Role of Individual Cysteine Residues and Disulfide Bonds in the Structure and Function of *Aspergillus* Ribonucleolytic Toxin Restrictocin†

Surendra K. Nayak, Dharmendar Rathore,‡ and Janendra K. Batra*

Immunochemistry Laboratory, National Institute of Immunology, Aruna Asaf Ali Road, New Delhi-110067, India

Received January 29, 1999; Revised Manuscript Received May 6, 1999

ABSTRACT: Restrictocin, produced by the fungus *Aspergillus restrictus*, belongs to the group of ribonucleolytic toxins called ribotoxins. It specifically cleaves a single phosphodiester bond in a conserved stem and loop structure in the 28S rRNA of large ribosomal subunit and potently inhibits eukaryotic protein synthesis. Restrictocin contains 149 amino acid residues and includes four cysteines at positions 5, 75, 131, and 147. These cysteine residues are involved in the formation of two disulfide bonds, one between Cys 5 and Cys 147 and another between Cys 75 and Cys 131. In the current study, all four cysteine residues were changed to alanine individually and in different combinations by site-directed mutagenesis so as to remove one or both the disulfides. The mutants were expressed and purified from *Escherichia coli*. Removal of any cysteine or any one of the disulfide bonds individually did not affect the ability of the toxin to specifically cleave the 28S rRNA or to inhibit protein synthesis in vitro. However, the toxin without both disulfide bonds completely lost both ribonucleolytic and protein synthesis inhibition activities. The active mutants, containing only one disulfide bond, exhibited relatively high susceptibility to trypsin digestion. Thus, none of the four cysteine residues is directly involved in restrictocin catalysis; however, the presence of any one of the two disulfide bonds is absolutely essential and sufficient to maintain the enzymatically active conformation of restrictocin. For maintenance of the unique stability displayed by the native toxin, both disulfide bonds are required.

The fungus *Aspergillus* produces a group of ribonucleolytic toxins called ribotoxins, which includes restrictocin and mitogillin (produced by *Aspergillus restrictus*) and α-sarcin (produced by *Aspergillus giganteus*) (*1*). Restrictocin and mitogillin both contain 149 amino acids and are identical except for the difference of a single amino acid residue at position 25, which is serine in restrictocin and asparagine in mitogillin (*2*, *3*). They have 86% homology with α-sarcin, which has 150 amino acids and was the first ribotoxin to be discovered (*4*). Ribotoxins strongly inhibit eukaryotic protein synthesis by cleaving a single phosphodiester bond in a highly conserved domain of 28S rRNA which is crucially involved in translation (*5*, *6*). Because of its potent cytotoxic activity, restrictocin has been successfully used in the construction of immunotoxins for targeted therapy both by chemical means and by genetic engineering (*7*−*9*). It has been shown that the potent cytotoxic activity of restrictocin is associated with the specific cleavage of the 28S rRNA (*10*). The structure of restrictocin has been solved by X-ray crystallography (*11*). On the basis of structure, several amino acids have been predicted to be critically involved in the catalysis by restrictocin. However, the precise molecular mechanism of ribotoxin catalysis and toxicity in terms of their substrate specificity, membrane interaction, and RNA hydrolysis is not very well understood.

All three fungal ribotoxins have four cysteine residues at identical positions which are involved in two disulfide bonds. In the case of restrictocin, Cys 5 and Cys 147 form one disulfide bond bringing the two termini of the molecule close together, whereas cysteine residues at positions 75 and 131 are involved in the formation of the second internal disulfide bond. *Aspergillus* ribotoxins have 34% sequence homology with another fungal ribonuclease, RNase U2, produced by *Ustilago sphaerogina*. The organization of both disulfide bonds in *Aspergillus* ribotoxins is similar to two of the three disulfide bonds in RNase U2, with one bringing the carboxyl and amino terminal regions together (*2*). Chemical modification of cysteine residues has been found to result in 100-fold reduction in the activity of restrictocin and mitogillin, implying that either some cysteine residue(s) and/or disulfide bond(s) are important for catalysis (*12*). Better et al. (*13*) have shown that in mitogillin substitution of Cys 5 and Cys 147 to alanine individually and in combination, removing one disulfide bond, results in a 2-fold decrease in the protein synthesis inhibitory activity of the toxin. These data suggest that at least these residues, as well as this disulfide bond, are not essential for in vitro activity.

In the current study we have investigated the role of each individual cysteine residue and disulfide bond in restrictocin catalysis by constructing mutants in which all four cysteine residues are substituted with alanine individually and in

† This work was supported by grants to the National Institute of Immunology from the Department of Biotechnology, Government of India.

* To whom correspondence should be addressed. Phone: 91-11-6163009/6162281. Fax: 91-11-6162125. E-mail: jkbatra@yahoo.com; janendra@nii.res.in.

‡ Present address: Laboratory of Parasitic Diseases, NIAID, NIH, Bethesda, MD 20892-0425.

10.1021/bi990222d CCC: $18.00
Published on Web 07/16/1999

different combinations, so as to remove either one or both the disulfide bridges. The mutants have been expressed in *Escherichia coli*. Removal of either one of the disulfide bonds while keeping the other one intact does not affect the ribonucleolytic activity of the toxin, but when both the disulfide bonds are removed, the toxin becomes inactive. Thus, either one of the two disulfide bonds is sufficient for the formation of the enzymatically active conformation of restrictocin. However, the mutant proteins containing a single disulfide bond were not as resistant to trypsin cleavage as the native toxin. This study should be useful in further engineering of restrictocin-based chimeric toxins designed for targeted therapy of various diseases.

EXPERIMENTAL PROCEDURES

Mutagenesis. The cloned cDNA of restrictocin was used as the template to mutate the cysteine residues to alanine either individually or in different combinations using PCR[1] (*14*). For construction of double mutants, the single mutants were used as the template for creating the second mutation. The primers were designed such that the amplified restrictocin fragments carrying mutations C5A, C75A, C131A, C147A, C5:147A, and C75:131A had recognition sites for Nde I at the 5′ and Eco RI at 3′ end. Fragments were digested with Nde I and Eco RI, purified by gel electrophoresis, and cloned into a T7 promoter-based *E. coli* expression vector pVex 11, restricted with the same enzymes. pVex 11 is a pUC based vector that has a phage T7 promoter, multiple cloning site and a T7 transcription terminator. *E. coli* strain DH5α was used for DNA manipulation for cloning. The mutations were confirmed by DNA sequencing done using dideoxy method (*15*). To create C5:75:131:147A, a mutant in which all four cysteine residues are mutated to alanine, DNA encoding C75:131A was used as template and PCR performed with primers containing appropriate mutations to convert C5 and C147 to alanine.

Expression and Purification of Recombinant Proteins. Restrictocin and its mutants were expressed in *E. coli* strain BL21 (λDE3). Bacterial cells, transformed with the appropriate plasmids, were grown at 37 °C with vigorous shaking in superbroth containing 100 μg/mL ampicillin. Superbroth per liter contains the following: tryptone, 12 g; yeast extract, 24 g; glycerol, 6.3 g; K_2HPO_4, 12.5 g; KH_2PO_4, 3.8 g; $MgSO_4$, 0.4 g; glucose, 4 g at pH 7.2. At an OD_{600} of 2.0, cultures were induced with 1mM IPTG and grown for further 2 h. Like restrictocin, all mutants except C5:75:131:147A accumulated in inclusion bodies (*14*) from which the proteins were purified by following the procedure described by Buchner et al. (*16*). The purified inclusion bodies were subjected to denaturation in guanidine hydrochloride, reduction by dithioerythritol, and renaturation in a buffer containing arginine and oxidized glutathione. The renatured proteins were dialyzed and purified by successive cation-exchange (S-Sepharose) and gel filtration (TSK 3000) chromatography as described earlier for recombinant restrictocin and α-sarcin (*14*, *17*). Mutant C5:75:131:147A was localized in the cytosol in soluble form from where it was purified by loading the cytosol, after dialysis, on to a S-Sepharose column followed by a gel filtration column. As a control, S-Sepharose purified mutant C5:75:131:147A was also denatured, renatured, and purified like the rest of the mutants.

Carboxymethylation of Restrictocin. Carboxymethylation was carried out as described by Waxdal et al. (*19*). Restrictocin was incubated at 37 °C for 1 h in 1 M Tris, pH 8.0, containing 2 mM EDTA and 6 M guanidinium hydrochloride. It was further incubated for 90 min at 37 °C after adding 25 mM DTT. Subsequently, 250 mM iodoacetamide was added and the mixture incubated in the dark for 1 h at 37 °C. The protein was purified from the other reaction components by gel filtration on a Sephadex G25 column in 50 mM PBS.

Characterization of Proteins by Circular Dichroism. Proteins were dissolved in 10 mM sodium phosphate buffer, pH 7.0, and their CD spectra were recorded on a Jasco J710 spectropolarimeter in the far-UV range at 25 °C. A cell of 1 cm optical path was used to obtain spectra at a scan speed of 50 nm/min with 50 mdeg sensitivity and a response time of 1 s. The spectra were averaged over 10 accumulations, and results were presented as mean residue ellipticity.

Assay of Specific Ribonucleolytic Activity of Restrictocin and Mutants. Restrictocin and its cysteine mutants were assayed for their ribonucleolytic activity on rabbit reticulocyte lysate as the source of 28S rRNA as described (*20*). Rabbit reticulocyte lysate was treated with various proteins in 40 mM Tris-HCl (pH 7.5) containing 10 mM EDTA for 15 min at 37 °C. The reaction was stopped by the addition of 0.4% SDS, and total RNA was extracted using RNAstat-60 (Tel-Test Inc.) solution. RNA was dissolved in 0.5% SDS, electrophoresed on 1.5% agarose gel, and visualized by ethidium bromide staining.

In Vitro Translation Assay. Rabbit reticulocyte lysate was used to assay the ribonucleolytic activity of restrictocin and its mutants by investigating their effect on in vitro protein synthesis. Reticulocyte lysate was prepared from rabbit blood and assay was carried out as previously described (*10*). The lysate was incubated with different concentrations of various proteins, and incorporation of [^{3}H] leucine, in the newly synthesized proteins, was quantitated. Activity was expressed as percent of control, where no toxin was added.

Cytotoxic Activity of Restrictocin and Its Mutants. To assess the cytotoxic activity of restrictocin and its cysteine mutants, HeLa cells permeabilized by adenovirus infection were used as described by Puentes and Carrasco (*21*). HeLa cells, grown at a density of 2×10^4 cells/well in 96-well culture plates for 12 h at 37 °C, were incubated with virus and different concentrations of the toxin/mutants at 37 °C for 5 h. Cells were labeled with 0.1 μCi [^{3}H]leucine/well for an additional 2 h, followed by harvesting onto filtermats and counting using a LKB β-plate counter.

Sensitivity of Proteins toward Trypsin Cleavage. The recombinant proteins (20 μg) were treated with 200 ng of trypsin (Sigma) either in 20 mM Tris-HCl, pH 7.4, or in 200 mM sodium acetate, pH 5.2, containing 1 mM $CaCl_2$ at 37 °C for indicated time periods. The reaction was stopped by adding SDS-gel loading buffer. The digested products were analyzed by SDS−PAGE on 16.5% polyacrylamide gels using the Tris-Tricine buffer system (*22*).

[1] Abbreviations: aa, amino acid(s); CD, circular dichroism; IPTG, isopropyl β-D-thiogalactopyranoside; PCR, polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; MRE, mean residue ellipticity.

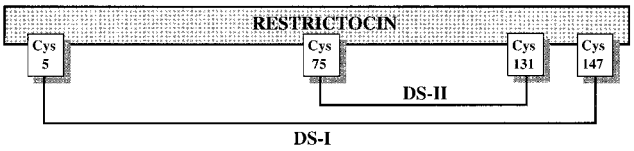

FIGURE 1: Organization of disulfide bonds in restrictocin. The diagramatic representation shows the position of the cysteine residues and disulfide bonds in restrictocin. The disulfide bonds have been designated as DS-I and DS-II.

RESULTS

Restrictocin contains two disulfide bonds, one between Cys 5−Cys 147 and the other between Cys 75−Cys 131, designated as DS-I and DS-II for convenience in this study (Figure 1).

Construction of Cysteine Mutants of Restrictocin. The cysteine residues in restrictocin were mutated to alanine individually and in various combinations to investigate the role of individual cysteines and disulfides in the catalytic activity of the protein. Alanine was chosen because it eliminates the side chain beyond the β-carbon without altering the main conformation. PCR-based site-directed mutagenesis was done to construct various restrictocin mutants, and the mutations were confirmed by DNA sequencing. In restrictocin mutants C5A, C147A, and C5:147A the outer disulfide bond, DS-I, has been removed, whereas mutants C75A, C131A, and C75:131A have lost the inner disulfide bond, DS-II (Figure 1). The mutant C5:75:131:147A contains no cysteines and thus no disulfide bonds.

Expression, Purification, and Characterization of Restrictocin Mutants. The mutants were overexpressed in *E. coli* strain BL21 (λDE3), and except for C5:75:131:147A, the proteins were found to accumulate in inclusion bodies. Proteins were isolated from the inclusion bodies by denaturation and renaturation and purified by successive cation-exchange and gel filtration chromatography. The fractions containing the monomeric protein were pooled after gel filtration. The mutant C5:75:131:147A was purified directly from the cytosol, where it was localized, using the same chromatography scheme followed for the other mutants.

The purified proteins were analyzed by SDS−PAGE under reducing and nonreducing conditions. Like restrictocin, all mutant proteins migrated as a single band under reducing conditions (Figure 2A). A polyclonal antibody against restrictocin reacted equally well with all cysteine mutants of restrictocin except C5:75:131:147A, as shown by Western blotting (Figure 2B). A comparatively weaker reactivity of antirestrictocin antibody was observed with the mutant C5:75:131:147A. Furthermore, the antibody revealed the presence of dimers in single cysteine mutants containing a single disulfide bond and a free unpaired cysteine (Figure 2B). Under nonreducing conditions, dimers of the protein were clearly visible on SDS−PAGE by Coomassie staining of those single disulfide mutants that had a free cysteine (Figure 2C) and on a Western blot probed with a polyclonal antirestrictocin antibody (Figure 2D).

The effect of substitution of cysteines with alanines on the restrictocin structure was investigated by CD spectral analysis. The molar ellipticity curves obtained for the different cysteine mutants lacking either one of the two disulfide bonds were quite similar to that obtained for the native protein (Figure 3A,B) indicating a modest alteration in the secondary structure of these mutants. However, a drastic change in the spectra was observed in case of mutant where both the disulfides have been removed (Figure 3C). This mutant appears to have only β sheets with no helix and turns (Figure 3C).

Table 1: Effect of Cysteine Mutations on in Vitro Protein Synthesis Inhibitory Activity of Restrictocin[a]

protein	disulfide removed	ID_{50} ng/mL	ID_{50} pM
restrictocin	none	2.8	166
C5A	DS-I	2.5	149
C147A	DS-I	1.5	89
C75A	DS-II	1.3	77
C131A	DS-II	2.4	143
C5:147A	DS-I	3.5	208
C75:131A	DS-II	2.1	125
C5:75:131:147A	DS-I & DS-II	8.6	512
CM restrictocin	DS-I & DS-II	>50.0	>2976

[a] Rabbit reticulocyte lysate was treated with various concentrations of cysteine mutants of restrictocin at 30 °C for 1 h. Incorporation of [^{3}H]leucine in the newly synthesized polypeptides was measured. The amount of toxin/mutant needed to inhibit protein synthesis by 50% was expressed as ID_{50}. CM restrictocin is carboxymethylated restrictocin.

Ribonucleolytic Activity of Restrictocin Mutants. The ability of restrictocin to inhibit protein synthesis arises from its capacity to specifically cleave a single phosphodiester bond in the 28S rRNA that releases a fragment of about 400 nucleotides, called the α-fragment. The role of individual cysteine residues and the two disulfide bonds in this unique ribonuclease activity of the toxin was investigated using the various mutants of restrictocin constructed. Rabbit reticulocyte lysate, used as the source for the 28S rRNA, was treated with different mutants. As seen in Figure 4, similar to restrictocin, mutants C5A, C147A, C75A, C131A, C5:147A, and C75:131A were able to produce the α-fragment indicating that none of the cysteines or disulfide bonds per se is crucial for the ribonucleolytic activity of restrictocin. However, the mutant C5:75:131:147A that lacks both the disulfide bonds did not produce the α-fragment even at a very high concentration (Figure 4). Carboxymethylated restrictocin also had no ribonucleolytic activity in the same assay (Figure 4). Three other double mutants C5:131A, C75:147A, and C131:147A were also constructed which have one cysteine from each disulfide bond mutated to alanine, thereby knocking off both disulfide bonds. These mutants, purified from the inclusion bodies after denaturation and renaturation, also behaved like the C5:75:131:147A and did not produce the α-fragment (data not shown).

Effect of Cysteine Replacements on Protein Synthesis Inhibitory Activity of Restrictocin. The capacity of the cysteine mutants of restrictocin to inhibit protein synthesis was tested in an in vitro assay using rabbit reticulocyte lysate. Translation of the endogenous globin mRNAs in rabbit reticulocyte lysate was studied by quantitating the incorporation of [^{3}H]leucine in the nascent polypeptides synthesized in the absence and presence of restrictocin or its mutants. Removal of DS-I or DS-II individually by single or double cysteine mutations did not affect the protein synthesis inhibitory activity of restrictocin (Table 1). C5:75:131:147A with both DS-I and DS-II removed was found to have a lower

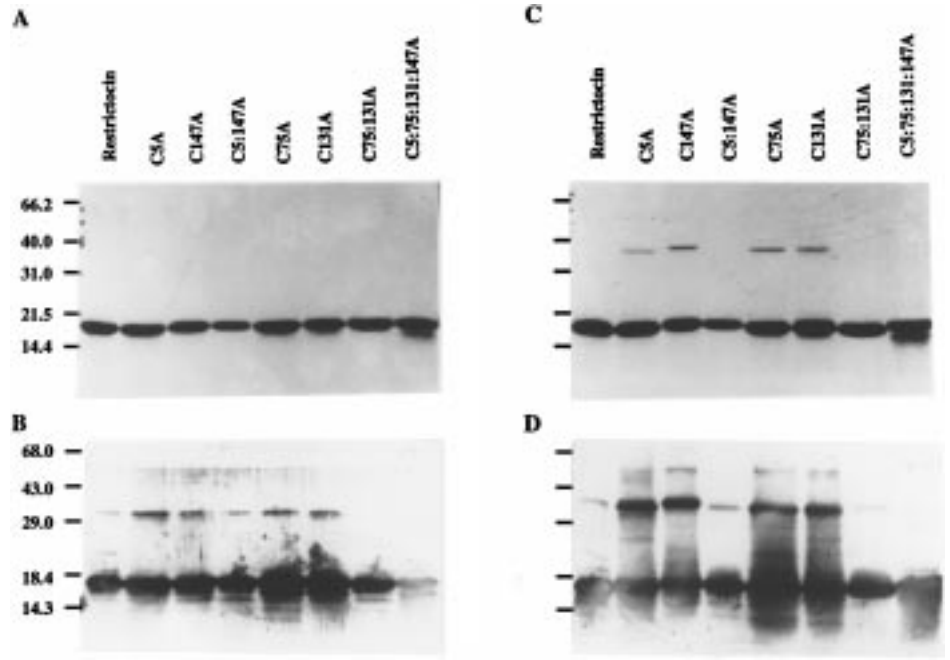

FIGURE 2: SDS−PAGE and Western blot analysis of restrictocin and its cysteine mutants. (A) SDS−PAGE analysis of the cysteine mutants under reducing conditions. Various constructs were expressed in BL21 (λDE3) and purified from inclusion bodies through ion-exchange and gel filtration chromatography. The proteins were subjected to SDS−PAGE (12.5%) under reduced condition and stained with Coomassie blue following the procedure described by Laemmli (*18*). (B) Western blot analysis of the mutants using antibody raised against restrictocin. The different lanes correspond to the same proteins as in panel A. (C) Nonreducing SDS−PAGE of various cysteine mutants after purification from *E. coli*. The proteins were dissolved in loading dye without the reducing agent, β-mercaptoethanol. The gel was stained with Coomassie blue. (D) Western blot analysis of the restrictocin mutants following SDS−PAGE under nonreducing condition.

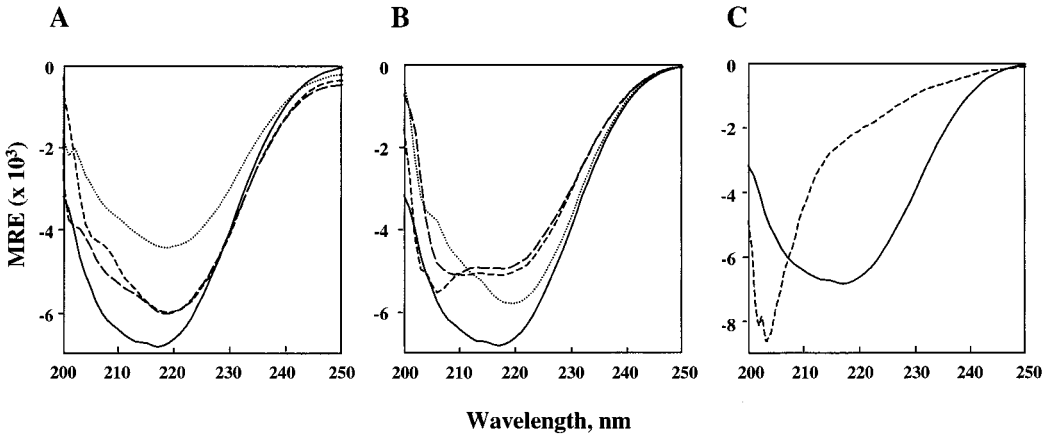

FIGURE 3: CD spectral analysis of restrictocin mutants. (A) CD spectra of restrictocin mutants lacking DS-I. The CD spectra of restrictocin (—), C5A (− −), C147A (- - -) and C5:147A (•••) were recorded in the far-UV region (200−250 nm) at 25 °C. The spectra are presented as mean residue ellipticity (MRE), expressed in (deg•cm^2)/dmol × 10^{-3}. (B) CD spectra of mutants lacking DS-II. The spectra of restrictocin (—), C75A (− −), C131A (- - -), and C75:131A (•••) were obtained as in (A). (C) CD spectrum of mutant lacking both DS-I and DS-II, C5:75:131:147A (− −), was analyzed similarly and compared with that of restrictocin (—).

protein synthesis inhibitory activity (Table 1).[2] Similarly when both the disulfides were removed by mutating one of the cysteines involved in each disulfide (C5:131A, C75:147A, or C131:147A), the toxin had a much lower in vitro protein synthesis inhibitory activity (data not shown). Carboxymethylation of cysteine residues also rendered the toxin inactive as had been reported earlier (*12*). A low protein synthesis inhibitory activity was observed with C5:75:131:147A, which appears to be due to some contamination as the protein was purified from the cytosol unlike the rest of the proteins which were isolated from the inclusion bodies.

Effect of Cysteine Replacements on the Cytotoxicity of Restrictocin. Restrictocin cannot enter eukaryotic cells because it does not have any cell binding activity. However, on permeabilized cells it manifests a potent cytotoxic activity. The cytotoxic activity of cysteine mutants of restrictocin was evaluated on HeLa cells permeabilized by adenovirus infection. The mutant lacking both cysteines of disulfide DS-II compared to the native toxin had full cytotoxic activity, but the mutant lacking DS-I appeared to have a slightly reduced activity (Table 2). The single cysteine mutants had a varied range of cytotoxic activity with C5A having about the same activity as restrictocin, whereas the other three mutants

[2] Restrictocin mutant C5:75:131:147A, partially purified from cytosol, was also denatured, renatured, and purified to homogeneity. The protein synthesis inhibitory activity, cytotoxicity, and CD spectrum of the refolded protein were found to be very similar to that of C5:75:131:147A purified directly from the cytosol (data not shown).

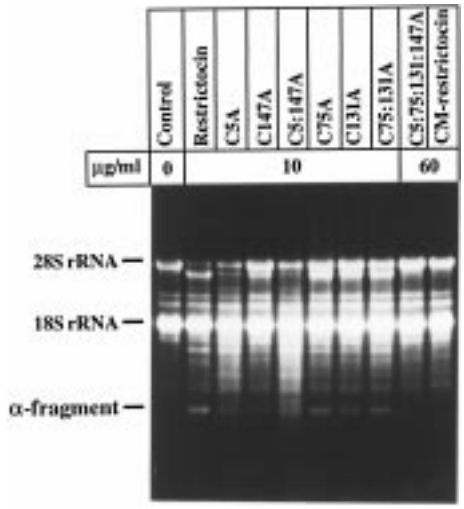

FIGURE 4: Specific ribonuclease activity on 28S rRNA. Rabbit reticulocyte lysate was treated with various mutants of restrictocin at the indicated protein concentrations. The reaction was carried out in 40 mM Tris-HCl, pH 7.5, buffer containing 10 mM EDTA at 37 °C for 15 min followed by termination of reaction using 0.4% SDS. Total RNA was extracted and resolved by electrophoresis on a 1.5% agarose gel. CM-restrictocin refers to carboxymethylated restrictocin. Carboxymethylation was performed as described (*19*). Restrictocin was treated with iodoacetamide under denatured and reduced condition. The chemically modified protein was separated from other reactants by gel filtration on a Sephadex G25 column.

Table 2: Cytotoxic Activity of Cysteine Mutants of Restrictocin in Adenovirus-Infected HeLa Cells[a]

protein	disulfide removed	ID_{50} μg/mL	ID_{50} nM
restrictocin	none	1.6	95
C5A	DS-I	4.2	250
C147A	DS-I	7.8	464
C75A	DS-II	15.0	893
C131A	DS-II	11.5	684
C5:147A	DS-I	3.7	220
C75:131A	DS-II	0.7	43
C5:75:131:147A	DS-I, DS-II	>100	>5952

[a] HeLa cells were infected with adenovirus and treated with different concentrations of the toxin and mutants. Incorporation of [^{3}H]leucine in the newly synthesized proteins was measured. ID_{50} refers to the amount of toxin required to inhibit protein synthesis by 50%.

namely C147A, C75A, and C131A had much lower activity (Table 2). C5:75:131:147A contained no cytotoxic activity (Table 2) as did the other mutants C5:131A, C75:147A, and C131:147A, which lack both the disulfide bonds (data not shown).

Sensitivity of Cysteine Mutants of Restrictocin toward Proteolytic Digestion. The stability of restrictocin mutants was compared with that of the native protein by studying the resistance of these proteins toward proteolytic degradation under different conditions. Restrictocin at neutral pH was resistant to trypsin digestion up to 12.5 ng of trypsin/μg of protein; however, mutants lacking DS-I or DS-II were not as resistant. A marginal difference was observed in the trypsin sensitivity of mutants lacking the disulfide DS-I (C5:147A) and DS-II (C75:131A). Whereas mutant lacking DS-I was almost completely degraded with 12.5 ng of trypsin, only a small fraction of mutant lacking DS-II appeared to have degraded (data not shown). Therefore, for subsequent characterization 10 ng of trypsin was used. Restrictocin at

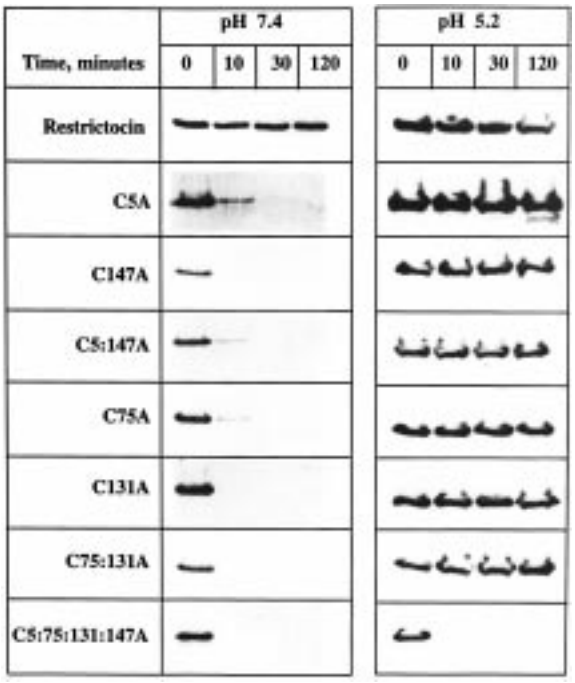

FIGURE 5: Trypsin sensitivity of cysteine mutants of restrictocin. Susceptibility of restrictocin mutants to trypsin digestion was assayed at neutral and acidic pH. Restrictocin and the cysteine mutants were digested with 10 ng of trypsin/μg of protein either in 20 mM Tris-HCl, pH 7.4 (left panel), or 200 mM sodium acetate, pH 5.2, containing 1 mM $CaCl_2$ (right panel) at 37 °C for various time durations as indicated in the figure. The digested products were then electrophoresed in 16.5% polyacrylamide gels using the Tris-Tricine buffer system.

neutral pH was resistant to trypsin digestion up to 2 h, while the cysteine mutants lacking either one of the two disulfide bonds were rapidly and extensively digested and within 30 min the proteins were totally degraded (Figure 5). The mutant lacking both the disulfide bonds was even more sensitive toward proteolytic degradation and was found to be totally degraded within 10 min of digestion with trypsin at pH 7.4 (Figure 5). However, under acidic condition (pH 5.2), the restrictocin mutants lacking one disulfide were as resistant to trypsin digestion as the native toxin (Figure 5), whereas the mutant with both disulfides removed was readily digested within 10 min (Figure 5).

Thermal Stability of Restrictocin Mutants. Restrictocin is thermally quite stable and when heated at 80 °C for 2 h retains 60% of its ribonucleolytic activity (Figure 6). Active mutants C5:147A and C75:131A, lacking DS-I and DS-II, respectively, were also found to be as resistant to heat denaturation as the native protein (Figure 6).

DISCUSSION

Restrictocin consists of a single polypeptide chain of 149 residues and contains two disulfide bonds. The individual cysteines do not seem to be playing a critical role in the enzymatic activity of restrictocin, since all single mutants replacing one cysteine at a time were able to produce the typical α-fragment and inhibit protein synthesis in vitro. However, not all of them were as cytotoxic as the native protein, which could be due to the presence of aggregates or the protein itself being prone to aggregation in the cell. Individually both DS-I and DS-II appear to be dispensable

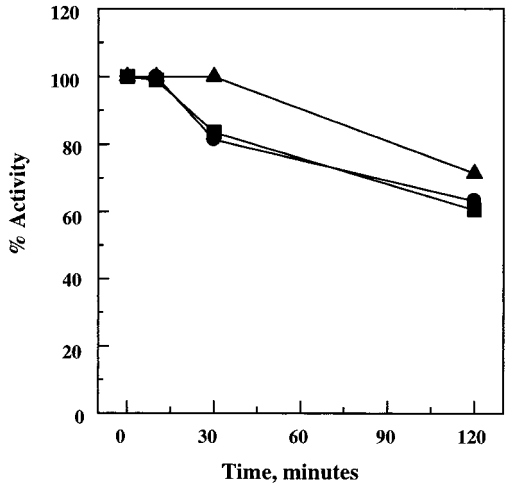

FIGURE 6: Evaluation of thermal stability of restrictocin and its cysteine mutants. Thermal stability of the mutants lacking DS-I or DS-II was compared with that of restrictocin. Restrictocin (●) and the mutants C5:147A (■) and C75:131A (▲) were heated at 80 °C for 0, 10, 30, and 120 min each, and their ribonucleolytic activity was measured in an in vitro translation assay.

for the functional activity of restrictocin. Mutants where a single disulfide bond was removed by replacing both cysteines of that disulfide had full enzymatic activity as well as the cytotoxic activity. Removal of both disulfide bonds simultaneously resulted in complete loss of protein structure and function. The mutants lacking DS-I or DS-II, although functionally as active as the native protein, were relatively less stable toward proteolytic degradation than the native protein. Removal of both DS-I and DS-II made the molecule even more susceptible to proteolytic degradation. The study clearly demonstrates that the two disulfide bonds in restrictocin play a role in maintaining the catalytically functional and protease resistant conformation of the protein.

Cysteine residues at positions 5 and 147 of mitogillin had been earlier mutated to alanine individually and in combination so as to obtain a free unpaired cysteine residue that can be utilized for construction of immunotoxin (*13*). The mutants were active in inhibiting protein synthesis in vitro, with at most a 2-fold reduction in potency. Restrictocin is a $\alpha + \beta$ structure containing seven β strands (*11*). A unique structural feature of the restrictocin molecule is the presence of six long loops connecting the β strands. DS-I (Cys 5−Cys 147) links the N-terminal β1 and C-terminal β7, stabilizing the two termini. A 40 residue glycine-rich loop (L3) between strands β3 and β4 forms an independent structural domain which is linked to the structural core by DS-II (Cys 75−Cys 131). In the crystal structure, His 49, Glu 58, Arg 120, and His 136 coming from different β strands form the active site and the side chains of these residues cluster together toward the open end of the cleft (*11*). Removal of DS-I and DS-II individually does not seem to alter the local structure of the active site cleft, though the overall structure of the molecule appears to be moderately altered.

Restrictocin is a fairly stable protein and is resistant to heat and proteolytic degradation. These properties reflect a tight folding of the protein for which the two disulfide bonds appear to be playing a crucial role. The present study shows that with either one of the disulfide bonds intact the enzymatically active and thermally stable conformation of restrictocin is retained, implying that the overall structure of the protein remains nativelike in the absence of one of the native disulfide bonds. However, protein containing a single disulfide is apparently loosely folded, which may result in local structural alterations thereby exposing some protease recognition sites and making the molecule more susceptible to proteolytic cleavage. The antiparallel β sheet and an adjacent long α helix which form the structural core of restrictocin also represent a common structural motif found in other ribonucleases including T1, RNase Sa, and barnase (*11*). RNase T1 also contains two disulfides, one internal and one linking the N and C terminus of the protein which aligns well with that of restrictocin. The two disulfides in RNase T1 have been found to be extremely important for the stability of the native protein; however, they do not determine the folding mechanism and the structure of the folded protein (*23*). The process of folding has not been studied in ribotoxins. The cysteine mutants of restrictocin could be employed to infer the role of disulfide bonds in the folding of this unique ribonuclease.

Restrictocin and other members of the ribotoxin family are promising candidate toxins for construction of immunotoxins. Recently we have generated recombinant chimeric toxin using restrictocin (*9*). Restrictocin mutants containing only a single disulfide bond were found to be resistant to proteolytic degradation at acidic pH mimicking the environment in the endosomes. A restrictocin mutant with a single disulfide may prove more useful in the development of chimeric toxins as it may facilitate optimal folding of the fusion protein.

ACKNOWLEDGMENT

We thank Dr. S. Srividya for critically reviewing the manuscript. S.K.N. is a Senior Research Fellow of National Institute of Immunology.

REFERENCES

1. Lamy, B., Davies, J., and Schindler, D. (1992) *Genetically Engineered Toxins* (Frankel, R. E., Ed.), pp 237−258, Marcel Dekker Inc., New York.
2. Lopez-Otin, C., Barber, D., Fernandez-Luna, J. L., Soriano, F., and Mendez, E. (1984) *Eur. J. Biochem. 143*, 621−634.
3. Fernandez-Luna, J. L., Lopez-Otin, C., Soriano, F., and Mendez, E. (1985) *Biochemistry 24*, 861−867.
4. Sacco, G., Drickamer, K., and Wool, I. G. (1983) *J. Biol. Chem. 258*, 5811−5818.
5. Schindler, D. G., and Davies, J. E. (1977) *Nucleic Acids Res. 4*, 1097−1110.
6. Endo, Y., and Wool, I. G. (1982) *J. Biol. Chem. 257*, 9054−9060.
7. Orlandi, R., Canevari, S., Conde, F. P., Leoni, F., Mezzanzanica, D., Ripamonti, M., and Colnaghi, M. I. (1988) *Cancer Immunol. Immunother. 26*, 114−120.
8. Rathore, D., and Batra, J. K. (1996) *Biochem. Biophys. Res. Commun. 222*, 58−63.
9. Rathore, D., and Batra, J. K. (1997) *Biochem. J. 324*, 815−822.
10. Nayak, S. K., and Batra, J. K. (1997) *Biochemistry 36*, 13693−13699.
11. Yang, X., and Moffat, K. (1996) *Structure 4*, 837−852.
12. Fando, J. L., Alaba, I., Escarmis, C., Fernandez-Luna, J. L., Mendez, E., and Salinas, M. (1985) *Eur. J. Biochem. 149*, 29−34.
13. Better, M., Bernhard, S. L., Lei, S.-P., Fishwild, D. M., and Carroll, S. F. (1992) *J. Biol. Chem. 267*, 16712−16718.

14. Rathore, D., Nayak, S. K., and Batra, J. K. (1996) *FEBS Lett. 392*, 259−262.
15. Sanger, F., Niklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
16. Buchner, J., Pastan, I., and Brinkmann, U. (1992) *Anal. Biochem. 205*, 263−270.
17. Rathore, D., Nayak, S. K., and Batra, J. K. (1997) *Gene 191*, 31−35.
18. Laemmli, U. K. (1970) *Nature 227*, 680−685.
19. Waxdal, M. J., Konigsberg, W. H., Henley, W. L., and Edelman, G. M. (1968) *Biochemistry 7*, 1959−1966.
20. Lacadena, J., Martinez del Pozo, A., Barbero, J. L., Mancheno, J. M., Gasset, M., Onaderra, M., Lopez-Otin, C., Ortega, S., Gracia, J., and Gavilanes, J. G. (1994) *Gene 142*, 147−151.
21. Fernandez-Puentes, C., and Carrasco, L. (1980) *Cell 20*, 769−775.
22. Schagger, H., and vonJagow, G. (1987) *Anal. Biochem. 166*, 368−379.
23. Mucke, M., and Schmid, F. X. (1994) *Biochemistry 33*, 14608−14619.

BI990222D