the native wt *Pvu*II, confirming the covalent linkage between the subunits in the sc version. Gel-filtration on Superdex 75 column performed under native conditions showed similar molecular masses for the covalent sc and for the non-covalently associated, homodimeric wt *Pvu*II (Figure2(d)). Interestingly, the sc enzyme eluted somewhat later (at 10.38 ml) than the wt (10.29 ml), despite its theoretical higher molecular mass due to the extra GlySerGlyGly linker. A possible explanation of this behaviour is that the subunits in the sc enzymes are associated more tightly than in the native enzyme. The gel-filtration profile did not show higher molecular mass proteins, which may theoretically arise in the sc enzyme as cross-folded structures, e.g. as non-covalently associated dimers of the sc molecules formed through intermolecular association of the subunits. The molecular mass determined by electron spray mass spectrometry were 36,669 Da (calculated 36,668 Da) and 18,212 Da (18,214 Da) for the sc and the wt enzymes, respectively. The calculations assumed Ser2 to be the N-terminal amino acid residue in both cases.

Catalytic and DNA-binding properties

The *in vitro* activities of the two enzymes were tested using several DNA substrates. Cleavage of λ

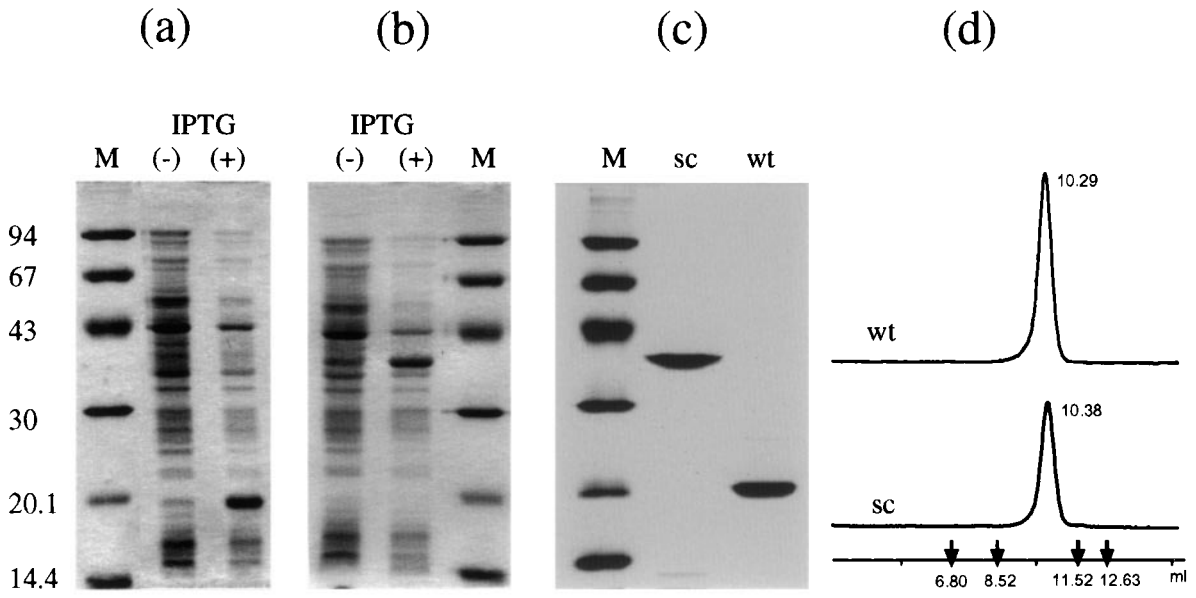

Figure 2. (a) Expression of wt *Pvu*II and (b) expression of sc*Pvu*II in *E. coli* using the pRIZ′ expression vector. (c) Analysis of the purified proteins by SDS-12.5 % PAGE. Lanes M contain molecular mass standards, shown in (a) in kDa. (d) Gel-filtration of the purified proteins on a Superdex 75 column. Arrows show the elution positions of calibration standards: blue dextran (6.80 ml), bovine serum albumin (8.52 ml, 67 kDa), chymotrypsinogen A (11.52 ml, 25 kDa) and ribonuclease A (12.63 ml, 13.7 kDa).

Table 1. Steady-state kinetic parameters for DNA cleavage by the wt and sc *Pvu*II

Enzyme	k_{cat} (min^{-1})	K_M (nM)	k_{cat}/K_M ($min^{-1}M^{-1}$)
wt	12.3	3.51	3.50×10^9
sc	5.84	5.28	1.11×10^9

DNA was performed to compare the specificities and the specific activities of the purified enzymes. Serial tenfold dilutions of the enzymes were tested in the concentration range of 0.1 to 100 nM by using λ DNA as substrate. Figure 3(a) shows that at low enzyme concentrations the extent of the cleavage reaction obtained with the sc enzyme was somewhat lower than that with the wt enzyme. The patterns of the complete cleavages were identical, while those of the partial cleavages (at 0.1 nM concentration) were very similar, indicating similar relative preferences of the sc and wt enzymes for the 14 cognate sites of the λ DNA. At the highest concentration (100 nM), trace amounts of extra bands could be detected in the wt but not in the sc enzyme reaction (Figure 3(a)). Such bands could be generated due to the ''star'' activity (i.e. substrate cleavage at sites differing only by 1 bp from the recognition sequence) observed generally under certain buffer conditions and/or at high enzyme concentrations.[3,27,28] After longer incubation times, the cleavage patterns obtained for λ, pBR322 and pUC19 substrates with 100 nM wt enzyme revealed significant amounts of star cleavage products. Similar cleavage patterns could be obtained with the sc enzyme only at higher (250 nM) concentration (data not shown). These results indicate that the sc *Pvu*II is a somewhat less efficient catalyst than the wt *Pvu*II, but it discriminates the cognate and non-cognate DNA sequences under standard buffer conditions at least as efficiently as the wt enzyme.

Cleavage of pBR322 containing a single site for *Pvu*II was performed under partial cleavage conditions. The time-course of the reactions showed that both the wt and the sc enzyme cleaved the supercoiled substrate without accumulation of the open-circle form (Figure 3(b)) This implies that the sc enzyme, like its wt counterpart, cleaves both DNA strands in one concerted reaction, each subunit acting on one DNA strand. Figure 3(b) also shows that the cleavage with the sc enzyme is slower.

A 258 bp fragment containing a single *Pvu*II site was used to determine the steady-state kinetic parameters. The velocity of the dsDNA cleavage followed the Michaelis-Menten kinetics (Figure 4). The kinetic parameters show that the k_{cat}/K_M value of the sc *Pvu*II is about threefold lower than that of the wt enzyme, and the decreased catalytic activity is due to the decrease of both the turnover number and the affinity compared to the corresponding parameters of the wt enzyme (Table 1).

Electrophoretic mobility shift assay (EMSA) was used to determine the binding affinities of the sc and wt *Pvu*II for cognate DNA. *Pvu*II was reported to bind to cognate DNA under non-catalytic conditions, in the presence of Ca^{2+} with high affinity and specificity.[17] By using a synthetic 42-mer ds oligonucleotide with centrally located recognition

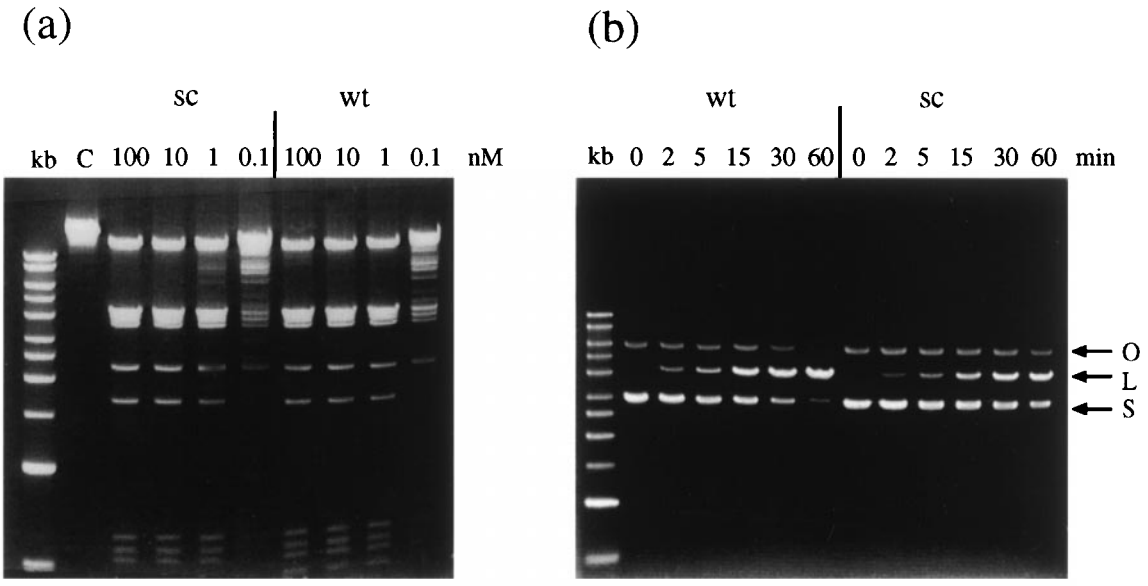

Figure 3. DNA cleavage by sc *Pvu*II and wt *Pvu*II. Samples were analyzed by electrophoresis on 0.6% agarose gel. (a) Cleavage of λ DNA at 37°C for 30 minutes, enzyme concentrations (nM) are shown on top of the corresponding lanes, kb is Kilobase DNA Marker (Pharmacia Biotech), lane C is control incubation without enzyme. (b) pBR322 cleavage performed by using 10 nM substrate and 0.1 nM enzymes at 37°C for the indicated time-periods. Arrows show the positions of the supercoiled (S), linear (L) and open-circle (O) forms of pBR322.

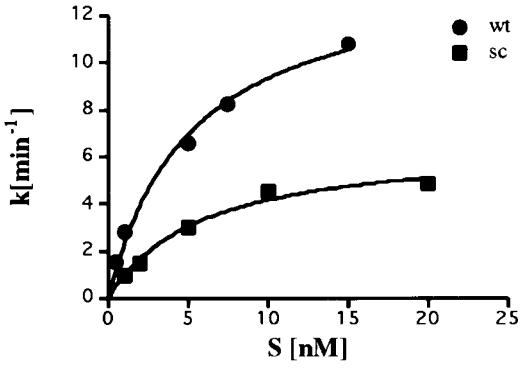

Figure 4. Steady-state kinetic analysis for the cleavage of a linear 258 bp DNA substrate by the wt and sc *Pvu*II. Kinetic parameters obtained by a best fit to the Michaelis-Menten equation are summarized in Table 1.

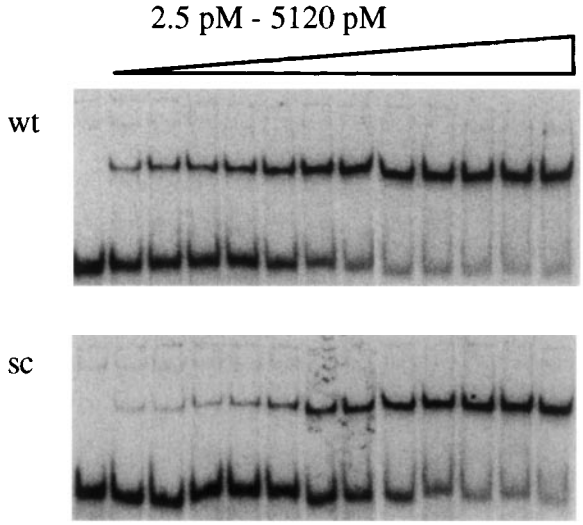

Figure 5. Comparison of the specific DNA-binding affinities of wt and sc *Pvu*II under non-catalytic conditions using protein titration and EMSA. Protein concentrations were increased in consecutive lanes, from left to right, in twofold increments. Concentrations for the wt enzyme are given in dimer equivalents.

site for *Pvu*II as a probe, protein titration experiments were performed with both enzymes (Figure 5). Based on these and several other titration experiments, half-maximal binding was calculated to take place at 32 pM (wt *Pvu*II) and 120 pM (sc *Pvu*II) protein concentrations. The lower binding affinity of the sc *versus* wt enzyme under non-catalytic conditions correlates qualitatively with the relative affinities observed under steady-state catalytic conditions. We note that somewhat lower affinity (approximately 110 pM) was reported for wt *Pvu*II;[17] the difference could be due to slightly different assay conditions and/or to different DNA probes. Figure 5 shows that the sc and wt enzymes cause the same extent of shift, indicating identical binding stoichoimetries: two protein subunits per DNA-binding site, irrespective of the nature of subunit dimerization.

Insights into the mechanism of substrate binding and catalysis studied by using heterodimeric sc mutants

The results of the gel-filtration experiments of the free enzymes and the DNA-binding assays performed under non-catalytic conditions both suggest that the sc enzyme is in the predicted conformation (Figure 1(b)) and the subunits of the sc enzyme interact in the same way as in the native, non-covalent dimer. These assays did not reveal higher molecular mass proteins that could, in principle, be formed through intermolecular subunit associations between two sc molecules. Our results do not exclude the existence of such aggregates under catalytic conditions. It is theoretically possible that the first (or N-terminal) subunits of two sc molecules form a non-covalently associated catalytic unit and the second (or C-terminal) subunits serve just as passive C-terminal extensions. In such a case, mutations in the C-terminal subunit should not influence the activity of the putative catalytic unit composed of two wt N-terminal subunits (Figure 6). The D34G mutation was shown to cause a 10^4 fold reduction in the activity of the native homodimeric enzyme.[17] When this mutation was introduced into the C-terminal subunit of the sc *Pvu*II, the heterodimeric sc enzyme cleaved pBR322 substrate substantially slower than the nonmutated sc enzyme (Figure 7). These results can be explained only if the covalently linked subunits communicate in DNA substrate binding and cleavage. A similar decrease was observed with the sc heterodimeric enzyme containing the D34G mutation in the N-terminal subunit. We also observed that different mutations in one subunit had different effects on the activity of heterodimeric sc *Pvu*II variants (not shown), in qualitative agreement with the results obtained for non-covalently formed *Eco*RV heterodimers.[29–31] These studies showed that mutations affecting base-specific DNA binding in one subunit strongly influenced the activities of both subunits, while substitution of a catalytic residue in one subunit did not significantly affect the activity of the other subunit. In this latter case, preferential cleavage of one DNA strand or plasmid DNA nicking was observed. The D34 residue of *Pvu*II, based on the structure of D34G *Pvu*II/DNA complex, was shown to have both recognition and catalytic roles,[11] therefore the D34G mutation in one subunit of the sc *Pvu*II was assumed to cause a drastic activity decrease. Figure 7 shows that plasmid DNA cleavage with the wt/D34G heterodimeric sc *Pvu*II, in comparison with the unmodified sc enzyme, is substantially slower and that a significant amount of open circular plasmid is accumulated as an intermediate. The pronounced but asymmetric effect of a single

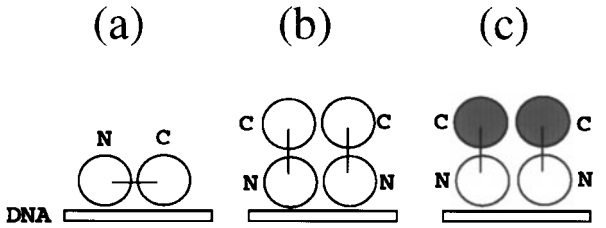

Figure 6. Theoretically possible DNA-binding modes of sc *Pvu*II. The wt subunits are shown as open circles, the mutant subunits are grey shaded circles. N and C indicate the N or C-terminal subunits, respectively.

D34G substitution on the activities of both subunits is in accord both with the postulated dual role of D34 in *Pvu*II[11] and with the general principles of intersubunit communication observed by using non-covalent *Eco*RV heterodimers.[29–31]

Conclusions

Two subunits of the naturally homodimeric restriction endonuclease *Pvu*II have been joined in a single polypeptide chain. The sc enzyme has been shown to be functional in *in vivo* assays. The *in vitro* DNA binding and cleavage assays performed with various DNA substrates showed that the wt and sc enzymes behave in a very similar manner, although the covalent linkage slightly impaired the enzyme function. Nevertheless, the sc *Pvu*II is a highly active enzyme to be used in structure-function studies. By using the sc arrangement, it is possible to perform amino acid changes in only one of the subunits or different modifications in the two subunits, providing a powerful approach to obtain heterodimeric enzymes or even libraries of such enzymes for affinity or functional selection of novel properties. The heterodimeric structure offers novel strategies to study the fundamental aspects of DNA binding and cleavage, coupling of substrate binding and catalysis, subunit cooperativity and the role of cofactors in these processes. The covalent subunit linkage allows for the creation of novel fusions, which facilitates practical applications, e.g. immobilization, surface display, *in vivo* and *in vitro* targeting, when co-presentation of the two subunits as a functional unit is desirable.

It could be interesting to test the covalent joining of the subunits of other homodimeric enzymes. Since only a very small fraction of the available restriction enzymes is characterized structurally, there may be many other cases when the appropriate subunit ends are close in space and can be linked with short peptide linkers without causing structural disturbances and loss of function. Several examples of sc DNA-binding proteins[20–22] show that even longer linkers may be suitable to connect distal termini. The general applicability of the sc arrangement to restriction endonucleases remains to be determined.

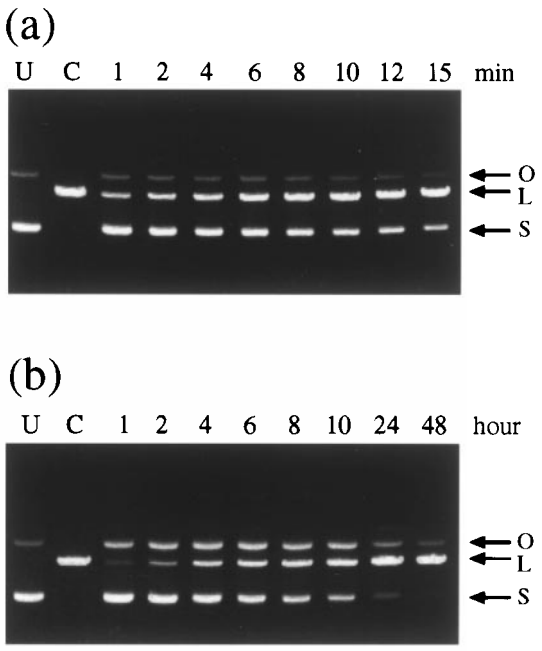

Figure 7. Comparison of pBR322 cleavage (a) by sc *Pvu*II and (b) by the heterodimeric wt/D34G sc *Pvu*II. Reactions were performed at 37 °C using 10 nM substrate and 1 nM enzyme concentrations in both cases. Aliqouts of the reaction mixtures were analyzed by electrophoresis on 0.5 % agarose gel. Time-points of sample withdrawal are given in minutes in (a) and in hours in (b) on top of the gels. U is uncleaved plasmid; C is fully cleaved linear plasmid DNA obtained by wt *Pvu*II cleavage. The positions of the supercoiled (S), linear (L) and open-circle (O) forms of pBR322 are shown by arrows.

Materials and Methods

Plasmids, DNA substrates and *E. coli* strains

Plasmid vectors pRIZ′,[22] pLG339[23] and pGB2[26] have been described. The pPvuII-3.4 vector contains the complete *Pvu*II restriction-modification system between the *Eco*RI and *Hin*dIII sites of pUC19.[32] The unmethylated λ, pBR322 and pUC19 DNA substrates were from Pharmacia Biotech. The *E. coli* strains used were HB101 (Gibco BRL), XL1 MRF′ (Stratagene), GM2929[33] and ER1398.[34]

Plasmid constructions

The *Pvu*II methyltransferase and endonuclease genes were obtained by PCR using pPvuII-3.4 as template.[32] The restriction sites incorporated into the PCR primers are underlined in the sequences and their names are given in parentheses.

The *Pvu*II methyltransferase gene was cloned on a 1.44 kb *Eco*RI-*Bam*HI fragment, obtained by using the PCR primers TACTTTCTTGAATTCAGGCATTTGCTA TTCGCTCA (*Eco*RI) and TTCACATTTGGATCCAA TTCCTTGAAGTGTCACCGT (*Bam*HI), into the low copy number pLG339 plasmid.[23] The resulting pLGM

plasmid was maintained in the presence of kanamycin in HB101 and XL1 MRF′ hosts and these transformed strains were used during the construction and expression of the endonuclease genes.

The gene coding for wt *Pvu*II endonuclease was obtained by using the PCR primers TCTTCTTTCA TGAGTCACCCAGATCTAAAT (*Rca*I) and TCTTA TTTATAAGCTTAGTAAATCTTTGTCCCATGT (*Hin*dIII) followed by cloning the *Rca*I/*Hin*dIII-cleaved fragment into the *Nco*I-*Hin*dIII sites of pRIZ′O_{lac} expression vector[22] to obtain pRIZ′-wtPvR. The tandem gene coding for the sc *Pvu*II was constructed in two steps. The first copy was obtained by PCR using the TCTTCTTTC ATGAGTCACCCAGATCTAAAT (*Rca*I) and TTTCTAT GGATCCGTAAATCTTTGTCCCATGTTC (*Bam*HI) primers and by cloning the *Rca*I/*Bam*HI-fragment into *Nco*I and *Bam*HI cleaved pRIZ′O_{lac}. The intermediate vector (pRIZ′-PvR NBt) obtained was then used to clone (*Bam*HI and *Hin*dIII sites) the second gene copy obtained as a *Bam*HI-*Hin*dIII fragment of a pUC19 clone, which was originally derived from an independent PCR amplification of the endonuclease gene by the TTTATTAGGATCCGGAGGAAGTCACCCAGATCTAA ATAAAT (*Bam*HI) and TCTTATTTATAAGCTTAGTA AATCTTTGTCCCATGT (*Hin*dIII) primers. The resulting vector is named pRIZ′-scPvR. The second gene copy codes also for the GlySerGlyGly peptide linker at the 5′ end of its sequence, including the *Bam*HI site. The sc gene codes for the sequence (1-157)-GlySerGlyGly-(2-157) where the numbers (1-157) and (2-157) stand for the encoded amino acid residues of the endonuclease gene and represent the first and the second subunit, respectively. Mutant sc *Pvu*II derivatives were obtained by overlap extension PCR using cloned templates in pRIZ′ or pUC vector. The constructed genes were sequenced by using a T7 sequencing kit (Pharmacia Biotech).

The pPvuII-3.4 vector containing the native *pvuIIRCWM* gene cluster was converted by cloning the *Xba*I-*Xba*I fragment of the sc gene (coding for residues (121-157)-GlySerGlyGly-(2-120)) into the unique *Xba*I site of pPvuII-3.4. The resulting pPvu12 contains the modified *pvuIIR^{sc}CWM* gene cluster. The natural and the modified systems were transferred into the low copy number vector pGB2 (between *Eco*RI and *Hin*dIII sites) to obtain pGB-Pvu15 and pGBPvu-16, respectively.

In vivo restriction assays

For viability tests, *E. coli* HB101 cells carrying either the pLG339 or the pLGM plasmid were transformed with pRIZ′-wtPvR or pRIZ′-scPvR vector, as well as with the empty pRIZ′ expression vector for control. The transformed cells were plated onto LB agar plates containing 75 mg/l ampicillin and 25 mg/l kanamycin and the plates were incubated at 37 °C. For λ phage restriction assays, unmodified and *Pvu*II-specifically modified λ_{vir} were prepared by growing the phage on *E. coli* ER1398 and on ER1398(pLGM), respectively. The restriction ratios were determined as described.[35] The results are the average of six titrations performed at 37 °C.

Protein expression, purification and analysis

HB101 or XL1 MRF′ *E. coli* strains containing the pLGM plasmid and the corresponding expression vector (pRIZ′-wtPvR or pRIZ′-scPvR) were grown in LB medium containing 75 mg/l ampicillin and 25 mg/l kanamycin at 37 °C. The protein expression was induced by adding IPTG to 0.5 mM concentration when the abosrbance of the culture at 600 nm reached 0.6 and the culture was shaken for a further three to four hours at 37 °C. The purification scheme was similar to that described[32] but with modifications as follows. Cells were disrupted by sonication in 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 10 mM 2-mercaptoethanol, 50 mM KCl and Complete.™ protease inhibitor cocktail (Roche Molecular Biochemicals). After the polyethyleneimine and ammonium sulfate precipitation steps, the pellets were dissolved and dialysed against 20 mM potassium phosphate (pH 7.0) buffer containing 1 mM EDTA, 10 mM 2-mercaptoethanol and 10 mM KCl. Purification on HiTrap^{R} SP-Sepharose column (Pharmacia Biotech) was performed in this buffer by applying a linear gradient of KCl (10 mM-500 mM). The peak fractions were further purified, after dialysis, on HiTrap.^{R} Q-Sepharose column (Pharmacia Biotech) in 20 mM Tris-HCl (pH 7.9), 1 mM EDTA, 10 mM 2-mercaptoethanol, 10 mM KCl using a linear gradient of 10 mM-500 mM KCl. The sc enzyme eluted between 120 and 150 mM, and the wt between 150 and 180 mM salt concentrations. The purified proteins were stored at 4 °C.

Protein purities were checked by SDS-12.5 % PAGE. The protein concentrations were determined by using the calculated molar extinction coefficient 71120 $M^{-1}cm^{-1}$ for the sc molecule and for the non-covalent wt dimer at 280 nm. Gel-filtration was performed on a Superdex 75 HR 10/30 column (Pharmacia Biotech) in 20 mM Tris-HCl (pH 7.9), 1 mM EDTA, 10 mM 2-mercaptoethanol, 100 mM KCl. The molecular masses were determined by electron spray mass spectrometry on an API 150 EX instrument (Perkin Elmer).

Cleavage experiments and kinetic analysis

DNA cleavages were performed at 37 °C in 10 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiotreitol (buffer 2 of New England Biolabs) containing 0.1 mg/ml bovine serum albumin. The substrate concentrations, calculated in terms of *Pvu*II site equivalents, were 45 nM (50 μg/ml) for λ and 10 nM (28.6 μg/ml) for pBR322, respectively. Reactions were terminated by adding a mixture of EDTA (to 50 mM concentration) and loading buffer and the reaction mixtures were analyzed by 0.6 % agarose gel electrophoresis.

For kinetic studies, a 258 bp region of the pUC19 (between positions 246 and 503) was obtained by large-scale PCR amplification using CCATTCAGGCTGCG-CAACT and GTGAGCGGATAACAATTTCACAC primers followed by purification on HiTrap^{R} Q-Sepharose column. The same PCR product was obtained as radioactive tracer in an independent reaction by using the first primer in 5′-^{32}P phosphorylated form. The cleavages were performed as described above by using 20 pM enzyme and 0.5 to 50 nM substrate concentrations. Samples taken at defined time-points were analyzed by electrophoresis on 8 % polyacrylamide gel (19:1 (w/w) acrylamide/bisacrylamide) to separate the 63 bp labeled product from the uncleaved substrate. The gel was fixed in 10 % (v/v) acetic acid, dried and evaluated by the Cyclone™ Phosphor Storage System (Packard). Data were analyzed to obtain the best fit to the Michaelis-Menten equation by using the KaleidaGraph™ software (v.3.08, Synergy Software).

Electrophoretic mobility shift assay (EMSA)

The 42 bp dsDNA probe corresponding to the TTAATTCTTAGTCTGTAGGCAGCTGCCTACAGAC-TAAGAATAA sequence was obtained by self-annealing of the 5′-^{32}P-TTAATTCTTAGTCTGTAGGCAGCTGCCT oligonucleotide (the *Pvu*II site is underlined) followed by Klenow fill-in reaction in the presence of dNTPs. The probe concentration was under 2 pM in the binding reactions. Binding and electrophoresis buffers were as described.[17] Binding was performed at room temperature for 30 minutes and 15% polyacrylamide gels (29:1 (w/w) acrylamide/bisacrylamide) were run at 4°C and 25V/cm. Dried gels were evaluated as described above and the data obtained were analyzed by curve-fitting to the binding isotherm $\Theta = 1/(1 + K_d/P_t)$, where Θ is the fraction of the bound DNA probe, K_d is the equilibrium dissociation constant and P_t is the total protein concentration.

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