Insertion of a Reversible Redox Switch into a Rare-Cutting DNA Endonuclease†

Karen L. Posey and Frederick S. Gimble*

Center for Genome Research, Institute of Biosciences and Technology, The Texas A&M University System Health Science Center, 2121 West Holcombe Boulevard, Houston, Texas 77030

Received November 14, 2001; Revised Manuscript Received December 17, 2001

ABSTRACT: Target sites for homing endonucleases occur infrequently in complex genomes. As a consequence, these enzymes can be used in mammalian systems to introduce double-strand breaks at recognition sites inserted within defined loci to study DNA repair by homologous and nonhomologous recombination. Using homing endonucleases for gene targeting in vivo would be more feasible if temporal or spatial regulation of their enzymatic activity were possible. Here, we show that the DNA cleavage activity of the yeast PI-*Sce*I homing endonuclease can be turned on and off using a redox switch. Two cysteine pairs (Cys-64/Cys-344 and Cys-67/Cys-365) were separately inserted into flexible DNA binding loop(s) to create disulfide bonds that lock the endonuclease into a nonproductive conformation. The cleavage activities of the reduced Cys-64/Cys-344 and Cys-67/Cys-365 variants are similar or slightly lower than that of the control protein, but the activities of the proteins in the oxidized state are decreased more than 30-fold. Modulating the activity of the proteins is easily accomplished by adding or removing the reducing agent. We show that defects in DNA binding account for the decreased DNA cleavage activities of the proteins containing disulfide bonds. Interestingly, the Cys-67/Cys-365 variant toggles between two different DNA binding conformations under reducing and oxidizing conditions, which may permit the identification of structural differences between the two states. These studies demonstrate that homing endonuclease activity can be controlled using a molecular switch.

The sequence of the human genome will help identify genetic mutations that cause inherited diseases, and in the postgenomic era, one of the central goals will be to develop tools to correct these errors. This need may be partially met using homing endonucleases, which can be used to induce DNA repair by introducing double-strand breaks at defined loci (*1*). Homing endonucleases recognize long target sequences (14−40 bp) and cleave DNA with extreme specificity (*2*). They are encoded by selfish DNA elements that are associated with introns or inteins (*3*). These enzymes have been used successfully in vitro in their native state to facilitate the mapping and cloning of complex genomic DNA (*4*−*7*). Their ability to cleave DNA in vivo at inserted target sites has enabled the study of double-strand break repair by homologous and nonhomologous recombination in plant, insect, and mammalian cells (*8*−*13*).

The temporal and/or spatial control of homing endonucleases would add greatly to their value by enabling them to become activated in specific organs or at distinct stages of development. As an initial step toward controlling the activity of these enzymes, we engineered a reversible, molecular switch into the PI-*Sce*I homing endonuclease from yeast (*14*). PI-*Sce*I is a 454-amino acid, intein-encoded homing endonuclease that generates a 4 bp 3′-overhang within a ≥31 bp DNA recognition sequence (*15*, *16*). The 2.4 Å X-ray structure of the PI-*Sce*I apoprotein indicates that it is divided into two domains that separately contain the protein splicing and endonucleolytic active sites (*17*). Both domains contain amino acid residues that make specific contacts to the DNA substrate (*18*, *19*). A comparison of two crystal structures of the apoprotein revealed that several surface loops involved in contacting the DNA may be flexible (*20*). Affinity cleavage and affinity photo-cross-linking mapping studies of the PI-*Sce*I−DNA complex suggest that they become ordered upon DNA binding (*20*−*23*). Here, we use protein engineering to insert redox switches into these loops that can be used to reversibly turn on and off the DNA cleavage and binding activities of the enzyme. Furthermore, we present evidence that one of these proteins can switch between two stable conformations of the PI-*Sce*I−DNA complex that have been previously reported (*15*, *16*). This work demonstrates a new means of controlling the activity of DNA endonucleases, and establishes a basis for the further development of reagents to use in repairing genetic lesions within complex genomes.

MATERIALS AND METHODS

Materials. The synthetic oligonucleotides used for site-directed mutagenesis were obtained from Sigma-Genosys or Operon Technologies, Inc. Restriction and DNA-modifying enzymes were purchased from New England Biolabs, Inc. Cobalt metal affinity resin (TALON resin) was obtained from CLONTECH, and SP-Sepharose was purchased from Amersham Pharmacia Biotech. 4-Azidophenacyl bromide and piperidine were obtained from Sigma. All other chemicals were reagent grade and were obtained from commercial sources.

† This work was supported by a grant from the Welch Foundation (BE-1452).

* To whom correspondence should be addressed. Phone: (713) 677-7605. Fax: (713) 677-7689. E-mail: fgimble@ibt.tamu.edu.

10.1021/bi015944v CCC: $22.00
Published on Web 01/22/2002

Mutagenesis. Duplex oligonucleotide cassette mutagenesis was used to introduce the K60C mutation into vector pET PI-*Sce*I(−5Cys), which encodes a PI-*Sce*I variant that contains a single solvent-inaccessible cysteine residue (Cys-75) (*21*). Vector pET PI-*Sce*I(−6Cys), which lacks all endogenous cysteines, was created from vector pET PI-*Sce*I-(−5Cys) by inserting a C75S mutation into the gene using a PCR[1] mutagenesis strategy (*24*). The same protocol was used to insert the S64C/T344C and V67C/A365C double-cysteine mutations into pET PI-*Sce*I(−6Cys). All mutations were verified by DNA sequencing.

Protein Purification. To express the PI-*Sce*I derivative proteins, the plasmid DNA was transformed into BL21(DE3) cells. The proteins were overexpressed and purified by Co^{2+}−metal affinity and SP-Sepharose ion exchange as described previously (*18*) except that the reducing agent was omitted during purification and storage.

Phenyl Azide-Mediated Photo-Cross-Linking. Derivatization of PI-*Sce*I(−5Cys) and the K60C variant with 4-azidophenacyl bromide and cross-linking of the modified proteins to 5′-end-labeled DNA containing a single PI-*Sce*I recognition site were performed as described previously (*20*). Modification of the proteins was effected by incubating them with 4-azidophenacyl bromide at a 20:1 molar ratio in the dark for 3 h at room temperature. To remove unreacted reagent, the proteins were dialyzed overnight against 25 mM HEPES (pH 8.0), 100 mM KCl, 0.1 mM EDTA, and 5% glycerol. PI-*Sce*I−DNA complexes were formed by combining the derivatized proteins (200 nM) with either a 158 or 187 bp DNA fragment (∼0.5 nM) containing a single PI-*Sce*I recognition sequence that is 5′-end-labeled on the top or bottom strand, respectively. Cross-linking was accomplished by UV irradiating the complexes for 2 min using a Fotodyne transilluminator (312 nm) from a distance of ∼13 cm. The reaction mixtures were heated at 70 °C for 10 min and extracted with phenol and chloroform (4:1, v/v), and the phenolic phase was washed with 1 M Tris-HCl (pH 8.0) and 1% SDS. The DNA was recovered by ethanol precipitation, cleaved with piperidine (1 M) for 30 min at 90 °C, and resolved by high-resolution gel electrophoresis on an 8 M urea, 6% polyacrylamide denaturing gel.

Oxidation and Reduction of Proteins. Purified proteins were reduced by treating them with 5 mM dithiothreitol (DTT) for 30 min prior to using them in DNA cleavage or DNA binding experiments. Oxidation of reduced proteins was performed by dialyzing the samples against buffer that lacks a reducing agent [10 mM KPO_i (pH 7.6), 5% glycerol, and 50 mM KCl].

Trypsin Digestion and Analysis by SDS−Polyacrylamide Gel Electrophoresis. The purified proteins (10 μg) were digested with TPCK-treated trypsin (U.S. Biochemicals, Inc.) at a 6000:1 ratio for 30 min at 37 °C in 100 mM Tris-HCl (pH 8.5). Digested and undigested samples were analyzed by SDS−PAGE on a 12.5% gel using protein sample buffer containing or lacking 0.7 M β-mercaptoethanol (*25*).

Assay of DNA Cleavage Activity. To assay DNA cleavage activity, PI-*Sce*I protein variants (100 nM) were incubated at 37 °C with a linearized plasmid substrate (7 nM), and aliquots were withdrawn at different times for analysis by electrophoresis on a 0.9% agarose gel. PI-*Sce*I binds tightly to one of the two DNA cleavage products, and single-turnover conditions exist in this experiment (*15*, *26*). The oxidized proteins were assayed in standard cleavage buffer lacking a reducing agent [100 mM KCl, 25 mM Tris-HCl (pH 8.5), and 2.5 mM $MgCl_2$], or they were treated with DTT and assayed in the same buffer containing 5 mM DTT.

Assay of DNA Binding Activity by the EMSA. Gel mobility shift assays were performed as reported previously (*18*) using a radiolabeled 219 bp DNA duplex containing a single PI-*Sce*I recognition site. Oxidized proteins were assayed in the absence of a reducing agent, and reduced proteins were assayed with 5 mM β-mercaptoethanol present in the gel and in the gel electrophoresis buffer.

PI-*Sce*I binds specifically to its recognition sequence in the absence of Mg^{2+}, but does not cleave the DNA (*15*, *16*). The relationship between free PI-*Sce*I (P_f), free DNA (D_f), and the two stable PI-*Sce*I−DNA complexes that are formed, one of high mobility (termed the lower complex, PD^{LC}) that contains minimally distorted DNA and one of low mobility (termed the upper complex, PD^{UC}) that contains severely distorted DNA, can be represented as follows:

$$P_f + D_f \overset{K_1}{\longleftrightarrow} PD^{LC} \overset{K_2}{\longleftrightarrow} PD^{UC} \quad (1)$$

The thermodynamic parameters K_1 and K_2 that describe these equilibria can be expressed as follows:

$$K_1 = \frac{[P_f][D_f]}{[PD^{LC}]} \quad (2)$$

$$K_2 = \frac{[PD^{LC}]}{[PD^{UC}]} \quad (3)$$

Values for these parameters were determined as described previously (*18*) from at least four EMSA experiments by nonlinear regression of the data using KaleidaGraph software (Abelbeck Software).

Selection of Amino Acid Positions for the Insertion of Disulfide Bonds. The PAIRWISE algorithm (*27*) was used to predict the suitability of proximal amino acid positions in PI-*Sce*I for formation of a novel disulfide bond.

RESULTS

Cross-Linking of a Loop in the PI-SceI Splicing Domain to DNA. Several regions of the PI-*Sce*I apoprotein are believed to be flexible because they adopt alternative conformations in two different PI-*Sce*I crystal structures (*20*). One is a loop comprised of amino acid residues 53−71 between β6 and β7 within the protein splicing domain, and alanine-scanning mutagenesis of some of these residues indicates that they are involved in DNA binding (K. L. Posey and F. S. Gimble, data not shown and ref *28*). A model of the PI-*Sce*I-DNA complex based on biochemical, mutational, and structural data indicates that residues 55−66 of the 53/71 loop extend outward from the body of the protein away from the DNA (*20*), and it has been suggested that the loop must undergo a conformational change to contact the DNA (*28*). To investigate the proximity of the loop to the DNA,

[1] Abbreviations: PCR, polymerase chain reaction; DTT, dithiothreitol; TPCK, L-1-4′-tosylamino-2-phenylethyl chloromethyl ketone; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay.

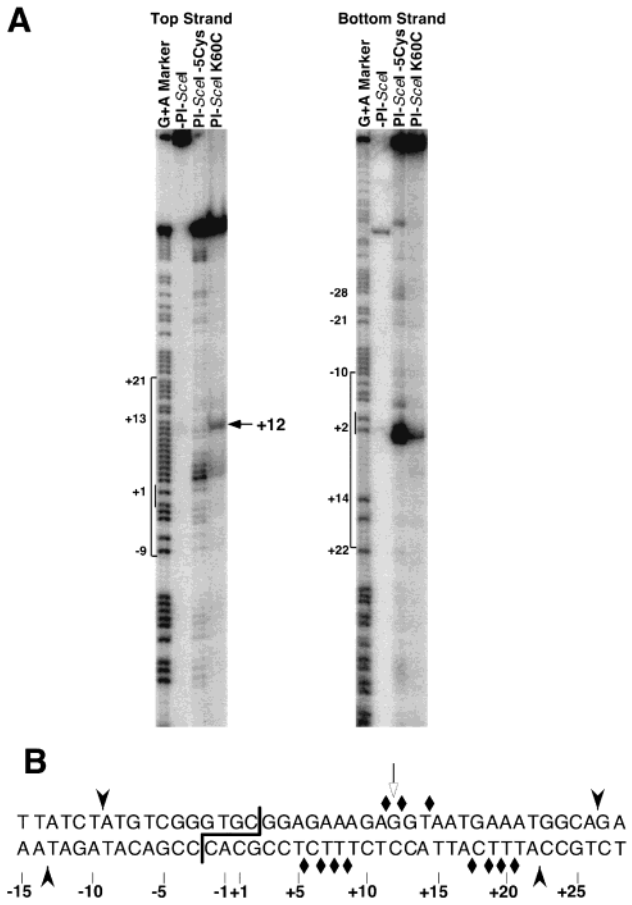

FIGURE 1: Affinity photo-cross-linking of DNA fragments containing a single PI-*Sce*I recognition sequence to phenyl azide-conjugated Lys60Cys PI-*Sce*I protein. (A) Phenyl azide-derivatized Lys60Cys PI-*Sce*I was incubated with fragments end-labeled with ^{32}P on either the top or bottom strand, and cross-linked to the DNA by UV irradiation. An arrow denotes the location of the photo-cross-linked product at G^{+12}. The bracket defines the PI-*Sce*I recognition sequence, and the vertical line indicates the 4 bp cleavage site. A G+A ladder is shown for reference. (B) The position of the cross-link (indicated with an empty arrow) is shown within the PI-*Sce*I recognition sequence. Solid arrowheads mark the boundaries of DNaseI protection, and diamonds indicate ethylated phosphate groups that interfere with PI-*Sce*I binding (taken from ref *15*).

we substituted Lys-60 within the loop with a cysteine residue, derivatized this side chain with a photoactivatable phenyl azide moiety, and attempted to cross-link this group to the DNA. If the modified PI-*Sce*I residue is within ~9−12 Å of the DNA, UV irradiation can cross-link it to the substrate DNA, and the exact position of the cross-link is determined after DNA strand scission with piperidine and electrophoresis on a denaturing gel. Figure 1 shows that a specific cross-link occurs between the phenyl azide on Cys-60 and G^{+12} on the top strand of the DNA, but no cross-linking is observed on the bottom strand at this position. The cross-link between Cys-60 and G^{+12} supports data from hydroxyl radical footprinting and ethylation interference experiments that show that PI-*Sce*I contacts this part of the substrate (*15, 26*). PI-*Sce*I(−5Cys), which encodes a PI-*Sce*I variant that contains a single solvent-inaccessible cysteine residue (Cys-75), and the K60C variant also cross-link to nucleotide position 3 on both strands. As stated previously (*20*), this probably results from modification of a non-cysteine residue elsewhere in the PI-*Sce*I molecule. Taken together, our data

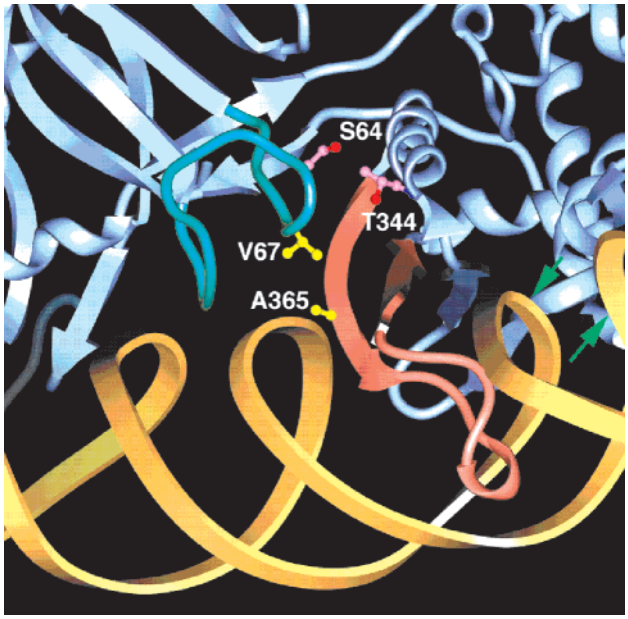

FIGURE 2: Positions of amino acid residues in PI-*Sce*I where cysteine residues were introduced to yield intramolecular disulfide bonds. The 1DFA crystal structure of PI-*Sce*I is shown docked to a modeled DNA substrate (*20*), colored in the following manner. Green represents the PI-*Sce*I 53/71 loop, red the PI-*Sce*I β-hairpin loop of residues 360−386, orange the DNA, and light blue the remainder of PI-*Sce*I. Amino acid side chains are shown for the Ser-64/Thr-344 pair (violet) and the Val-67/Ala-365 pair (yellow). Green arrows indicate the positions of the two phosphodiester bonds hydrolyzed by PI-*Sce*I.

are consistent with the 53/71 loop being in the proximity of the phosphate backbone of the substrate near G^{+12} in the PI-*Sce*I−DNA complex.

Insertion of Redox Switches into PI-SceI. If the 53/71 loop undergoes a conformational change to contact the DNA, we reasoned that it should be possible to inactivate the protein by using a disulfide bond to lock the loop into a conformation that does not bind DNA. The PAIRWISE algorithm (*27*) and manual methods were used to identify residue pairs that are in an appropriate geometrical configuration and the correct distance apart to form a disulfide bond. Furthermore, residues were chosen that are unlikely to contact the DNA based on mutational data and/or on their position in the model of the PI-*Sce*I−DNA complex (*20*). One residue pair identified by PAIRWISE with the potential to form a disulfide bond includes Ser-64 within the 53/71 loop and Thr-344, which is at the N-terminal end of α8 within the endonuclease domain (Figure 2). Although Thr-344 is adjacent to His-343, which is involved in DNA binding (*18*), it is predicted to point away from the DNA according to the model. The second pair includes Val-67 within the 53/71 loop and Ala-365, which lies at the C-terminal end of β21 (Figure 2). Ala-365 is positioned at one end of an extended β-hairpin loop (residues 360−386) that has been mapped to the major groove of the protein−DNA complex position adjacent to the cleavage site [Figure 2 (*20*)]. Substitution of Val-67 with methionine does not affect PI-*Sce*I activity (*29*), and the Ala-365 methyl group side chain is not expected to be involved in DNA binding. The α-carbons of the Ser-64/Thr-344 and Val-67/Ala-365 pairs are 6.81 and 6.69 Å distant, respec-

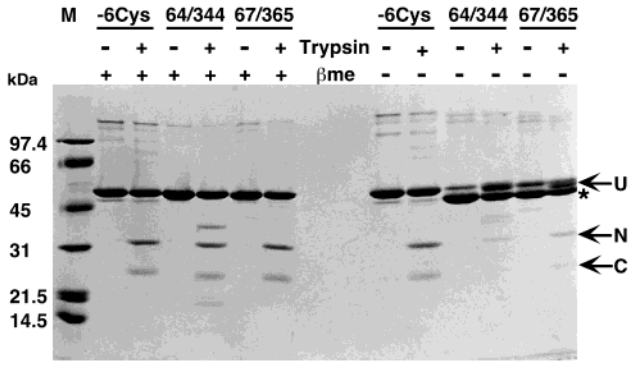

FIGURE 3: Analysis of disulfide bond formation by SDS−PAGE of trypsinized and undigested PI-*Sce*I variants. Purified PI-*Sce*I(−6Cys), PI-*Sce*I Cys-67/Cys-365, and PI-*Sce*I Cys-64/Cys-344 either were treated with trypsin or were not treated, and the proteolytic fragments were resolved from intact PI-*Sce*I by SDS−PAGE under reducing or nonreducing conditions (see Materials and Methods). The positions of undigested PI-*Sce*I (U) and of the two major proteolytic fragments corresponding to digestion at Arg-277 (N and C) are indicated by filled arrows. An asterisk indicates the position of intact proteins that contain a disulfide bond. Molecular mass standards are shown in the first lane.

tively, distances which are favorable for disulfide bond formation [Cα−Cα′ distance of 4.6−7.4 Å (*30*)].

DNA Cleavage Activities of the Oxidized and Reduced PI-SceI Variants. We predicted that the disulfide bonds might form immediately upon cell lysis in the native proteins if the cysteines were properly oriented and proximal to each other. The C67/C365 and C64/C344 variants and a control PI-*Sce*I(−6Cys) protein lacking all cysteines were purified to homogeneity by conventional methods (*18*). To determine whether a disulfide bond is present in the two PI-*Sce*I variants, the purified proteins were analyzed by SDS−PAGE under reducing or nonreducing conditions after mild trypsin digestion. Trypsin cleaves PI-*Sce*I at Arg-277 (*31*) and would yield a 22 kDa C-terminal fragment and a 31.6 kDa N-terminal fragment for the protein derivative used here. Approximately equal amounts of the two trypsin fragments are observed for the control protein under both reducing and nonreducing conditions (Figure 3). By contrast, the amounts of the fragments observed for the C67/C365 and C64/C344 variants are significantly smaller if the protein is not reduced following trypsinization. This indicates that a disulfide bond exists in the unreduced protein between two cysteines located on either side of Arg-277. Furthermore, a band that migrates slightly faster than either the full-length C67/C365 or C64/C344 proteins that appears only under nonreducing conditions is likely to be the oxidized form of the full-length proteins. Quantitation of the doublet shows that >70% of the C67/C365 and C64/C344 proteins are oxidized. Treatment of the proteins with oxidized glutathione does not further decrease the amount of protein in the upper band (data not shown), and it is possible that this band contains protein that cannot be oxidized. No species corresponding to PI-*Sce*I dimers are observed in the undigested samples, which indicates that intermolecular disulfide bonds do not form. This result is not unexpected since PI-*Sce*I is a monomer (*16*, *32*). It is also noteworthy that the appearance of ∼39 and ∼20 kDa digestion products for the C64/C344 variant indicates that trypsin cleaves at a residue besides Arg-277, which may be caused by conformational changes near the introduced cysteine substitutions.

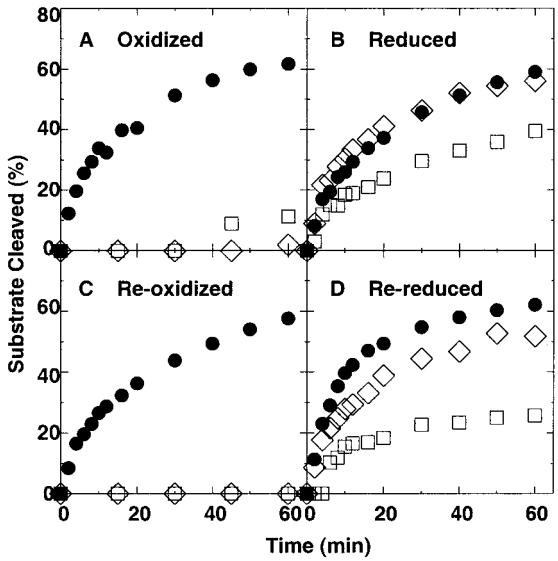

FIGURE 4: DNA cleavage activities of oxidized, reduced, and reoxidized PI-*Sce*I variant proteins. (A) Oxidized PI-*Sce*I(−6Cys) (●), PI-*Sce*I Cys-67/Cys-365 (◇), and PI-*Sce*I Cys-64/Cys-344 (□) proteins (100 nM) were incubated with a linearized plasmid substrate (7 nM) containing a single PI-*Sce*I recognition sequence in the absence of reducing agent as described in Materials and Methods. The extent of cleavage was quantitated after separation of the digestion products by gel electrophoresis. The data represent the averaged values of at least three experiments. (B) The same experiment that is depicted in panel A except that the proteins were pretreated with a reducing agent and assayed under reducing conditions. (C) The reducing agent was removed by dialysis from the DTT-treated proteins used in the experiments depicted in panel B, and the proteins were re-assayed in the absence of any reducing agent. (D) An aliquot of the proteins assayed in the experiments depicted in panel C were treated with DTT and assayed under reducing conditions.

Table 1: Relative DNA Cleavage Rates of PI-*Sce*I Variants[a]

variant	oxidized protein	oxidized protein with DTT	reoxidized protein	reoxidized protein with DTT
PI-*Sce*I(−6Cys)	1[b]	1[b]	1[b]	1[b]
Cys-64/Cys-344	0.03	0.66	0.01	0.31
Cys-67/Cys-365	0.01	1.2	0.01	0.74

[a] Relative DNA cleavage rates are the ratio of the mutant to PI-*Sce*I(−6Cys) rates. Values represent the means from at least three experiments. [b] The rates are expressed in units of moles of DNA cleaved per minute per mole of enzyme and are $(3.3 \pm 0.7) \times 10^{-3}$ for the oxidized protein, $(2.8 \pm 0.1) \times 10^{-3}$ for the oxidized protein with DTT, $(2.6 \pm 0.1) \times 10^{-3}$ for the reoxidized protein, and $(3.9 \pm 0.06) \times 10^{-3}$ for the reoxidized protein with DTT. The wild-type value is 3.9×10^{-3} mol of DNA cleaved min^{-1} (mol of enzyme)$^{-1}$ (*21*).

PI-*Sce*I(−6Cys) cleaves ∼62% of the substrate during a 1 h time course (Figure 4) and cleaves ∼92% of the substrate after 6 h (data not shown). The measured cleavage rate is only slightly lower than that of wild-type PI-*Sce*I (Table 1). In marked contrast, the cleavage rates of the oxidized C64/C344 and C67/C365 variants are >30- and >85-fold lower than that of PI-*Sce*I(−6Cys), respectively (Figure 4 and Table 1), and only 23% and 15% of the substrate is cleaved, respectively, by the two proteins after 6 h (data not shown). To test whether the low activity of the double cysteine mutants is due to disulfide bond formation, the same experiments were performed under reducing conditions. The activity of PI-*Sce*I(−6Cys) is the same in the presence and absence of a reducing agent (Table 1); however, the cleavage

activities of the C64/C344 and C67/C365 variants are 18- and 88-fold higher, respectively, than under nonreducing conditions (Table 1). The reduced C67/C365 protein is as active as the control, whereas the C64/C344 protein exhibits slightly lower activity. Taken together, these results suggest that the disulfide bonds in the C64/C344 and C67/C365 variants dramatically lower the PI-*Sce*I cleavage activity.

Reversibility of the Redox Switch by Reoxidation. For a molecular switch to be practical in a variety of applications, it should be readily reversible. We attempted to reoxidize the reduced cysteine-substituted proteins by dialysis against buffer that lacks a reducing agent. Figure 4 shows that this treatment has no effect on the cleavage activity of PI-*Sce*I(−6Cys), but it dramatically decreases the activity of the C64/C344 and C67/C365 variants (>65-fold) relative to their reduced counterparts (Table 1). Thus, inactivation of the activity is simple to accomplish and does not require the manual addition of exogenous oxidizing agents. As a control, we also show that reduction of the reoxidized proteins restores their cleavage activities (Figure 4 and Table 1).

DNA Binding Activities of the Oxidized and Reduced PI-SceI Variants. The loss of DNA cleavage activity of the PI-*Sce*I variants likely results from defects in DNA binding because the introduced disulfide bonds are located within loops that are involved in contacting the DNA. In EMSA experiments, wild-type PI-*Sce*I forms two stable complexes, a lower complex in which the protein binds the DNA without severely distorting it and an upper complex in which the DNA is extensively distorted (*15*, *16*). Only the upper complex is competent to progress to the catalytic transition state when the essential Mg^{2+} cofactor is added, suggesting that the lower complex represents a prior intermediate in the catalytic pathway. PI-*Sce*I(−6Cys) forms both complexes regardless of whether it has been treated with a reducing agent (Figure 5). The level of formation of the upper complex by this protein, as reflected by the K_1K_2 values, is reduced only 3-fold relative to that of wild-type PI-*Sce*I (Table 2). Similarly, the reduced C67/C365 and C64/C344 proteins form both complexes, and their DNA binding activities are approximately 2-fold reduced compared to that of PI-*Sce*I(−6Cys). Thus, the DNA binding properties of PI-*Sce*I(−6Cys) and of the reduced double-cysteine proteins are not markedly different. In striking contrast, the DNA binding activities of the oxidized C64/C344 and C67/C365 proteins are ∼16- and ∼45-fold lower, respectively, than that of PI-*Sce*I(−6Cys) (Figure 5 and Table 2). The PI-*Sce*I C64/C344 variant, like wild-type PI-*Sce*I, predominantly forms the upper complex at protein concentrations where DNA binding is observed. The binding defect of this protein is due primarily to an elevated K_1 value, which reflects the initial interaction between the protein and DNA. Conversely, the C67/C365 protein yields mostly the lower complex species (Figure 5) which is reflected in the higher K_2 value (Table 2). Thus, the decreased cleavage activities of both disulfide variants can be attributed to large defects in DNA binding, but different steps of the binding pathway are affected for the two proteins.

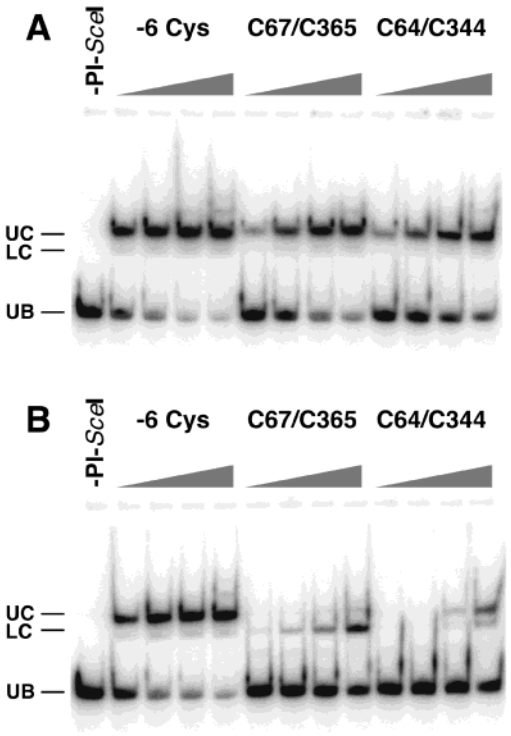

FIGURE 5: DNA binding activities of oxidized and reduced PI-*Sce*I variant proteins. (A) The DNA binding activities of purified PI-*Sce*I(−6Cys), PI-*Sce*I Cys-67/Cys-365, and PI-*Sce*I Cys-64/Cys-344 proteins determined by the electrophoretic mobility shift assay. Proteins that were pretreated with DTT were incubated with a radioactive 219 bp fragment containing a single PI-*Sce*I recognition sequence, and the protein−DNA complexes were resolved from unbound DNA by electrophoresis under reducing conditions. The migration positions of the unbound fragment (UB) and the lower (LC) and upper (UC) complexes are indicated. Protein concentrations of 1, 2.4, 6, and 15 nM were used for each variant. (B) The same assay was performed as for panel A except that the proteins were not treated with DTT and electrophoresis was performed under nonreducing conditions.

DISCUSSION

In this work, we demonstrate that the DNA cleavage activity of a site-specific endonuclease can be turned on and off using a redox switch. A single disulfide bond engineered into the PI-*Sce*I homing endonuclease at two different locations dramatically reduces its DNA binding and cleavage activities. In each of the two double-cysteine mutants, one or both of the introduced cysteines occur in flexible DNA binding loop(s), and we hypothesize that the disulfide bond constrains the loop(s), thereby interfering with DNA binding and cleavage. Introduction of double-strand breaks at defined loci by homing endonucleases greatly improves DNA recombination and repair (i.e., gene targeting) through gene conversion (*33*), and the ability to activate a pre-existing enzyme at will would facilitate gene targeting at a specific developmental stage or in a specific organ. Here, we identify locations in PI-*Sce*I where switches can be introduced to regulate its activity. Moreover, we show that the DNA binding and cleavage activities can be reversibly turned on and off. The ability to reverse the DNA binding activity of PI-*Sce*I may be advantageous because the wild-type enzyme remains tightly bound to one of the two DNA cleavage products, which may interfere with the recruitment of repair factors to the newly exposed DNA ends. Furthermore, deactivation of the endonuclease activity could be rapidly accomplished because the activity does not depend on transcriptional or translational regulation.

Table 2: Thermodynamic DNA Binding Parameters for Wild-Type PI-*Sce*I and PI-*Sce*I Variants[a]

	with reducing agent			without reducing agent		
	K_1 (nM)	K_2	K_1K_2 (nM)	K_1 (nM)	K_2	K_1K_2 (nM)
wild type	4.3 ± 0.3[b]	0.16 ± 0.01[b]	0.67 ± 0.05[b]	ND[c]	ND[c]	ND[c]
PI-*Sce*I(−6Cys)	17 ± 3.0	0.12 ± 0.02	2.0 ± 0.2	16 ± 3.6	0.13 ± 0.01	2.0 ± 0.4
Cys-64/Cys-344	48 ± 12	0.11 ± 0.02	4.1 ± 0.4	110 ± 27	0.28 ± 0.10	30 ± 5
Cys-67/Cys-365	30 ± 6	0.09 ± 0.02	3.1 ± 0.1	18 ± 3	4.9 ± 2.0	89 ± 50

[a] Values represent means ± standard deviations from four experiments. [b] Values taken from ref *18*. [c] Not determined.

The C67/C365 protein may be better suited to being a switchable endonuclease than the C64/C344 enzyme. The reduced form of the C67/C365 protein is as active as the control enzyme in cleaving DNA, while the reduced C64/C344 enzyme is clearly less active. In addition, trypsin digests the C64/C344 protein more extensively than the control or the C67/C365 protein, which may indicate subtle conformational differences.

Redox switches have evolved in naturally occurring proteins to regulate cellular functions and have also been artificially engineered into proteins. In *Escherichia coli*, disulfide bond formation in the Hsp33 chaperone and the OxyR transcription factor activates bacterial functions that protect the cell from oxidative damage. In the inactive Hsp33 chaperone, a four-cysteine cluster coordinates a zinc ion, which is released under conditions of oxidative stress when disulfide bonds form between the cysteines (*34*). This activates the chaperone to act as a "holdase" that prevents the irreversible aggregation of oxidatively damaged proteins. The OxyR transcription factor promotes gene expression of proteins involved in the repair and prevention of oxidative damage (*35*). Formation of a disulfide bridge between residues Cys-199 and Cys-208 as a consequence of oxidation activates OxyR to bind to target promoters, interact with RNA polymerase, and enhance transcription of targeted genes (*36*). In contrast to the PI-*Sce*I mutants described here, OxyR binds DNA only under oxidizing conditions.

Engineering disulfide bonds to regulate the DNA binding properties of sequence-specific transcriptional repressors has been reported previously, but this work is the first to use redox switches to regulate a DNA endonuclease. Via introduction of disulfide bonds into specific regions of the Tet repressor, for example, it was possible to limit the conformational changes that occur during induction of the protein by tetracycline binding and to trap the mutually exclusive tetracycline-bound and operator-bound conformational states of the protein (*37*). Similarly, the introduction of a disulfide bond into the hinge region of the Lac repressor disrupts the allosteric linkage between operator DNA and inducer binding, and results in a variant protein that binds DNA constitutively (*38*). In these cases, the engineered disulfide bond indirectly regulates DNA binding by disrupting a naturally occurring allosteric switch, whereas in PI-*Sce*I, the redox switch directly controls DNA binding.

Other types of switches for regulating endonucleases have been developed or are possible, but each has its disadvantages. Endonuclease activity can be regulated by altering the concentration of the metal ion cofactor. For reactions in vitro, the advantage of this method is that it is a very tight form of regulation, since the metal ion is essential for activity for PI-*Sce*I. Modulation of the Mg^{2+} concentration in vivo has been shown to regulate enzymatic activity. In yeast, overexpression of a putative Mg^{2+} transporter, the Msr2 protein, increases the mitochondrial Mg^{2+} concentration and restores splicing activity to mutant group II introns that require this cofactor (*39*). It is uncertain, however, how one would clear the Mg^{2+} from the cell to inactivate the endonuclease. Furthermore, the activities of any other enzyme that requires a metal cofactor would be disrupted by methods that alter the metal ion intracellular concentration. Temperature sensitive mutants of the *Eco*RI restriction endonuclease have been described that are inactive at 42 °C due to defects in protein folding or stability (*40*). However, controlling a temperature sensitive switch is impractical in vivo, and the switch is irreversible in vitro because the endonucleases cannot be easily renatured. Redox switches, unlike temperature sensitive switches, can be turned on or off in the existing protein population without requiring protein translation, allowing faster regulation of the activity. Moreover, as shown here, the redox switch is easily reversible in vitro. Currently, however, it is not possible to regulate an introduced redox switch in vivo without also controlling the redox state of a targeted cell, which has not been demonstrated. The cytosol of the cell is maintained as a reducing environment to prevent the deleterious effects of reactive oxygen species, and the PI-*Sce*I redox switch proteins would likely exist only in the active state in vivo. Thus, although the current versions of the PI-*Sce*I redox switch proteins have limited value in vivo, they identify residues in the protein where alternative switches can be engineered in the future. These next-generation switches, which may be photoactivatable and/or employ unnatural amino acids or ligands, would allow regulation of the endonuclease activity without affecting cell viability.

We also show in this report that it is possible to switch the PI-*Sce*I−DNA complex between two stable conformations observed in EMSA experiments. For wild-type PI-*Sce*I, more than 80% of the complexed protein and DNA is present in the upper complex, but specific mutations in the DNA substrate or in the PI-*Sce*I endonuclease domain markedly increase the proportion of the lower complex (*15*, *18*). In particular, PI-*Sce*I proteins with His377Ala and Lys378Cys mutations in the β-hairpin loop of residues 360−386 generate the lower complex predominantly in EMSA experiments and are defective in DNA cleavage (*18*, *21*). Interestingly, the oxidized form of the PI-*Sce*I Cys-67/Cys-365 protein behaves like these mutants and forms more lower complex (83% of total), but the reduced protein behaves like wild-type PI-*Sce*I and forms more upper complex (91% of the total). We conclude that constraining the β-hairpin loop with the Cys-67/Cys-365 disulfide bond eliminates its contacts to the major groove near the cleavage site that contribute to the formation of the upper complex. The ability to switch PI-*Sce*I between the two conformations may be useful in time-resolved

structural studies in analyzing the dynamics and mechanism of the transition. In contrast to what is observed for the Cys-67/Cys-365 variant, the ratio of upper to lower complex of the PI-*Sce*I Cys-64/Cys-344 mutant is similar to that of the control protein. From these results, we conclude that constraining the 53/71 loop does not greatly affect the partitioning of the complexes, but it markedly decreases the overall level of DNA binding. This finding confirms the critical role of the 53/71 loop in DNA binding and is consistent with the idea that the 53/71 loop undergoes a conformational change to contact the DNA.

ACKNOWLEDGMENT

We thank Amy Davidson for critically reviewing the manuscript and Randall Burton for help using the PAIRWISE program.

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BI015944V