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Photocaged Variants of the MunI and PvuII Restriction Enzymes

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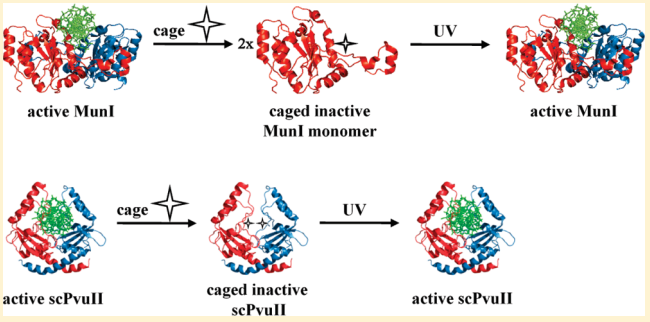
Supporting Information

ABSTRACT: Regulation of proteins by light is a new and promising strategy for the external control of biological processes. In this study, we demonstrate the ability to regulate the catalytic activity of the MunI and PvuII restriction endonucleases with light. We used two different approaches to attach a photoremovable caging compound, 2-nitrobenzyl bromide (NBB), to functionally important regions of the two enzymes. First, we covalently attached a caging molecule at the dimer interface of MunI to generate an inactive monomer. Second, we attached NBB at the DNA binding site of the single-chain variant of PvuII (scPvuII) to prevent binding and cleavage of the DNA substrate. Upon removal of the caging group by UV irradiation, nearly 50% of the catalytic activity of MunI and 80% of the catalytic activity of PvuII could be restored.



The ability to regulate biological processes by an external signal (e.g., light) seems to be very attractive and useful for many in vivo and in vitro applications and/or experiments. This could be achieved by caging of biomolecules with photoremovable compounds. The principle of this strategy is simple: the caged biomolecule is inactivated, while irradiation with light removes the cage resulting in reactivation of the biomolecule. When the biological process one wishes to regulate is an enzymatic reaction, each participant in this reaction (substrate, inhibitor, cofactor, and enzyme) could be targeted for caging.[1] Introduction of a photoremovable protecting group into a substrate could prevent its binding by the enzyme. A successful example exploiting this strategy made use of the incorporation of 6-nitropiperonyloxymethyl (NPOM)-caged thymine into DNA at a restriction enzyme recognition site, which dramatically decreased the level of DNA cleavage by the restriction enzymes EcoRI, BglII, and BamHI.[2] DNA cleavage was completely restored upon removal of the caging group by UV irradiation. In the case of the Src tyrosine kinase, bivalent peptide-derived inhibitors containing a photocleavable group were synthesized.[3] Inhibitors associate with the active site of the SH1 domain and the regulatory SH2 domain blocking Src activity; photolysis splits the inhibitors and releases the active enzyme. Cofactors such as $NADP^+$ and NAD^+, ATP, or Mg^{2+} have been caged, allowing regulation of various enzymatic reactions.[4,5]

Several caging strategies are available when the target is an enzyme or protein.[1] Various functional groups of a protein (carboxylates, amines, and thiols) can be caged by chemical modification or introduction of unnatural (caged) amino acids by in vitro or in vivo translation. Furthermore, different functionally important regions (active site, substrate binding site, and oligomerization interface) of the enzymes can be selected for modification to abolish their activity. Chemical caging of the active site cysteines of the SssI DNA (cytosine-C5)-methyltransferase with 4,5-dimethoxy-2-nitrobenzyl bromide (DMNBB) decreases the activity by up to 95%, while irradiation of the caged methyltransferase restored 60% of its activity.[6] The *Taq* polymerase was inactivated by sterically blocking the space reserved for incoming dNTPs.[7] In this case, a tyrosine residue containing the 2-nitrobenzyl group was introduced at position 671 using an in vivo translation system. Irradiation with UV light regenerated the wild-type (wt) *Taq* polymerase and thus restored DNA polymerization activity. When an enzyme is active in a particular oligomeric state, there is a possibility of disrupting protein−protein interaction or damaging communication between different monomers by introducing a bulky photoremovable compound (see below as an example the restriction endonuclease BamHI).

Photoremovable caging compounds could be used to regulate the activity of DNA-cutting enzymes, which could have potential applications in gene targeting and gene therapy.[8] Homing endonucleases that recognize and cleave large (>12 bp) DNA sequences were successfully used as scaffolds for generating molecular scissors for a variety of genomic applications.[9−12] The type II restriction endonucleases that specifically act on

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short (4−8 bp) DNA sequences could be adapted for gene targeting purposes by fusing them to a DNA binding module recognizing long DNA sequences. The nonspecific DNA cleavage domain of the type IIS restriction endonuclease FokI was extensively used for fusion with various DNA binding modules such as Zn-finger motifs,[13−17] TALE repeats,[18−20] a noncleaving mutant of the homing endonuclease I-SceI,[21] and other DNA binding domains.[22−27] Triplex-forming oligonucleotides may serve as DNA binding modules as demonstrated for the PvuII restriction endonuclease.[28] However, despite the advances made in the construction of specific DNA scissors, cleavage of off-targets remains a major limitation for their application in gene therapy (in vivo),[16,17,29] which could be minimized by controlling the activity of the nucleases. For example, formation of the DNA triple helix is quite slow; therefore, the active enzyme in the restriction enzyme−triple-helix-forming oligonucleotide fusion protein could start to cleave nonspecific DNA target sites that are not addressed by triple helix formation. Caging with a photoremovable compound could help to regulate activity of these designed enzymes and prevent possible nonspecific activity.

To date, most of the work on caging of DNA-cleaving enzymes with photosensitive compounds was conducted with the type IIP restriction endonuclease BamHI. In the case of BamHI, which is active as a dimer, the dimerization interface was chosen as the target for caging to control the enzyme activity.[30−32] Unnatural amino acids, a lysine derivative with a photoremovable compound (6-nitroveratryloxycarbonyl) and alanine derivatives such as azophenylalanine or 4′-carboxyphenylazophenylalanine, which undergo *cis*−*trans* isomerization upon illumination, were incorporated at the dimer interface of BamHI via substitution of amino acid Lys132 using an in vitro translation system with special codons and the corresponding tRNA.[30−32] These light sensitive compounds destroy the intricate salt bridge network at the dimer interface, thereby inactivating the enzyme. Irradiation of the caged enzymes with UV light restores their catalytic activity.

Here we demonstrate activity regulation of two restriction endonucleases, MunI and PvuII, using near-UV light. We used two different strategies. We chemically introduced a photoremovable cage compound, 2-nitrobenzyl bromide (NBB), at the dimer interface of MunI, converting the nuclease into an inactive monomer form, or at the DNA binding site of PvuII, preventing substrate binding and cleavage. Irradiation of the caged restriction endonucleases with near-UV light restores up to 50% of MunI's cleavage ability and 80% of PvuII's cleavage ability as compared with those of the uncaged enzymes.

MATERIALS AND METHODS

Strains and Plasmids. *MunI.* Plasmid pUHE25-2-MunIR (Ap^r) containing the restriction endonuclease gene munIR was used for protein expression in *Escherichia coli* ER2267 cells containing a compatible plasmid pMunIM 6.2 (Tc^r, Cm^r) harboring the methyltransferase gene *munIM*.[33]

PvuII. We have chosen the single-chain variant of the naturally homodimeric restriction enzyme PvuII (scPvuII) for our study.[34] The plasmid encoding the C-terminal His_6-tagged variant scPvuII(S81C)$_2$, harboring a cysteine residue at position 81 in both "halves" of the pseudodimer, was generated using plasmid pRIZ′-scPvuII (Ap^r)[34] as a template and employing a PCR-based site-directed mutagenesis method.[35] The sequence of the mutated gene was confirmed by sequencing. For protein expression, pRIZ′-scPvuII(S81C)$_2$ was used to transform *E. coli* XL10-Gold cells, which contain the pLGM plasmid (Cm^r) encoding the PvuII methyltransferase.

Protein Expression and Purification. ER2267 cells containing the pUHE25-2-MunIR plasmid were grown in LB medium containing 50 mg/L Ap and 30 mg/L Cm at 37 °C. After being induced with 0.2 mM IPTG for 4 h, cells were harvested by centrifugation.

MunI was purified according to the method described in ref 36 and stored at −20 °C in storage buffer [10 mM Tris-HCl (pH 7.4 at 25 °C), 100 mM KCl, 1 mM EDTA, 1 mM DTT, and 50% (v/v) glycerol]. The enzyme was >95% homogeneous as shown by SDS−PAGE analysis. The concentration of protein was determined spectrophotometrically at 280 nm using an extinction coefficient of 45720 M^{-1} cm^{-1}.

scPvuII(S81C)$_2$ was expressed in *E. coli* XL10-Gold cells, purified as described previously,[37] and finally stored at −20 °C in 20 mM sodium phosphate (pH 7.4), 300 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 0.01% (w/v) Lubrol, and 50% (v/v) glycerol. The enzyme was >95% homogeneous as shown by SDS−PAGE analysis. The concentration of protein was determined spectrophotometrically at 280 nm using an extinction coefficient of 71240 M^{-1} cm^{-1}.

GdmCl-Induced Protein Unfolding of MunI. We conducted protein denaturation experiments by mixing 1 μM (for fluorescence and AUC analysis) or 8 μM (for CD analysis) MunI with various concentrations of GdmCl in 10 mM Tris-HCl (pH 7.5 at 25 °C), 200 mM KCl, and 1 mM DTT. Samples were incubated overnight at 25 °C.

Fluorescence spectra were recorded at 25 °C in the thermostatically controlled cell holder of a Perkin-Elmer LS 50 luminescence spectrophotometer. The excitation wavelength was 295 nm, and the emission was recorded from 310 to 380 nm using a 5 nm bandwidth. Differences in fluorescence intensity between native and denatured protein were measured at a fixed emission wavelength of 335 nm. Data reported here represent the average of three spectra.

CD spectra were recorded at 25 °C in the thermostatically controlled cell holder of a Jasco J-710 spectropolarimeter, using a cylindrical cuvette with a light path of 0.05 cm. CD spectra were recorded between 185 and 250 nm. To monitor the denaturation of MunI, 10 readings of the CD signal at 220 nm were taken (with a 20 s averaging time) and the data averaged.

Analytical Ultracentrifugation of MunI. GdmCl-induced protein denaturation was monitored with an analytical ultracentrifuge by determining apparent molar masses from sedimentation equilibrium experiments. Samples contained 5.4 μM MunI, 10 mM Tris-HCl (pH 7.4 at 25 °C), 200 mM KCl, and different concentrations of GdmCl. A Beckman XLA analytical ultracentrifuge equipped with UV absorption optics scanning the samples at 280 nm, an eight-hole analytical rotor, and six-channel charcoal filled Epon centerpieces was used. Samples were spun at 16000 rpm and 20 °C and scanned continuously until no change in the concentration profile could be detected over a period of 12 h. Scans collected over these 12 h were averaged and evaluated assuming a single sedimenting species, yielding apparent molar masses.[38]

Caging of MunI and PvuII. Before being caged, the MunI protein was dialyzed against 10 mM sodium phosphate (pH 7.4 at 25 °C) and 0.1 M NaCl. Caging of MunI was performed by overnight incubation of 60 μM protein (monomer concentration) with 500 μM 2-nitrobenzyl bromide (NBB) in the

presence of 1 M GdmCl at 4 °C. After the reaction, unreacted NBB was quenched with 1 mM DTT. The reaction mixture was dialyzed against 10 mM sodium phosphate (pH 7.4 at 25 °C) and 0.1 M NaCl. The caged MunI was purified by gel filtration using a Superdex 200 HR column (Amersham Biosciences) equilibrated with 20 mM Tris-HCl (pH 7.5 at 25 °C) and 0.2 M KCl. Fractions containing caged MunI were pooled, dialyzed against 10 mM Tris-HCl (pH 7.4 at 25 °C), 100 mM KCl, 1 mM EDTA, and 50% (v/v) glycerol, and stored at −20 °C.

The scPvuII(S81C)$_2$ protein was dialyzed against 20 mM sodium phosphate (pH 7.4 at 25 °C), 100 mM NaCl, and 0.5 mM EDTA to remove DTT from the storage buffer. Caging of scPvuII(S81C)$_2$ was performed by incubation of 10 μM protein with 900 μM NBB for 2 h at 25 °C in 20 mM Tris-HCl (pH 8.5 at 25 °C), 100 mM NaCl, and 0.5 mM EDTA. The caging reaction was stopped by the addition of 9 mM DTT. Finally, a Vivaspin 500 spin column (Satorius, Goettingen, Germany) was used to remove unreacted NBB and to change the buffer to 20 mM sodium phosphate (pH 7.4 at 25 °C), 100 mM NaCl, 0.5 mM EDTA, and 1 mM DTT.

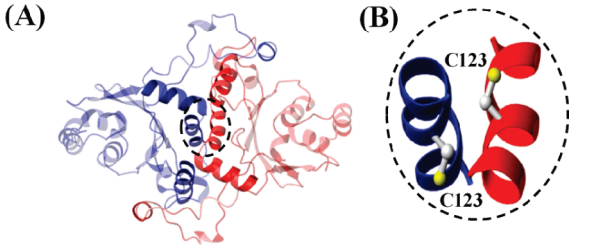

Figure 1. Dimer interface of MunI. (A) Crystal structure of the MunI restriction endonuclease. Ribbon representation of the two monomers colored blue and red, respectively. Helices involved in protein dimerization are shown in more intense colors. The region magnified in panel B is circled. (B) Close-up of a region of the MunI dimerization interface showing the cysteines residues (C123) subjected to caging.

Decaging of MunI and PvuII. Decaging of caged MunI (4 μM monomer) was performed by irradiation with UV light of different wavelengths for 1 h in 100 mM sodium acetate (pH 5.8 at 25 °C), 0.2 M KCl, and 20 mM DTT at 4 °C. The Fluoromax-3 (Jobin Yvon, Stanmore, U.K.) spectrofluorimeter equipped with a Xe lamp was used as the UV source. After irradiation, the protein sample was analyzed by gel filtration with a Superdex 200 HR column (Amersham Biosciences) equilibrated with 20 mM Tris-HCl (pH 7.5 at 25 °C) and 0.2 M KCl. The best efficiency of decaging was obtained by irradiation with a wavelength of 330 nm (29 nm bandwidth).

Decaging of caged scPvuII(S81C)$_2$ (1 μM) was performed in 20 mM sodium phosphate (pH 7.4 at 25 °C), 100 mM NaCl, 0.5 mM EDTA, and 9 mM DTT by irradiation at 325 nm using a 40 mW helium−cadmium laser (Laser 2000). After different time intervals, aliquots were withdrawn and tested for PvuII activity.

DNA Cleavage Activity. DNA cleavage activity of MunI was tested using bacteriophage λ DNA as a substrate. Protein (400 nM dimer concentration) was incubated in 50 μL with 1 μg of λ DNA in 10 mM Tris-HCl (pH 7.0 at 25 °C), 10 mM $MgCl_2$, 50 mM NaCl, and 0.1 mg/mL BSA for 15 min at 37 °C. The reaction was quenched with a loading dye solution containing 50 mM EDTA (pH 8.0 at 25 °C), 0.1% SDS, 50% (v/v) glycerol, and 0.01% bromophenol blue and analyzed by agarose gel electrophoresis.

The pUCGK-4 plasmid (provided by G. Kruckas) containing a single copy of the MunI recognition sequence was used in DNA cleavage experiments. DNA cleavage was performed by incubation of 0.75 nM wt MunI and 1.5 nM caged and decaged MunI with 1.5 nM plasmid DNA in reaction buffer at 25 °C.

To analyze the PvuII cleavage activity, we used plasmid pAT-PEB, a derivative of pAT153 with one PvuII site, as a substrate. The efficiency of the caging reaction was tested by determining the DNA cleavage activity using an excess of caged scPvuII-(S81C)$_2$ (20 nM) over plasmid DNA (9 nM) in 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl, and 0.1 mg/mL

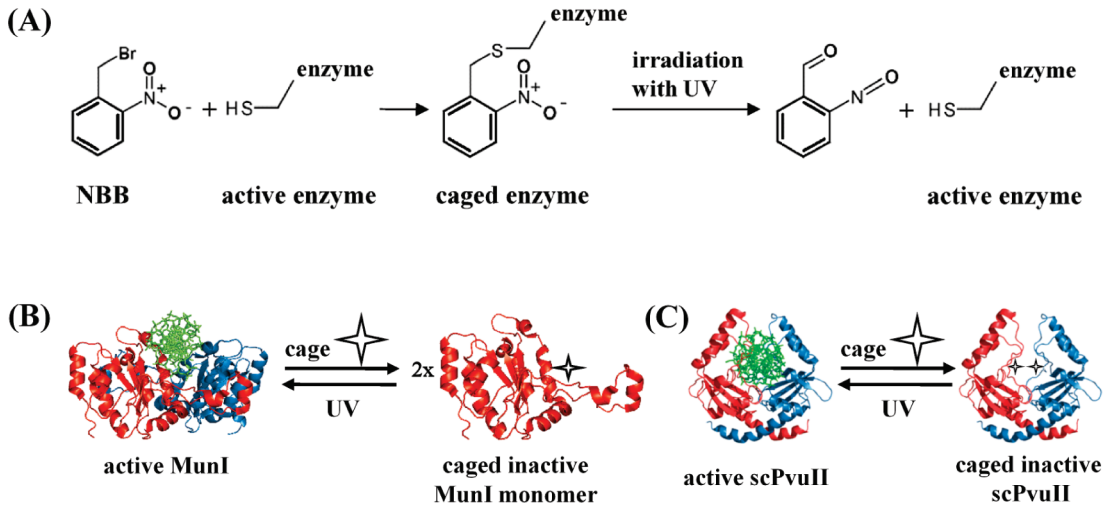

Figure 2. Generation of photoswitchable restriction endonucleases. (A) Caging of the enzyme with 2-nitrobenzyl bromide (NBB) and reactivation by irradiation with UV light. (B) Generation of photoswitchable MunI. A cysteine residue located at the dimer interface of wt MunI (residue 123) is modified using NBB. The resulting caged enzyme is an inactive monomer that is unable to bind to DNA. Irradiation at 330 nm leads to the release of the caging group and formation of the active MunI dimer. (C) Generation of the photoswitchable scPvuII. A single cysteine residue in each "half" of scPvuII located at the enzyme−DNA interface (residue 81) is modified using NBB. The resulting caged enzyme is not able to bind to scPvuII recognition sites. Illumination at 325 nm leads to the release of the caging group and reactivation of scPvuII.

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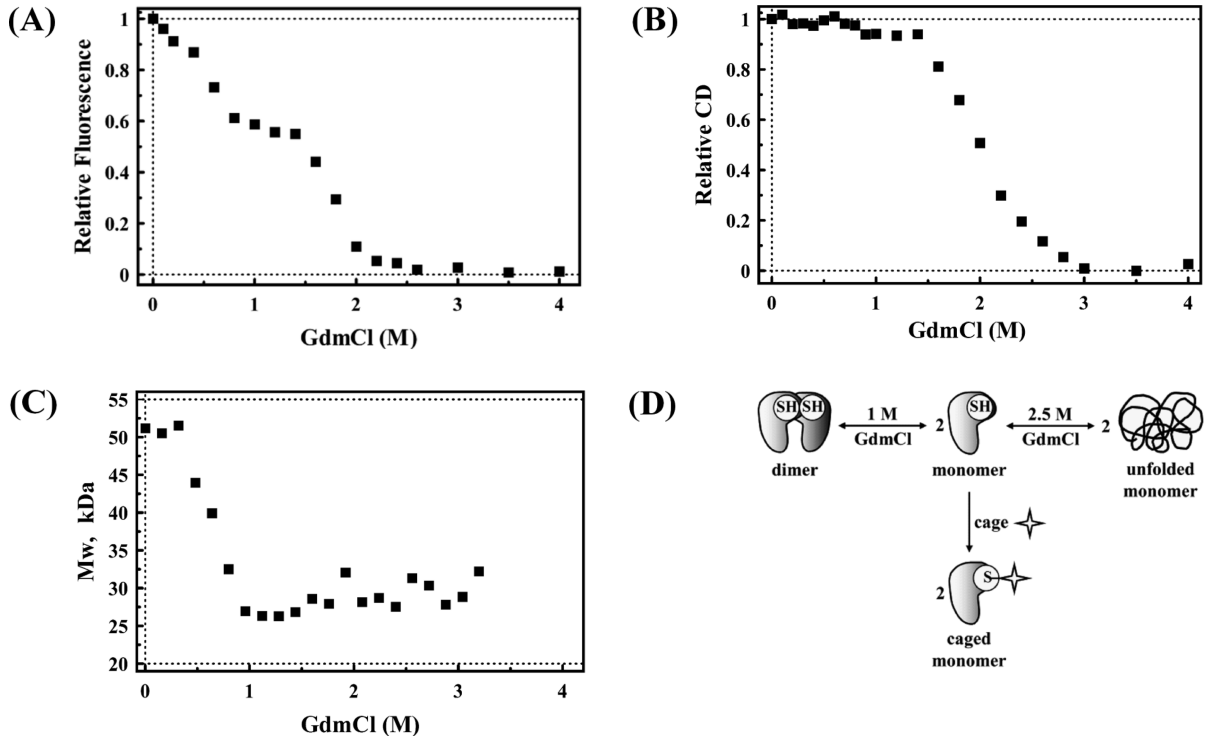

Figure 3. Unfolding of MunI induced by GdmCl. Unfolding was monitored by (A) fluorescence emission at 335 nm (λ_{exc} = 295 nm), (B) far-UV CD at 222 nm, and (C) analytical ultracentrifugation. (D) Schematic representation of the MunI denaturation pathway in the presence of GdmCl. The monomeric state of MunI at 1 M GdmCl allows caging of native cysteines at the dimer interface.

BSA at 37 °C. The recovery of activity during the decaging of caged scPvuII(S81C)$_2$ was analyzed with 2 nM enzyme and 6 nM plasmid substrate.

Samples were collected at given time intervals, and reactions were quenched with the loading dye solution and analyzed by agarose gel electrophoresis. The amounts of supercoiled (SC), open-circular (OC), and linear (FLL) DNA forms were evaluated by densitometric analysis of ethidium bromide-stained gels.

RESULTS AND DISCUSSION

Strategy. To generate photoswitchable restriction enzymes, we selected the MunI and PvuII restriction endonucleases as model systems. Both enzymes are well-characterized both biochemically and structurally. MunI recognizes the sequence 5′-C/AATTG-3′ and cuts the phosphodiester bond after the C (as indicated by the slash) to generate a double-strand break.[39] Two MunI monomers dimerize via a bundle of α-helices forming a functional dimer (Figure 1A) that symmetrically binds and cleaves the C/AATTG target site in both DNA strands.[40] The MunI dimer contains two cysteine residues at position 123 (one per monomer) at the dimer interface (Figure 1B). These cysteines are located in the dimerization helices close to each other (Figure 1B). One can assume that chemical modification of the Cys123 residues with a bulky photoremovable cage would disrupt the functionally important dimer via formation of inactive monomers (Figure 2A,B). Reactivation of the caged protein could be performed with near-UV light. Irradiation of the caged MunI removes the cage from the dimerization interface, allowing association of the monomers into the active dimer. For caging, we used 2-nitrobenzyl bromide (NBB), which dissociates from the protein after irradiation leaving unmodified cysteine residues (Figure 2A).

A different strategy was used to cage the PvuII restriction endonuclease. On the basis of the structure of PvuII bound to its specific substrate,[41] we selected different positions in the DNA binding site of the protein, which presumably prevent DNA binding if modified with a bulky photoremovable caging group (Figure 2C). To modify the protein at the selected positions, we produced single-cysteine variants using the single-chain variant of the PvuII enzyme (scPvuII).[34] scPvuII was chosen because this variant had been used before for the production of the scPvuII−triple-helix-forming oligonucleotide fusion protein.[28] For the modification studies, we selected the S81C scPvuII variant with the Ser → Cys substitution in both halves of the pseudodimer; scPvuII(S81C)$_2$ shows only slightly reduced DNA cleavage activity compared to that of scPvuII (data not shown), which in turn has a 3-fold lower activity (k_{cat}/K_m) than wt PvuII.[34]

Accessibility of Cys123 at the Dimer Interface of MunI. Titration of MunI with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)[42] showed that the native cysteines are not accessible at the dimer interface (Figure 1 of the Supporting Information). One way to make cysteines accessible for chemical modification is to induce dissociation of the MunI dimer into monomers using denaturing agents. Therefore, we investigated unfolding of MunI by guanidinium chloride (GdmCl).

MunI unfolding induced with increasing GdmCl concentrations was monitored by the intrinsic protein fluorescence and circular dichroism (CD) and by performing analytical ultracentrifugation (AUC) experiments (Figure 3). Intrinsic MunI fluorescence was measured at a wavelength of 335 nm, where the difference is largest between the native and fully denatured

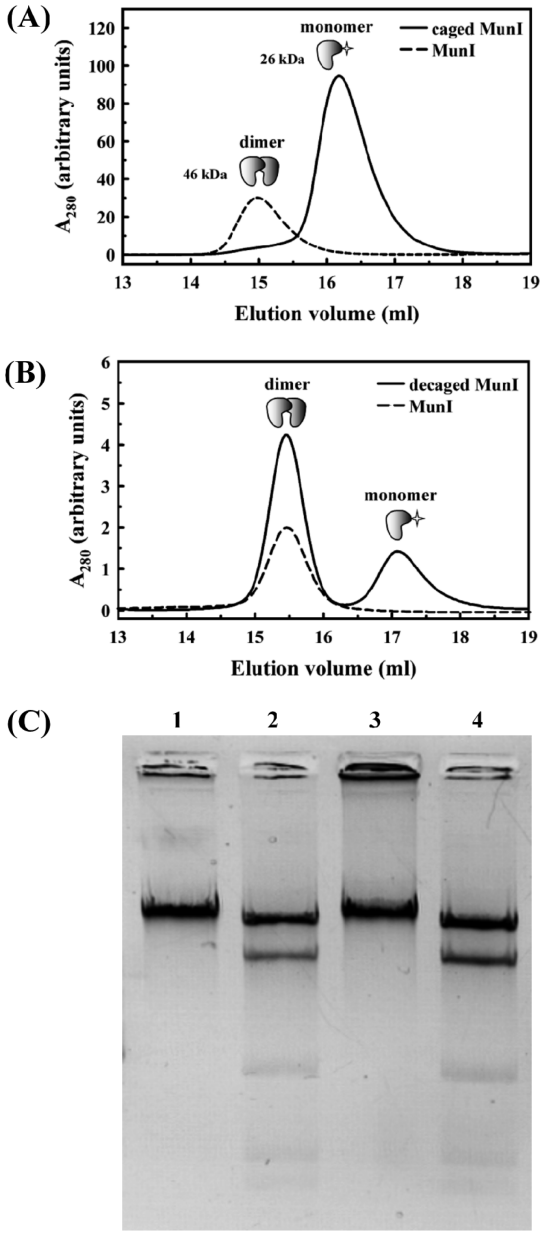

Figure 4. Caging of MunI with 2-nitrobenzyl bromide (NBB) (A) and decaging with UV light (B) monitored by gel filtration. (A) Caging of MunI with NBB at the dimer interface generates a monomer in solution. The solid line indicates reaction products after caging of MunI; the dashed line indicates the profile of wt MunI. (B) Irradiation of caged monomeric MunI with UV light promotes formation of the dimer. The solid line indicates reaction products after decaging; the dashed line indicates the profile of wt MunI. (C) Cleavage of λ DNA with caged and decaged MunI: lane 1, substrate DNA only; lane 2, cleavage with wt MunI; lane 3, cleavage with caged MunI; lane 4, cleavage with decaged MunI.

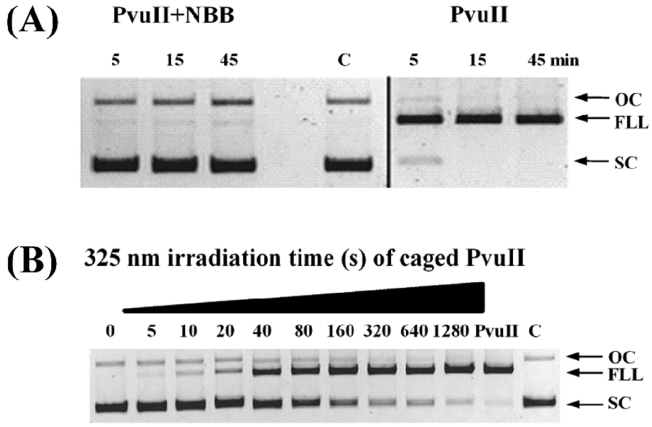

Figure 5. (A) Analysis of the cleavage activity of NBB-treated and untreated scPvuII. Experiments were performed using an excess of enzyme (20 nM) over plasmid DNA (9 nM). Samples after 5, 15, and 45 min were analyzed. (B) Decaging reaction of caged scPvuII. The plasmid cleavage activity of the caged scPvuII was analyzed after irradiation with with 325 nm light for different times (0−1280 s). The cleavage reaction was performed for 30 min with 2 nM enzyme and 6 nM plasmid substrate: uncleaved plasmid (C), untreated PvuII (PvuII), supercoiled DNA (SC), open-circular DNA (OC), and full-length linear DNA (FLL).

forms of the protein. The fluorescence of MunI decreases in two discrete stages: the first between 0 and 1 M GdmCl and the second between 1.5 and 2.5 M GdmCl (Figure 3A). Far-UV CD spectra at 222 nm showed that the molar ellipticity of MunI changed in the range from 1.5 to 3 M GdmCl (Figure 3B). The changes in the CD signal indicate loss of secondary structures by the protein accompanied by its unfolding. Therefore, the decrease in fluorescence between 1.5 and 2.5 M GdmCl corresponds to MunI unfolding (Figure 3A,B). AUC data showed that the molecular mass of MunI decreased from 51 to 26 kDa between 0 and 1 M GdmCl, indicating dissociation of the dimer into monomers (Figure 3C). Thus, the first stage of the fluorescence decrease in Figure 2A correlates with dissociation of the MunI dimer. Taken together, our data indicate that the MunI dimer first dissociates into native monomers followed by monomer unfolding with increasing GdmCl concentrations (Figure 3D). Additional studies revealed that the GdmCl-induced denaturation of MunI is completely reversible and that the refolded MunI has the same catalytic and DNA binding properties as the native protein (data not shown).

In the case of an oligomeric protein, the dissociation into monomers might occur prior to unfolding of the polypeptide chain,[43] or both processes can occur concertedly.[44] GdmCl-induced denaturation studies show that MunI first dissociates into monomers followed by monomer unfolding at increased GdmCl concentrations. A similar denaturation pathway has been reported for the tetrameric restriction endonuclease Bse634I.[45] On the other hand, dissociation and unfolding of the dimeric restriction endonucleases BfiI and PvuII occur simultaneously.[46,47]

According to the denaturation pathway of MunI (Figure 3D), the Cys123 residues located at the dimer interface should be accessible for chemical modification in the presence of 1.0−1.5 M GdmCl when the MunI dimer is dissociated into monomers. Indeed, titration of MunI with DTNB in the presence of 1.5 M GdmCl showed that the Cys123 residues are accessible and reactive (Figure 1 of the Supporting Information), allowing their caging with photochemical reagents.

Caging of MunI and PvuII with NBB. Caging of MunI was performed by overnight incubation with 2-nitrobenzyl bromide (NBB) in the presence of 1 M GdmCl at 4 °C. Reaction products were dialyzed to remove GdmCl and analyzed by gel filtration (Figure 4A). In contrast to wt MunI, which is a dimer in solution (~46 kDa), the caged MunI mainly elutes as a monomer with a molecular mass of ~26 kDa (Figure 4A and Figure 2 of the

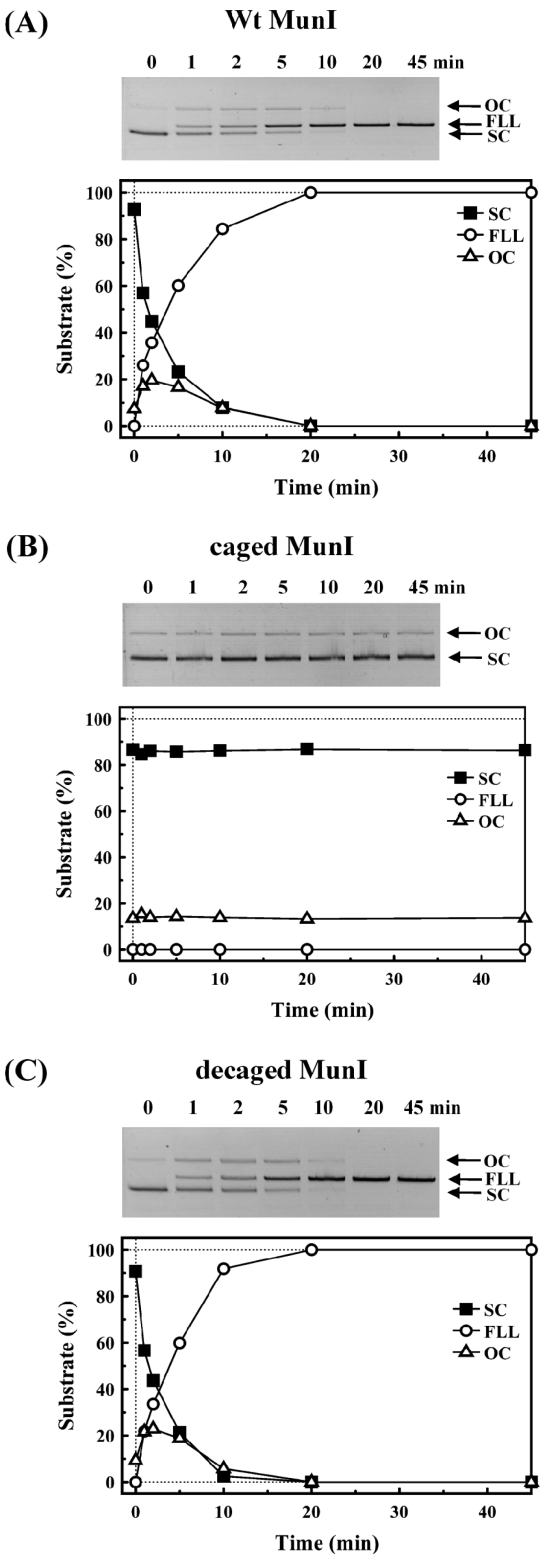

Figure 6. Photoactivation of MunI. (A−C) Cleavage of the plasmid DNA containing a single MunI recognition site by wt, caged, and decaged MunI, respectively. Kinetic experiments were performed by using 0.75 nM wt MunI and 1.5 nM caged and decaged MunI with 1.5 nM plasmid DNA. The following forms of DNA were observed during the reaction: supercoiled DNA (SC), open-circular DNA (OC), and full-length linear DNA (FLL).

Supporting Information). After caging, only a small fraction (∼10%) of MunI remains dimeric, presumably because of incomplete chemical modification (Figure 4A). Altogether, gel filtration analysis showed that the caging efficiency of MunI with NBB is high, yielding ∼90% of the caged monomer. Monomeric MunI showed no cleavage activity with phage λ DNA as the substrate (Figure 4C).

Caging of scPvuII(S81C)$_2$ was performed under native conditions using a 90-fold excess of 2-nitrobenzyl bromide over protein at 25 °C for 2 h. The reaction was stopped by the addition of DTT. After the unreacted NBB had been removed, the efficiency of the caging reaction was verified by a DNA cleavage assay (Figure 5A). The inactivity of the caged scPvuII preparation suggests that the yield of caged protein was close to 100%.

Decaging of Caged MunI and PvuII. Decaging of caged MunI was performed by irradiation with near-UV light using different wavelengths (312−350 nm) for 1 h at 4 °C. The decaging efficiency was determined by gel filtration by calculating the fraction of the MunI dimer formed after irradiation (Figure 4B). Irradiation at 330 nm yielded the largest amount (∼70%) of MunI dimer (Figure 3 of the Supporting Information). Decaged MunI showed the same λ DNA cleavage pattern (Figure 4C) as the wt enzyme.

The photoreactivation of caged scPvuII was performed by irradiation at 325 nm using a helium−cadmium laser at 4 °C. The efficiency of the decaging reaction was analyzed after defined illumination times by a DNA cleavage assay (Figure 5B).

DNA Cleavage Studies. To determine more precisely the catalytic activity of caged and decaged MunI, we performed DNA cleavage experiments using supercoiled plasmid DNA that contains a single copy of the MunI recognition sequence. At equimolar concentrations of protein and DNA, caged MunI exhibited no catalytic activity, not even nicking (Figure 6B). However, in the case of decaged MunI, the profile of plasmid DNA cleavage was similar to that of wt MunI with the exception that a ∼2-fold higher concentration of the decaged protein had to be used (Figure 6A,C). This result indicates that photoreactivation of MunI reaches ∼50%.

Kinetic experiments together with gel filtration data show that the chemical modification of the cysteine residues in MunI by NBB at the dimer interface inactivates the enzyme, preventing association of the caged monomers into the active dimer (Figures 4A and 6B). Electrophoretic mobility shift assay (EMSA) experiments demonstrated that in contrast to the wt enzyme the caged monomeric MunI is unable to bind a cognate DNA (Figure 4 of the Supporting Information).

The analysis of the caging reaction of scPvuII(S81C)$_2$ was performed using an excess of caged enzyme over supercoiled plasmid DNA to detect traces of remaining activity. No DNA cleavage activity was detected for the caged scPvuII(S81C)$_2$ preparation, whereas the enzyme incubated without 2-nitrobenzyl bromide exhibited full activity (Figure 5A). To test if the inactivity of caged scPvuII is due to a weakened DNA binding ability, the DNA binding of caged scPvuII(S81C)$_2$ and scPvuII was analyzed by fluorescence anisotropy. The results (Figure 5 of the Supporting Information) indicate that introducing the bulky NBB moiety at the DNA binding site at position 81 prevents specific DNA binding. The time course of the photoreactivation of caged scPvuII(S81C)$_2$ showed that after of irradiation at 325 nm for 1 min 50% and after 20 min 80% of the scPvuII activity is obtained as compared to that of the uncaged enzyme (Figure 5B).

CONCLUSIONS

Two strategies for controlling the activity of restriction enzymes by caging were presented: (1) preventing formation of the active dimer in the case of MunI and (II) preventing DNA binding in the case of PvuII.

(1) Selective caging of cysteine residues with NBB at the dimer interface of MunI generated inactive monomers. The yield of the caged protein obtained was ~90%. Activation of the caged enzyme was performed with near-UV light irradiation, resulting in removal of the cage compound and formation of the active MunI dimer. A decaging efficiency of up to 70% and regeneration of cleavage activity of up to 50% were achieved under our experimental conditions.

(2) Similar results were obtained by blocking the DNA binding site of PvuII. Two cysteine residues were introduced at the DNA binding site, which did not affect the DNA cleavage activity of PvuII. Modification of these cysteine residues with NBB resulted in a DNA binding deficient endonuclease. Photoreactivation of the caged PvuII by near-UV light restored the DNA binding and consequently the DNA cleavage activity up to 80%.

The strategies used in this work could be used to regulate activity of other enzymes. Native cysteine residues or cysteine residues introduced by mutagenesis at the oligomerization interface or at the substrate binding site could be selectively caged with NBB, resulting in an inactive enzyme, whose catalytic activity could be restored by irradiation with near-UV light. The procedures that we have developed are sufficiently general that they can be used for many other enzymes, for which it is desirable to turn them "on" by light. This is the case, for example, for so-called meganucleases that in addition to a cleavage module have a specific DNA binding module. After delivery to the cell, they can be turned "on" after the DNA binding module has located the target site, thus preventing premature DNA cleavage by the cleavage module.

ASSOCIATED CONTENT

Supporting Information. Graph representing accessibility of cysteine residues at the MunI dimer interface to DTNB reagent (Figure S1), calibration curve for determination of the molecular mass of wt and caged MunI (Figure S2), bar diagram showing the efficiency of MunI decaging at different wavelengths (Figure S3), gel mobility shift analysis of DNA binding by wt and caged MunI (Figure S4), and a graph representing scPvuII and caged scPvuII binding to the specific and nonspecific DNA analyzed by fluorescence anisotropy (Figure S5). This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

AUC, analytical ultracentrifugation; CD, circular dichroism; DMNBB, 4,5-dimethoxy-2-nitrobenzyl bromide; DTT, 1,4-dithiothreitol; EDTA, ethylenedinitrilotetraacetic acid; EMSA, electrophoretic mobility shift assay; FLL, linear (DNA); GdmCl, guanidinium monochloride; λ_{exc}, excitation wavelength; NBB, 2-nitrobenzyl bromide; OC, open-circular (DNA); PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; SC, supercoiled (DNA); scPvuII, single-chain PvuII; SDS, sodium dodecyl sulfate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; wt, wild type.

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