Converting *MlyI* endonuclease into a nicking enzyme by changing its oligomerization state

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N.BstNBI is a nicking endonuclease that recognizes the sequence GAGTC and nicks one DNA strand specifically. The Type IIs endonuclease, *MlyI*, also recognizes GAGTC, but cleaves both DNA strands. Sequence comparisons revealed significant similarities between *N.BstNBI* and *MlyI*. Previous studies showed that *MlyI* dimerizes in the presence of a cognate DNA, whereas *N.BstNBI* remains a monomer. This suggests that dimerization may be required for double-stranded cleavage. To test this hypothesis, we used a multiple alignment to design mutations to disrupt the dimerization function of *MlyI*. When Tyr491 and Lys494 were both changed to alanine, the mutated endonuclease, *N.MlyI*, no longer formed a dimer and cleaved only one DNA strand specifically. Thus, we have shown that changing the oligomerization state of an enzyme changes its enzymatic function. This experiment also established a protocol that could be applied to other Type IIs endonucleases in order to generate more novel nicking endonucleases.

INTRODUCTION

Few nucleases break just one strand of DNA thereby introducing a nick into DNA. Most such proteins are involved in DNA replication, DNA repair and other DNA-related metabolisms [e.g. gpII (Higashitani *et al.*, 1994) and MutH (Modrich, 1989)], and cannot easily be used to manipulate DNA. Indeed they usually recognize long sequences and associate with other proteins to form active complexes that are difficult to manufacture. None of these nicking enzymes is commercially available. Recently two nicking enzymes with unprecedented features were isolated from the thermophilic bacteria *Bacillus stearothermophilus*: *N.BstSEI* (Abdurashitov *et al.*, 1996) and its isoschizomer *N.BstNBI* (Morgan *et al.*, 2000). They differ from typical restriction enzymes in that they cleave only the top DNA strand (as the site is conventionally represented), which contains

the sequence 5'-GAGTC-3', four bases downstream from that sequence. These nicking enzymes can be used to introduce nicks and gaps into DNA substrates and thus are useful for studying mismatch repair (Wang and Hays, 2000). A more general potential application is in strand displacement amplification (SDA), which is a fast, isothermal DNA amplification technology (Walker *et al.*, 1992; Walker, 1993). SDA is being used as a diagnostic method for detecting infectious agents, such as *Chlamydia trachomatis* (Spears *et al.*, 1997). Currently SDA reactions require incorporation of α -thio-deoxynucleotides (dNTP α S) into the DNA, to prevent cleavage on one DNA strand. A restriction endonuclease will only cleave the unmodified strand in the primer region, producing a nick. This nick is then extended by a DNA polymerase, which displaces the parental DNA strand. Use of nicking enzymes could potentially eliminate the need for dNTP α S, increase the flexibility of the protocol, as well as lowering the cost and increasing the efficiency of SDA reactions.

It would be helpful if there were other nicking endonucleases available. The rarity of reported nicking enzymes might reflect their limited natural occurrence or the difficulty in unambiguously detecting their nicking activity. An alternative source of nicking endonucleases might be the engineering of existing restriction endonucleases. Interestingly, previous investigations suggested that *N.BstNBI* is a naturally mutated Type IIs endonuclease (Higgins *et al.*, 2001). To assess whether Type IIs enzymes can be engineered into nicking endonucleases, we have chosen the Type IIs endonuclease *MlyI* as a model for conversion because *MlyI* shares several similar features with the naturally existing nicking enzyme *N.BstNBI*. Both enzymes recognize the same DNA sequence, 5'-GAGTC-3', and share significant sequence similarity (Higgins *et al.*, 2001). In this paper we demonstrated the feasibility of converting a Type IIs endonuclease into a strand-specific nicking enzyme by changing its oligomerization state using PCR-mediated mutagenesis.

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Fig. 1. Multiple sequence alignment of the C-terminal domains of *PleI*, *MlyI*, *N.BstNBI* and *FokI*. Identical residues are shown as white on black, and conservative replacements are black on gray. Dots represent gaps introduced to improve the alignment. Filled circles indicate the residues that were changed to alanine by mutagenesis. Motif YGGK is conserved in *PleI* and *MlyI* but absent in *N.BstNBI*. In *FokI* this pattern seemed to be reversed as KGN Y. Both tyrosine (Tyr491) and lysine (Lys494) were changed to alanine in *MlyI* and are indicated by stars. The $\alpha 4$ and $\alpha 5$ helices of *FokI* dimerization interface are drawn under the *FokI* amino acid sequence. Pileup and Prettybox programs were used with a gap creation penalty of 3 and gap extension penalty of 1.

RESULTS

Disrupting the dimerization interface of *MlyI*

Previous gel filtration results showed that *MlyI* and *N.BstNBI* are both monomers in solution and that *MlyI* apparently dimerizes in the presence of specific DNA, whereas *N.BstNBI* remains as a monomer (Higgins *et al.*, 2001). This suggests that mutations may have disrupted the *N.BstNBI* dimerization interface, altering its double-stranded cleavage activity into a nicking activity. Therefore, if the dimerization function of a Type II restriction enzyme could be abolished by mutation, it might become a nicking enzyme. To engineer *MlyI* into a nicking endonuclease, the first step was to search for the potential dimerization interface of *MlyI*.

FokI crystallization studies showed that *FokI* dimerization is mediated primarily through helix $\alpha 4$ and possibly parallel helix $\alpha 5$ of the cleavage domain, located <10 amino acid residues away from its catalytic site PD-DTK (Wah, 1998). The amino acid sequences of the C-terminal half of *PleI*, *MlyI*, *N.BstNBI* and *FokI* endonucleases were aligned using the programs Pileup and Prettybox (Figure 1). The computer alignment of the last 200 amino acid residues of *PleI*, *MlyI* and *FokI* showed some clusters of conserved residues. Some charged residues, which seemed to be in a region corresponding to the *FokI* dimerization domain, were changed in *MlyI* (S446A, R450A, H451A, D454A, R457A, D460A, K471A, K471E, P473K, S436K and F480A/F481A). None of these mutations abolished the double-stranded DNA cleavage activity of *MlyI*, suggesting that none of these residues is essential for DNA cleavage or dimerization.

A closer look at the residues conserved in *PleI*, *MlyI* and *FokI*, but not in *N.BstNBI*, suggested a new potential mutagenesis

target. We focused on a short region (four amino acids) that seems to be missing in *N.BstNBI*, but is present in *PleI*, *MlyI* and *FokI* (Figure 1). The conserved residues of this region were a tyrosine, a lysine and a glycine. In *MlyI* and in *PleI* the conserved pattern was YGGK. Both Tyr491 and Lys494 in *MlyI* were changed to alanine. Preliminary activity assays using crude cell extracts containing the mutated *MlyI* showed specific DNA nicking activity and *MlyI*-Y491A/K494A was renamed *N.MlyI*.

N.MlyI is a specific nicking endonuclease with high specific activity

N.MlyI was purified to >95% homogeneity (see Methods). The purified *N.MlyI* was tested with λ DNA. No double-stranded DNA cleavage was detected. The nicking activity of *N.MlyI* was further examined using plasmid DNAs. The supercoiled form of an undigested plasmid can be converted into a nicked open circular form when one strand is cleaved by a nicking endonuclease, or into a linear form when both strands are cleaved in proximity by a restriction enzyme. When plasmid pNB1 containing one *MlyI* recognition site was used in the digestion assay, pNB1 was converted into a nicked open circular form by *N.MlyI* and the nicking enzyme *N.BstNBI*, and into linear form by wild-type *MlyI* (Figure 2). When plasmid pNB0 containing no *MlyI* site was used in the same assay, pNB0 remained in the supercoiled form following the digestions (Figure 2). This result suggested that the nicking activity of *N.MlyI* was sequence specific. *N.MlyI* activity was titrated using pNB1. One unit was defined as the amount of *N.MlyI* needed to achieve complete nicking of 1 μ g of pNB1 in 1 h at 37°C. The specific activity of the mutated *MlyI* was ~400 000 units per mg of protein, which is very similar to that of wild-type *MlyI*.

Fig. 4. Gel filtration results. The K_{av} value for each protein standard (on the linear scale) was plotted against the corresponding molecular weight (on the logarithmic scale). Those standard proteins (ovalbumin, 43 kDa; albumin, 67 kDa; aldolase, 158 kDa; catalase, 232 kDa) are represented by unfilled squares. The K_{av} of the proteins of interest (black diamonds) were located on this calibration curve and the corresponding molecular weights were calculated using the calibration curve equation: $K_{av} = -0.266 \log(\text{molecular weight}) + 1.658$.

1999). To cleave two DNA strands with one catalytic center, the endonuclease either has to form a dimer or undergo major conformational changes between the two cleavage events. Most of the Type II and Type IIs restriction endonucleases catalyze double-stranded cleavage by forming dimers (Wilson and Murray, 1991). Some restriction endonucleases exist as monomers in solution, but form dimers in the presence of DNA and divalent metal ion, e.g. *FokI* (Vanamee *et al.*, 2001), *Sau3AI* (Friedhoff *et al.*, 2001) and *MlyI* (Higgins *et al.*, 2001).

If the dimerization function of a restriction enzyme was eliminated by mutation, it might become a nicking enzyme. Pingoud and coworkers generated a nicking endonuclease from the Type II endonuclease *EcoRV* by combining a subunit with an inactive catalytic center with a subunit that had a defect in the DNA binding site. The mutated *EcoRV* is a heterodimer that only nicks DNA within the *EcoRV* recognition sequence (Stahl *et al.*, 1996). However, since the recognition site is palindromic this type of heterodimer does not have strand-specific activity. Type IIs endonucleases recognize non-palindromic sequences. Thus, abolition of their dimerization function may convert them into strand-specific nicking endonucleases. When the dimerization function of the Type IIs endonuclease *FokI* was abolished by site-directed mutagenesis, the resulting monomeric *FokI* showed very low DNA nicking activity, probably due to the fact that *FokI* cleaves two DNA strands concertedly (Bitinaite *et al.*, 1998).

In contrast, N.*MlyI* shows very high DNA nicking activity (~400 000 units per mg of protein). This high specific activity not only eliminates suspicion that N.*MlyI* might be a near-dead *MlyI*

variant, which was not active enough to perform double-stranded cleavage, but also sheds some light on the mechanism of the cleavage reaction catalyzed by Type IIs endonucleases. A previous study showed that *MlyI* cleaves DNA in two sequential steps: DNA is nicked on one strand and then the nicked DNA is further cleaved via dimerization of *MlyI* (Higgins *et al.*, 2001). Results from this study are consistent with the two-step cleavage theory. Mutation in the YGGK motif of *MlyI* resulted in a monomeric N.*MlyI*, which can still catalyze the first step of the cleavage reaction but completely fails to catalyze the second cleavage step.

This mechanistic distinction between *MlyI* and *FokI* may be the reason why the monomeric N.*MlyI* is a much more active nicking enzyme than the monomeric *FokI*. Mutating the dimerization function of *MlyI* blocks only the second step reaction, which by hypothesis is mediated by dimerization; but lack of dimerization has little (if any) effect on the first-step DNA nicking reaction. In contrast, abolishing the *FokI* dimerization function may affect DNA cleavage on both DNA strands because the two cleavage steps are concerted and coupled.

In addition, N.*MlyI* specifically cleaves only the top strand containing its recognition sequence 5'-GAGTC-3', as in N.*BstNBI*. Based on these results, we suggest that *MlyI* binds to its asymmetric recognition sequence 5'-GAGTC-3' in an orientation enabling it to cleave only the top strand, with the bottom strand cleaved normally by a second *MlyI* molecule interacting with the first enzyme molecule.

In this study, we successfully demonstrated that a Type IIs endonuclease, such as *MlyI*, could be converted into a strand-specific nicking enzyme by changing its oligomerization state (from dimer to monomer). More generally, we have shown that changing the oligomerization state of an enzyme changes its enzymatic function. This study suggests that the Type IIs endonucleases, which generate nicking intermediates before complete digestion on both DNA strands, may be the preferred candidates for conversion. Our data also support the idea that N.*BstNBI* is a mutated Type IIs enzyme, whose dimerization function was probably limited by mutations. It would be interesting to test whether the double-stranded cleavage activity of N.*BstNBI* can be restored by protein engineering.

METHODS

Strains and plasmids. *Escherichia coli* strain ER2502 [*fhuA2 ara-14 leu D(gpt-proA)62 lacY1 glnV44 galK2 rpsL20 endA1 R(zgb210::Tn10) Tet S xyl-5 mtl-1 D(mcrC-mrr) HB101*] was obtained from E. Raleigh and M. Sibley (New England Biolabs). To express the gene encoding *MlyI* endonuclease (*mlyIR*) cloned into pUC19 without damaging the host DNA, a pSYX20 plasmid carrying the gene encoding N.*BstNBI* methylase was transformed into *E. coli* strain ER2502. The pNB0 and pNB1 plasmids contain zero or one N.*BstNBI* site, respectively. To construct those pNB0 and pNB1 plasmids, six or five N.*BstNBI* sites were, respectively, removed from Litmus28 plasmid by site-specific mutagenesis (Morgan *et al.*, 2000).

Gel filtration. Gel filtration was performed on an Amersham Pharmacia Akta FPLC system using a Pharmacia Superdex 200 HR 10/30 column. The column was equilibrated with a buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM CaCl₂, 0.1 mM EDTA, 7 mM 2-mercaptoethanol, 5%

glycerol. Protein elution was detected by recording the absorption at 280 nm. The linear standard curve was generated by using the calibration kits (low and high molecular weight) from Pharmacia. Four nanomoles of purified *MlyI* or *N.MlyI* were mixed with an excess of either non-specific or specific DNA duplexes, which were generated by annealing two complementary oligodeoxynucleotides (27mer) in a buffer containing 50 mM potassium acetate, 20 mM Tris–acetate pH 7.9, 10 mM calcium chloride, 1 mM dithiothreitol (DTT). The samples were incubated for 5 min at 37°C before being loaded onto the column. As controls the protein or the oligodeoxynucleotides were loaded alone. A similar experiment was carried out with *N.BstNBI* with a few changes: a buffer containing 10 mM Tris–HCl, 150 mM KCl, 10 mM CaCl₂, 1 mM DTT was used and the samples were incubated for 5 min at 55°C.

Construction of dimerization-deficient mutated *MlyI*. *MlyI* residues that were conserved among *MlyI*, *PleI* and *FokI*, and that aligned with residues thought to be important for *FokI* dimerization, were changed into alanine by PCR-mediated site-directed mutagenesis (Morrison and Desrosiers, 1993). Two residues, conserved among the three Type IIs restriction endonucleases but not found in *N.BstNBI*, were changed to alanine as well. The following forward mutagenic primer was used for changing both Tyr491 and Lys494 to alanine: 5′-ACATGCTGG-TGGAGCAACAAAGATTATTC-3′. The constructs were sequenced to confirm the mutations and to check that no additional mutations were introduced during PCR.

Expression and purification of *N.MlyI*. *Escherichia coli* ER2502 cells containing pUC19-*mlyI*R-Y491A/K494A (pUC19-*N.MlyI*) were grown at 37°C in LB medium supplemented with 0.1 mg/ml ampicillin. The cells were harvested by centrifugation. The following procedures were performed on ice or at 4°C. One hundred grams of cell pellet (wet weight) were resuspended in 327 ml of buffer A (20 mM potassium phosphate buffer pH 6.9, 0.1 mM EDTA, 7 mM 2-mercaptoethanol, 5% glycerol) supplemented to 50 mM NaCl, and broken with a Manton-Gaulin homogenizer. Sigma Protease Inhibitor solution (25 ml) was added after the first pass. The extract was centrifuged at 15 000 *g* for 40 min at 4°C. The supernatant was passed through a 392 ml Heparin HyperD AP5, an 80 ml (3.5 × 8.3 cm) Source-15Q Finesline 35, a 6 ml (1.6 × 3.0 cm) Source 15S and an 8 ml (1 × 11.3 cm) Heparin TSK5pw column. The purification steps were all performed using Pharmacia AKTA FPLC system. The activity assays were carried out on T7 DNA in order to detect specific nicking activity at *MlyI* sites (Morgan *et al.*, 2000).

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