The seeds of the subtropical leguminous climber 

Abrus precatorius contain the potent toxic lectin abrin and the relatively less toxic Abrus agglutinins (13). Abrin and agglutinin-I (APA-I) consist of a toxic subunit, the A chain of 30 kDa and the galactose-binding B subunit of 31 kDa (14). A single disulfide bond covalently links the two chains.

In our earlier studies we have demonstrated that apart from inhibiting protein synthesis abrin also triggers apoptosis via the mitochondrial pathway of cell death (15). In spite of \( \sim 70\% \) overall sequence identity seen between abrin and APA-I, the striking differences in the toxicity exhibited by the toxic A subunit and APA-I on different cells urged us to look at the structure-function relationship of these proteins. Studies were therefore initiated to compare the activities of abrin and APA-I with respect to the kinetics of protein synthesis inhibition and apoptosis. Data obtained showed that APA-I is manyfold less toxic than abrin on the cell lines tested. Apoptosis induced by APA-I follows the mitochondrial pathway of cell death like abrin.

The three-dimensional structures of various type II RIPs have been reported, e.g. abrin (8), ricin (9), mistletoe lectin (16), ebulin (17), and Himalayan RIP (18). The complete amino acid sequence of APA-I has been determined (19) and APA-I is needed for the same degree of inhibition. Like abrin, agglutinin-I also induced apoptosis in the cells by triggering the intrinsic mitochondrial pathway, although at higher concentrations as compared with abrin. The reason for the decreased toxicity of agglutinin-I became apparent on the analysis of the crystal structure of agglutinin-I obtained by us in comparison with that of the reported structure of abrin. The overall protein folding of agglutinin-I is similar to that of abrin-a with a single disulfide bond holding the toxic A subunit and the lectin-like B subunit together, constituting a heterodimer. However, there are significant differences in the secondary structural elements, mostly in the A chain. The substitution of Asn-200 in abrin-a with Pro-199 in agglutinin-I seems to be a major cause for the decreased toxicity of agglutinin-I. This perhaps is not a consequence of any kink formation by a proline residue in the helical segment, as reported by others earlier, but due to fewer interactions that proline can possibly have with the bound substrate.

Proteins capable of inactivating ribosomes, termed as ribosome-inactivating proteins (RIPs), are RNA N-glycosidases that depurinate the universally conserved α-sarcin loop of the 28S rRNA of eukaryotes at the most widespread position A4324 (1, 2). The depurination prevents the binding of ribosomes to elongation factors thereby arresting protein synthesis causing cytotoxicity (3). RIPs are of two types: type I (single subunit) and type II (two subunits) (4, 5). Type I RIPs are single polypeptide proteins consisting of only the toxic A subunit with a molecular mass of \( \sim 30 \) kDa, e.g. momordin (6) and gelonin (7). Type II RIPs are two polypeptide proteins with the toxic A subunit and a lectin-like B subunit, e.g. abrin (8) and ricin (9). The latter bind to the cell-surface receptors containing terminal galactose through the B subunit, enter the cells by receptor-mediated endocytosis (10), and are transported to the endoplasmic reticulum (ER) by the retrograde pathway (11). The reduction of the A-B intersubunit disulfide bond essential for the cytotoxicity takes place in the ER followed by translocation of the A chain to the cytosol. In addition to inhibition of protein synthesis, RIPs are also capable of inducing apoptosis (12).

The atomic coordinates and structure factors (code 2AMZ) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/)

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The abbreviations used are: RIPs, ribosome inactivating proteins; APA-I, A. precatorius Agglutinin I; ER, endoplasmic reticulum; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell scan.

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model of APA-I was refined using a data set at 3.5 Å resolution collected at room temperature using a single crystal. The three-dimensional model thus obtained has provided a structural basis for understanding the decreased toxicity of APA-I in relation to abrin.

**EXPERIMENTAL PROCEDURES**

**Purification of Toxins Abrin and A. precatorius Agglutinin**

The proteins were purified from the seeds of *A. precatorius* as described previously (13). The seed kernels were soaked in 5% acetic acid overnight and homogenized. The crude extract was subjected to 30% ammonium sulfate precipitation, and the supernatant was subsequently subjected to 90% ammonium sulfate precipitation. Proteins precipitated were dissolved in 10 mM phosphate buffer containing 150 mM NaCl, pH 7.4 (PBS), and dialyzed extensively against water, and lyophilized. The peak corresponding to abrin or APA-I in 200 l of leucine-free RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 µg/ml streptomycin was centrifuged at 10,000 rpm at 4 °C for 15 min, and the supernatant was loaded onto lactamyl-Sepharose affinity column pre-equilibrated with 20 mM PBS. The bound proteins were then eluted with 0.4 M lactose. The fractions containing abrin and APA-I were pooled separately and loaded onto Sephadex G-100 gel filtration column pre-equilibrated with 20 mM PBS. The fractions corresponding to peak I (~120 kDa) and peak II (~60 kDa) were pooled, dialyzed extensively against water, and lyophilized. The peak corresponding to APA-I was subjected to DEAE ion-exchange chromatography to exclude contamination by abrin, if any. Purified proteins were analyzed on 7.5% native gel. As reported earlier, the proteins Abrin and APA-I eluted with 0.4 M lactose, and hence the lactose eluate has been used as a standard for checking the mobility of Abrin and APA-I on the native gel.

**Cell Lines Used**

JR4, J16 Bcl2 (overexpresses Bcl2), and A3 I9.2 (caspase 8 mutant) clones of Jurkat were cultured in RPMI 1640, and MCF-7, the human breast cancer cell line, was cultured in Dulbecco’s modified Eagle’s medium. The treatment period the cells were kept under leucine starvation for 2 h after which 1 µCi of [3H]leucine was added and incubated for 1 h. The total cell protein was precipitated with 5% trichloroacetic acid, washed twice with 20% ethanol, dried, and dissolved in 200 µl of 0.1 N NaOH in 1% SDS. The incorporated radioactivity was measured in a scintillation counter.

**FITC Binding Assay**

The conjugates abrin-FITC and APA-I-FITC were prepared as described elsewhere (23). Cells were harvested and washed once with ice-cold PBS, and 0.5 × 10⁶ cells were incubated with different concentrations of the FITC conjugates on ice for 45 min. The cells were washed in ice-cold PBS, and bound fluorescence was analyzed by a fluorescence-activated flow cytometric analysis (FACScan, BD Biosciences).

**Acridine Orange-Ethidium Bromide Staining**

Briefly, Jurkat cells were cultured in the presence or absence of varying concentrations of abrin or APA-I. At different time intervals, and the cells were harvested and centrifuged at 3000 rpm for 5 min at 4 °C. After draining the supernatant completely, the pellet was resuspended in 3 µl of dye mixture of acridine orange and ethidium bromide (100 µg/ml each in PBS) (24). The cells were observed under fluorescence microscope (Leica) using a blue filter.

**Propidium Iodide Staining and FACScan Analysis for Apoptosis**

As described elsewhere (25), Jurkat cells (1 × 10⁶ cells/ml) were treated with 10 ng/ml (0.16 nM) of abrin in RPMI 1640 medium supplemented with 10% FBS. At the end of each time interval as indicated, the cells were centrifuged at 300 × g for 5 min, resuspended in 100 µl of 50 mM PBS, and fixed with ice-cold 70% ethanol for 30 min at −20 °C. The cells were pelleted and washed once with PBS and stained with propidium iodide (50 µg/ml), staining solution containing 100 µg/ml RNase A, 1% Triton X-100, and 40 mM sodium citrate for 60 min at 37 °C. The cells were then analyzed by FACScan analysis. Similar assay was carried out using the caspase 8 mutant Jurkat cell line A3I9.2.

**DNA Fragmentation Assay**

As described earlier (25), cells (1 × 10⁶/ml) were cultured in RPMI 1640 medium supplemented with 10% FBS in the presence of abrin or APA-I. At various time intervals, cells were centrifuged at 1000 × g at room temperature for 5 min, suspended in 100 µl of 50 mM PBS, and fixed with ice-cold 70% ethanol for 60 min. After removing ethanol completely, the cells were resuspended in 0.1 M citrate phosphate buffer, pH 7.8, and incubated at room temperature for 60 min with occasional mixing. To the cell pellet 3 µl of 0.25% Nonidet P-40 and 1 µl of RNase A (10 mg/ml stock) was added and incubated at 37 °C for 3 h. Then 1 µl of proteinase K (10 mg/ml stock) was added, and the incubation was continued for 3 h at 37 °C. Ten µl of DNA loading dye (orange G) was added to the mixture and loaded onto a 1.5% agarose gel and electrophoresed at 30 V for 12 h. The gel was then observed under a UV trans-illuminator.
Crystallization and Data Collection

Initial crystallization conditions were screened by the hanging drop vapor diffusion method using commercial crystallization screening kits (Crystal Screen 1 and Crystal Screen 2) from Hampton Research. A protein concentration of 5 mg/ml in Tris-HCl buffer, pH 7.5, a reservoir volume of 400 μl, and a drop size of 6 μl (1:1 ratio of protein and reservoir solution) were used for initial screening. Truncated bipyramidal crystals of size 0.3 x 0.3 x 0.2 mm, suitable for x-ray diffraction analysis, appeared in 2–3 weeks from the crystallization condition No. 36 from Crystal Screen 1 (8% (w/v) polyethylene glycol 8000, 0.1 M Tris-HCl, pH 8.5). Crystals were damaged upon cryo-cooling despite trials with a variety of cryo-protectants. Hence, the x-ray diffraction data were collected at ambient temperature. The data were collected on X9A using synchrotron radiation source at beam line of National Synchrotron Light Source, at the Brookhaven National Laboratory. The wavelength of incident radiation was 0.978 Å. With an oscillation range of 1° for each image, a total range of 45° was covered. The total exposure time was 6 s in order to avoid radiation damage as the data were collected at room temperature. Auto-indexing routines gave solutions consistent with tetragonal space group P42212, which enabled unit cell dimensions to be refined to 41.122 Å, 105.253 Å, and 90°. The space group was subsequently confirmed by the successful structure solution and refinement. The value of Matthews’s coefficient (V_m) (26) was found to be 4.65 Å³ Da⁻¹, which is consistent with the presence of one dimer in the crystal asymmetric unit. The estimated solvent content is 73.3%.

Structure Solution and Refinement

Molecular Replacement—Molecular replacement for APA-I was performed using the coordinates of abrin-a from the Protein Data Bank at Brookhaven National Laboratory (access code 1ABR) and employing the molecular replacement program AmoRe (27). The rotation and translation searches were carried out assuming that the APA-I molecule contains two independent modules as follows: one represented by the abrin-a A chain and the other by abrin-a B chain. The best solution corresponding to a high correlation coefficient of 65.1% and an R factor of 36.8% was used to generate the two chains of APA-I. An x-ray diffraction data shell from 15 to 4 Å was used for the calculation of cross-rotation function. The disulfide bond linking A and B chains could be clearly located in the electron density maps, in the subsequent steps of refinement, and model building.

Crystallographic Refinement and Model Building—The structure determined using 3.5 Å data were refined with CNS (28). Iterations of rigid body refinement, positional refinement,
Structure-Function Relationship of A. precatorius Toxins

RESULTS

Toxins—Abrin and APA-I were purified by a four-step procedure as described under “Experimental Procedures.” The gel filtration column elution profile of the two proteins is seen in Fig. 1A. The 7.5% native gel profile of purified proteins is shown in Fig. 1B. APA-I eluted in the void volume of the column suggesting that it is a tetramer (~120 kDa). The mobility of APA-I is slower on the native gel as compared with that of abrin, the molecular mass which is ~62 kDa.

Inhibition of Protein Synthesis by Abrus Toxins—To determine the cytotoxicity of APA-I in comparison to abrin, Jurkat and MCF-7 cells were cultured with varying concentrations of the toxins for varying periods of time. The amount of protein synthesized was assessed by the incorporation of [3H]leucine in the cells and expressed as the percentage of [3H]leucine incorporated in the treated cells as compared with the untreated cells. Fig. 2 shows the kinetics of inhibition of protein synthesis. Abrin inhibited protein synthesis in both cell lines in a dose- and time-dependent manner. However, as is clearly seen, no toxicity was seen in the MCF-7 cells at 1 ng/ml even up to 4 h of incubation, indicating that the amount of protein is directly related to the kinetics of inhibition (Fig. 2A). APA-I also inhibited protein synthesis in both cell lines in a dose-dependent manner. Higher amounts of both the toxins showed faster kinetics. Jurkat cells appear to be more sensitive to abrin and APA-I as compared with MCF-7.

APA-I Binding Is Comparable with Abrin on Cells—The initial step in the cytotoxicity of toxins is binding to the cell surface. To find out whether the lower activity of APA-I is due to lower extent of binding to cells, binding of APA-I-FITC to Jurkat and MCF-7 cells was compared with that of abrin-FITC. Cells were incubated with different concentrations of the toxin conjugates for 1 h at 4°C. Data shown in Fig. 3 indicate that the binding of both toxin conjugates is comparable in both cell lines employed.

Triggering of Apoptosis by Abrin and APA-I—RIPs have been shown to induce apoptosis in different cell lines (36), and studies from our laboratory have shown that Jurkat cells undergo apoptosis when treated with abrin (15). To test whether APA-I is similar to abrin with respect to this activity as well, Jurkat and MCF-7 cells were treated with the toxins, and their DNA was analyzed by FACS and agarose gel electrophoresis. Fig. 4 shows that like abrin, APA-I also induces apoptosis in both cell lines, although the concentration of APA-I required to trigger apoptosis is more than 10-fold higher as compared with that of abrin. The onset of DNA fragmentation in Jurkat cells by both abrin and APA-I was earlier (6 h) than seen in MCF-7 (48 h). To determine the involvement of caspases in the apoptotic activity of APA-I, the cells were incubated with the pan-caspase

and simulated annealing were alternated with model building using COOT (29). The refinement of the model was completed using REFMAC5 (30). This step included the refinement of temperature factor using TLS (31). The APA-I molecule was divided into four TLS groups. Groups I and II consist of residues 1–165 and 146–248 of chain A and groups III and IV consist of residues 7–142 and 143–267 of chain B. Ten cycles of TLS refinement were carried out. Composite omit maps (32, 33) were calculated for the entire macromolecule under study, and the model was built into the omit density.

In the final refinement the R factor and Rfree converged to 19.4 and 25.4%, respectively. The final model was then subjected to PROCHECK (34) analysis. Only one non-glycine residue (Asn-140(B)) was present in the disallowed region. The PROCHECK parameters are given in Table 1. The shape complementary index was calculated using the software from the CCP4 (30) suite of programs. All the figures were generated using PyMOL molecular graphics package (35).
inhibitor N-benzylxycarbonyl-Val-Ala-Asp-(OMe)-fluoromethyl ketone before treatment with the toxins and then analyzed for the apoptotic population by FACSscan analysis. The inhibitor rescued both the cell lines from apoptosis by abrin and APA-I (Fig. 5).

Toxins like diphtheria toxin (37) and shiga toxin (38) have been reported to induce apoptosis by activating caspase 8, but our earlier studies (15) have shown that abrin-induced apoptosis does not involve the death receptor pathway. Cross-linking of cell-surface receptors has been shown to be essential for the manifestation of biological responses by lectins (39). APA-I is a multivalent lectin, and hence there exists a possibility that death receptors may be cross-linked and thereby get activated. To understand the apoptotic pathway initiated by APA-I, comparative studies were carried on Jurkat A3I9.2 cells that lack the expression of caspase 8. These cells were treated with APA-I and abrin for different time intervals and then analyzed by FACSscan (Fig. 6) and nuclear staining (Fig. 7). It was observed that APA-I-like abrin induces apoptosis in this cell line, and the extent of apoptosis induced is comparable with that of the Jurkat cells suggesting that lectin activity is not linked with the apoptosis induced by APA-I, and therefore APA-I-induced cell death is independent of caspase 8.

Abrin induces apoptosis in Jurkat cells via the intrinsic pathway of cell death (15); hence, we examined whether APA-I also induces apoptosis by the mitochondrial pathway. Bcl-2 is an anti-apoptotic protein that blocks the intrinsic pathway of apoptosis. When the J16 Bcl-2 clone of Jurkat cells was treated with different concentrations of APA-I, no apoptosis was observed as measured by flow cytometry for DNA fragmentation (Fig. 6) and nuclear staining (Fig. 7). These experiments further confirm that APA-I-induced apoptosis follows the intrinsic pathway of cell death.

Overall Structure of APA-I—APA-I structure has been solved by molecular replacement methods using the coordinates of Abrus agglutinin as a search model (Protein Data Bank accession code 1ABR). The crystallographic R/Rfree values for the refined model are 19.4/25.4% for a total of 11,360 unique reflections in the resolution range 30.0–3.5 Å. The summaries of data statistics, refinement statistics, and model parameters are tabulated in Table 1. The complete amino acid sequence of APA-I was determined by Liu et al. (19). A and B chains of APA-I have 66.9% sequence identity with abrin-a A chain and 80.2% with the abrin-a B chain, respectively. However, the sequence identities with other type II RIPs are considerably lower (40). APA-I shares 37% sequence identity with A chain of ricin and 58% with the B chain. Similarly, the corresponding values with mistletoe lectin are 39 and 54% for A chain and B chain, respectively. Although the sequence identities between respective chains of abrin and APA-I are high, the variations in their sequences are of great biological significance in view of the differences in their toxicity. Hence, determination of the three-dimensional structure becomes crucial for analyzing the major dissimilarities between abrin and agglutinin, which could help in exploring the biological potential of APA-I.

The overall folding of APA-I is similar to that of abrin. However, there exist some major biologically important substitutions that account for the less toxic nature of APA-I. The root square mean deviation calculated only for structurally related Cα atoms of abrin-a and APA-I is 1.14 Å. It is higher than that calculated separately for A chains and B chains, 0.78 and 0.57 Å respectively. The general architecture of APA-I is similar to that of other type II RIPs that include ricin, abrin, and mistletoe lectin. Fig. 8 shows the schematic representation of the APA-I molecule.

By reference to the description of the abrin-a A chain, APA-I A chain consists of three folding domains. Domain 1 is composed of residues 2–108 consisting of six stranded β-sheet, one β-hairpin, and two helices. The N terminus of APA-I is one residue shorter than that of abrin-a, and the first two residues are disordered. Domain 2 consists of residues from 109 to 196.
and is the most conserved part of the A chain of type II RIPs. It has a preponderance of helices with a total of five helices. Domain 3 consists of residues 197–261. It contains two helices and two anti-parallel strands and a random coil at the C terminus. The C terminus of APA-I is 10 residues longer than that of abrin-a. The electron density for the terminal residues was not clear, hence the C terminus of APA-I could be modeled until Asn-250.

The structure of B chain, the lectin chain, is highly conserved across the type II RIP family. The major differences may lie in the labile region or the loop structures. The lectin chain of APA-I is divided into two homologous domains B1 and B2, each of which consists of four subdomains namely \( \alpha/H_9251 \), \( \beta/H_9252 \), \( \beta/H_9253 \), and \( \gamma/H_9254 \). The three subdomains \( \alpha, \beta, \text{and } \gamma \) possess a pseudo 3-fold symmetry around a hydrophobic core. This type of folding has been classified as the \( \beta \)-Trefoil fold (41).

APA-I B chain contains three N-glycosylation sites, namely Asn-100, Asn-140, and Asn-214. The consensus sequence corresponding to the N-glycosylation sites in particular is Asn-Xaa-(Ser/Thr). In case of APA-I this sequence is Asn-Xaa-Thr, where Xaa is any amino acid. The crystal structure reveals the presence of two glycosylation sites Asn-100 and Asn-140 holding sugar chains in \( \beta \) and \( 2\beta \) subdomains. APA-I B chain contains two sugar-binding sites at Asn-51 and Asn-260 present in \( \alpha \) and \( 2\gamma \) subdomains, respectively. The architecture of sugar-binding pockets in APA-I is similar to that of abrin-a.

**Active Site Architecture**—The A chain of APA-I is associated with N-glycosidase enzymatic activity (1, 2). The overall structure of A chain consists of three domains. The active site is located in the cleft formed by these domains. The active site consists of Tyr-73, Tyr-112, Glu-163, Arg-166, Trp-197, Asn-71, Arg-123, Gln-159, Glu-194, and Asn-195 (Fig. 8), which are conserved among the type II RIPs. Based on recent crystal structure analyses of various type II RIPs, it has been suggested that the active site remains constant in all RIPs belonging to this class (8). Some deviation may occur because of orientation of...
the side chain, number of water molecules, water-mediated hydrogen bonding, and the overall interaction network in the active site region (8). In APA-I the position of Trp-197 is fixed by direct hydrogen bonds to the main chain NE1 (Trp-197) to O (Leu-241). The position of Arg-166 is fixed by side chain hydrogen bonds with NH1 (Arg-166) to OE2 (Glu-163), and the main chain hydrogen bond with N (Ala-240) to O (Arg-166). The hydroxyl group of Tyr-112 is seen interacting with OE1 of Glu-163 via hydrogen bonds, and OE2 of Glu-163 is hydrogen-bonded to NH1 of Arg-166. Hence the active site residues seem to be held strongly at their positions via a network of hydrogen bonds.

The orientation of two aromatic rings of Tyr-73 and Tyr-112 (Fig. 8) forms an important point of discussion. Fig. 9 shows the omit map density around these tyrosine residues. The almost parallel orientation proves to be the best for incorporating the planar adenine of the ribosomal unit. The main function of these two residues is to form a route for the substrate by keeping adenine strictly parallel to the plane of their aromatic rings. In case of ricin (9), the Tyr-80 must rotate about 40° to facilitate stacking. Like in abrin-a, these aromatics are almost parallel in APA-I. The studies on site-directed mutagenesis of invariant Tyr residues in the active site of ricin have revealed that Y80F and Y123F substitutions reduced the activity by 15- and 7-fold, respectively (42). This implies that the inability of Phe side chains to take part in the hydrogen bonding weakens their ability to hold the adenine molecule. Hence the presence and orientation of tyrosine residues play important roles in the catalytic activity of RIPs. Glu-163 and Arg-166 are the main residues involved in the hydrolysis of N-C-glycosidic bond.

Studies on mutants of abrin-a (43) (E164A, R167L, and the double mutant E164A/R167L) show marked decrease in the activity by 25-, 625-, and 1250-fold, respectively.

**Sugar Binding and N-Glycosylation Sites**—Two putative galactose-binding sites Asn-51 and Asn-260 are present in APA-I B chain as in abrin-a (19). These sugar-binding sites

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**TABLE 1**

Data collection, refinement statistics, and the model parameters

| Data collection |   |
|-----------------|---|
| Resolution limit (Å) | 30-3.5 (3.7-3.5)* |
| Total reflections  | 13,311 |
| Unique reflections | 11,360 |
| Completeness, %   | 95.7 (96.8) |
| Multiplicity      | 3.8 (3.8) |
| I/σ(I)           | 13.1 (2.3) |
| Rmerge, %        | 9.5 (53.9) |
| Mosaicity        | 0.25* |
| Number of molecules in A. U. | One dimer |

| Refinement statistics |   |
|-----------------------|---|
| No. of protein atoms  | 4029 |
| No. of carbohydrates  | 3 |
| Rcryst, %             | 19.4 |
| Rfree, %              | 25.4 |

| Root mean square deviations |   |
|-----------------------------|---|
| Bond distance (Å)           | 0.013 |
| Bond angles (°)             | 1.723 |

| Luzzati coordinate error (Å) |   |
|------------------------------|---|
| Working set                  | 0.525 |

| Ramachandran plot statistics (%) |   |
|----------------------------------|---|
| Residues in allowed regions      | 80.8 |
| Residues in additionally allowed regions | 18.5 |
| Residues in generously allowed regions | 0.7 |
| Residues in disallowed regions   | 0.2 |
| Mean B-factors (Å²)              | 51.88 |

* Parentheses show high resolution shell.
occur in the subdomains 1α and 2γ. In these subdomains the sugar molecule is expected to lie in a shallow pocket formed on one side by a kink in the chain, and a conserved aromatic residue forms the top of the pocket (44). For APA-I the sequence of the kink in subdomain 1α is Asp-Val-Ser, whereas for 2γ it is Asp-Val-Lys. The corresponding sequences in abrin are Asp-Val-Tyr and Asp-Val-Lys, respectively. The aromatic rings in the two subdomains of APA-I are Trp-42 and Trp-253. These side chains serve as a flat binding surface for sugar moieties but do not make specific interactions with the sugar. Hydrogen bonding occurs between galactose and homologous amides Asn-51 and Asn-260, which in turn are stabilized by hydrogen bonds to homologous amino acids Asp-27 and Asp-239. The superposition of sugar-binding pockets of abrin and agglutinin shows no marked differences (figure not shown).

The predicted potential glycosylation sites of APA-I are Asn-100, Asn-140, and Asn-214 in APA-I A chain and Asn-215 and Asn-250 in APA-I B chain (19). These five glycosylation sites occur at the 1β, 2β, and 1γ subdomains of the lectin chain, whereas one occurs in domain 3 of the toxin subunit of APA-I.

**Structural Basis for APA-I (with Pro at Position 199) Being Less Toxic than Abrin**—Despite sharing major similarities with the other type II RIPs, APA-I possesses some distinct features, which may be responsible for it being less toxic. A strikingly different behavior is seen at the substrate-binding site of APA-I. The nature of the residue at the substrate-binding site is found to play a major role. Comparison of the residues that actively participate in the binding of the substrate in abrin, ricin, and APA-I reveal that Asn-200 of abrin-a, corresponding to Arg-213 of ricin A, is required for the binding to the substrate in the α-sarcin loop, a GpApApAp tetranucleotide sequence located at the 3′-terminal region of 28 S rRNA (45, 46). In case of ricin, Arg-213 acts as a positively charged recognition group for the negatively charged substrate, possibly by forming a salt linkage with the phosphate backbone of RNA (47). In abrin-a, the corresponding Asn-200 has a polar group and can bind to the substrate effectively via strong interactions. However, a proline residue, Pro-199, is present at the corresponding position in APA-I. It was hypothesized by others earlier (19) that the Pro-199 in APA-I (Fig. 9A) could cause bending of the amphiphilic helix (Fig. 8) and thereby be a contributing factor for the decreased toxicity of APA-I.

The present analysis of x-ray structure model reveals no evidence of a pronounced kink in the helix induced by the proline residue in APA-I. The proline substitution takes place at the 3rd position of the amphiphilic α-helix from the N terminus. In the past, research has revealed that the highest preference of occurrence of proline is at the start of the α-helix (48). Pro at the N terminus is perhaps better described as the helix initiator than the helix breaker (49). The inability of proline to form main chain and side chain hydrogen bonds effectively results in weak binding of the substrate in this region and hence probably is the major cause of the decreased toxicity of APA-I as compared with abrin-a and ricin. Moreover, comparison of the amphiphilic helices in abrin-a, ricin, and APA-I provides no evidence of any kink in the helix caused by proline in APA-I (details not shown).

**Proposed Model for APA-I Existing as a Heterotetramer**—Fig. 10 shows the dimerization of APA-I molecule about the 2-fold axis, thereby depicting its organization as a heterotetramer, made up of two A and two B chains. This is also clearly evident from gel filtration and electrophoresis experiments shown in Fig. 1, A and B. Two A-B monomers are related by a crystallographic 2-fold symmetry and face each other at the N terminus of their respective A chains thereby forming a BAAB or B-s-s-A . . . A-s-s-B heterotetramer, where s-s is a disulfide bond. Unlike ricinus agglutinin (50), APA-I tetramer has no disulfide bonds at the interface region because of the absence of free cysteines in APA-I A chain. The interfacial residues lose their accessible surface area upon dimer formation and hence are buried at the contact surface. The total accessible surface area buried by the components in the contact site is 2287 Å², which is well within the range for a noncovalent interaction forming a protein-protein dimer (51). However, many polar residues are present at the interface perhaps implying that the oligomer is of the nonobligate type (52, 53).

Within the limits of accuracy of experimental measurement, the distances calculated at the putative tetramerization interface are approximately in the range of hydrogen bonds, salt bridges, and hydrophobic contacts. The shape complementary index (54) is used here to examine the shape complementarity of dimer-dimer interface as obtained by applying crystallographic 2-fold axis. The calculated value of the shape complementarity index for the tetrameric arrangement of APA-I is 0.729, which is well within the limits of 0.70 to 0.76 for oligomeric and protein/protein inhibitor interfaces as suggested by Lawrence and Colman (54).
By taking together the factors such as decrease in surface accessibility upon oligomerization, shape complementarity, high solvent content in the unit cell, and the presence of both short and long range cohesive forces at the interface region, we are able to provide a plausible tetramer model for APA-I. It may be suggested that the tetrameric arrangement of APA-I observed from the crystal structure presumably corresponds to that seen in the gel filtration experiments (Fig. 1B), even though the functional implications of the dimer of dimers are not clear at present.

**DISCUSSION**

Abrin, ricin, and diphtheria toxins are powerful cellular toxins; even a single molecule can kill a cell (55). The protein synthesis inhibitory activity of an *Abrus* agglutinin was reported to be weaker than abrin in *in vitro* assays (19). Both the molecules have similar therapeutic indices (56); however, the LD$_{50}$ for abrin is 20 $\mu$g/kg body weight, and agglutinin is 5 mg/kg body weight (57).

Experiments conducted in our laboratory revealed that the APA-I is less toxic to whole cells, and the reduction in the activity of the molecule could be related to the structural differences in the active sites of abrin and APA-I. The differences in protein synthesis inhibition observed between cell lines by APA-I is not due to lower binding of this molecule as shown by binding studies (Fig. 3).

Induction of apoptosis by RIPs has been known for a long time (58). APA-I apart from protein synthesis inhibition also induced apoptosis. Even though Jurkat and MCF-7 cells showed greater clumping (agglutination) upon addition of low concentrations of APA-I, apoptosis was observed only upon the addition of the amount of protein more than or equal to that of the IC$_{50}$ values, suggesting that inhibition of protein synthesis is required for apoptosis. Despite the agglutinating activity of APA-I, it does not induce any signals for apoptosis through the cell-surface death receptors. A Jurkat cell clone overexpressing Bcl-2 did not undergo apoptosis on treatment with APA-I, and hence it can be concluded that APA-I, like abrin, follows the mitochondrial pathway of cell death. To summarize, the biological studies shown here suggest that abrin and APA-I are toxic lectins in that they inhibit protein synthesis and induce apoptosis, and the reason for the lower cytotoxic effect by APA-I is because of the alterations in the structure, which is well represented in the crystal structure of APA-I.

The crystal structure of APA-I was analyzed to elucidate the most probable factors for the less toxicity of APA-I than abrin. The structure presented here revealed important findings. The less toxic nature of APA-I is perhaps not because of any proline-induced kink present in amphiphilic helix, as postulated previously (19), but because of the fewer interactions involved at the substrate-binding site. The presence of Pro-199 at the substrate-binding site (as opposed to Asn-200 in abrin-a and Arg-213 in the case of ricin) hinders the possibility of interactions involving the main chain and the side chain with the substrate adenine.

The toxicity assays and the apoptosis studies drive the attention toward the possibility of using APA-I, whose three-dimen-
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sional structure is reported here, in the construction of immu-
notoxins. Also, studies are in progress in our laboratory for
designing inhibitors that could modulate the toxicity of RIPS.

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