Primary Structure and Function Analysis of the Abrus precatorius Agglutinin A Chain by Site-directed Mutagenesis

Pro199 of AMPHIPHILIC α-HELIX H IMPAIRS PROTEIN SYNTHESIS INHIBITORY ACTIVITY*

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Abrus agglutinin (AAG), a low-toxicity protein from the plant Abrus precatorius, is less lethal than abrina (ABRa) in mice (LD50 = 5 mg/kg versus 20 μg/kg of body weight). Nucleotide sequence analysis of a cDNA clone encoding full-length AAG showed an open reading frame with 1641 base pairs, corresponding to a 547-amino acid residue preproprotein containing a signal peptide and a linker region (two amino acid residues) between the AAG-A and AAG-B subunits. AAG had homologous to ABRa (77.5%). The 13 amino acid residues involved in catalytic function, which are highly conserved among abrins and ricins, were also conserved within AAG-A. The protein synthesis inhibitory activity of AAG-A (IC50 = 3.5 nM) was weaker than that of ABRa-A (0.05 nM).

Molecular modeling followed by site-directed mutagenesis showed that Pro199 of AAG-A, located in amphiphilic helix H and corresponding to Asn200 of ABRa-A, can induce bending of helix H. This bending would presumably affect the binding of AAG-A to its target sequence, GpApGpAp, in the tetraloop structure of the 28S rRNA subunit and could be one of the major factors contributing to the relatively weak protein synthesis inhibitory activity and toxicity of AAG.

Ribosome-inactivating proteins (RIPs)1 compose a family of N-glycosidases that cleave a specific adenine residue from the α-sarcin-ricin loop of the 28S RNA. In rats, for example, RIPs were found to hydrolyze the C–G nuclease bond of the adenosine residue at position 4324 of the 28 S rRNA subunit (1, 2). RIPs can cleave a synthetic RNA structure with a short double-helical stem and a loop containing a centered GpApGpAp sequence, the first A being the cleavage site (3). The depurination blocks the ability of ribosomes to bind to elongation factor-2 (4), thereby inhibiting protein synthesis.

There are two categories of RIP, type I (single chain) and type II (two chains) (5). Type II RIPs contain two types of subunit: the B chain, a lectin with two α-galactose moiety-binding sites (6); and the A chain, which inhibits protein synthesis (7). The B chain facilitates translocation of type II RIPs into cells. Type I RIPs, such as trichosanthin and mormorchin (8, 9), carry the toxophoric A chain only. Because they lack the B chain and therefore cannot be easily translocated into cells, they are less toxic than type II RIPs (10).

Two kinds of type II RIP have been isolated from jequirity bean, the plant Abrus precatorius (5, 11). One kind, abrin (ABR), is extremely toxic to eukaryotic cells; the other kind, Abrus agglutinin (AAG), is of low toxicity. ABRs are heterodimeric glycoproteins, whereas AAG is a heterotetrameric glycoprotein. Both of these type II RIPs inhibit the growth of tumors in experimental animals (7, 12); ABR is 10–100 times more toxic to some transformed cell lines than to normal cells (13).

Many attempts have been made to study the structure and function of these toxic proteins; interest has been stimulated in part by the use of the A chain in the preparation of immunotoxins for cancer chemotherapy (14, 15). The therapeutic indexes of AAG and ABRs are similar (12). However, ABRa is extremely toxic, with an LD50 of 20 μg/kg of body weight; the LD50 of the relatively nontoxic AAG, on the other hand, is 5 mg/kg of body weight (10). The remarkable disparity prompted us to examine the causes for this difference in the toxicity of ABRa and AAG at the molecular level.

In this paper, we report the determination of the amino acid sequence of AAG by protein techniques and the molecular cloning of the cDNA encoding AAG. Using molecular modeling and site-directed mutagenesis, we found that Asn200 of ABRa-A and Pro199 of AAG-A are involved in the differences in the protein synthesis inhibition and toxicity of these two type II RIPs.

EXPERIMENTAL PROCEDURES

Materials—Taq DNA polymerase, the pGEM-T and pGEM-T-easy vectors, and the rabbit reticulocyte lysate system were obtained from Promega (Madison, WI). Restriction endonucleases and T4 ligase were purchased from New England Biolabs Inc. (Beverly, MA). Deoxyribonucleotide primers were synthesized by the phosphoramidite method in an Applied Biosystems automated DNA synthesizer. The Marathon cDNA amplification kit was from CLONTECH (Palo Alto, CA). The pGEX-2T expression vector and all columns used for protein purification were from Amersham Pharmacia Biotech (Uppsala, Sweden).

Purification of Abrus Agglutinin Subunits and Determination of Amino Acid Sequences—AAG was purified from extracts of mature A.
precatorius seeds as described previously (11). AAG-A and AAG-B were separated and purified by fractionation through a Sephadex G-150 column (1.8 × 90 cm), which was eluted with 0.01 M Tris (pH 8.6) in 6 M urea and 0.13 M 2-mercaptoethanol. AAG-A was found in the first protein peak, whereas AAG-B was found in the second protein peak. The homogeneity of purified lectins and their subunits was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (16), and the protein concentration was measured by the bicinchoninic acid method (17).

The amino acid sequences of AAG-A and AAG-B were determined by protein techniques as previously reported (18). AAG was cleaved with cyanogen bromide as described previously (19) to facilitate analysis of the primary structure. The cleavage products were separated by SDS-PAGE and subsequently electroblotted onto a polyvinylidene difluoride membrane. The blot was stained with 0.1% Coomassie Brilliant Blue and destained with 50% methanol. The sequences of the peptide bands were analyzed with an ABI 476A sequencer.

The peptides generated by 1-lysylamido-2-phenylthiolethyl chloroform methyl ketone-treated trypsin, Lys-C endoprotease, or Streptococcus aureus V8 endoprotease digestion and cyanogen bromide cleavage were purified by HPLC on a C18 reverse-phase column (4.6 mm × 20 cm), which was eluted with a linear gradient of acetonitrile (10–70%) in 0.1% trifluoroacetic acid. The amino acid sequences of the purified peptides were then determined as described above.

Expression of AAG in Escherichia coli—To construct the AAG-A expression vector, we obtained AAG-A cDNA by amplifying pTAAgA DNA with the sense primer GSP-1, which encodes the first seven N-terminal amino acid residues of AAG-A, and the antisense primer GSP-2, which encodes the last eight C-terminal amino acid residues and the stop codon of AAG-A. The PCR product was then ligated into the T vector for amplification and DNA sequencing. The amplified product was used as the template for the subsequent PCR steps.

The N-terminal and internal amino acid sequences of AAG were used to design degenerate oligonucleotide primers for amplification of AAG cDNA for 5′- and 3′-RACE. The PCR profile for each of the RACE PCR steps was as follows: 35 cycles of denaturation at 94 °C for 1 min, annealing at 45 °C for 1 min, and extension at 67 °C for 2 min. The PCR products were ligated to T vector for amplification and DNA sequencing (22, 23).

For the first PCR, AAG cDNA was amplified with the sense degenerate primer A and the antisense degenerate primer B, corresponding to amino acids 119–127 and 222–228 of AAG-B, respectively. The amplified product was sequenced and used to design specific antisense primer GSP-1, corresponding to amino acids 129–135 of AAG-B. GSP-1 was then used with the sense degenerate primer C, derived from amino acids 1–8 of AAG-A, for the second PCR. The product of the second PCR step was sequenced and used to design the specific antisense primer GSP-2, corresponding to amino acids 11–17 of AAG-A. In the third PCR step, GSP-2 was used along with the Marathon primer AP-1. The product of this step was used as the template for the fourth PCR step, in which GSP-2 was used along with the sense primer AP-2 to yield the 5′-end of the AAG cDNA.

The DNA sequence of the product obtained from the first PCR step was also used to design GSP-3, a specific sense primer corresponding to amino acids 174–182 of AAG-B. GSP-3 was then used along with the Marathon primers AP-1 and AP-2 in consecutive PCR steps, as described above, to yield the 3′-end of the AAG cDNA.

The full-length AAG cDNA was obtained by fusion of the corresponding 5′- and 3′-RACE fragments. The AAG cDNA encoding AAG-A, linker, and AAG-B was obtained by amplifying A. precatorius cDNA with the sense primer GSP-4, which encodes the first seven N-terminal amino acid residues of AAG-A, and the antisense primer GSP-5 (which encodes the last eight C-terminal amino acid residues and the stop codon of AAG-B). The PCR product was then ligated into the T vector pTAAgA DNA (Fig. 1 and Table I).

Expression of AAG in Escherichia coli—To construct the AAG-A expression vector, we obtained AAG-A cDNA by amplifying pTAAgA DNA with the sense primer GSP-6, which encodes the first seven N-terminal amino acid residues of AAG-A behind a BamHI restriction site and the antisense primer GSP-7, which encodes the last seven C-terminal amino acid residues of AAG-A with a stop codon following an EcoRI restriction site. The amplified BamHIEcoRI fragment of AAG-A was also ligated into the T-easy vector to form pTAAgA for amplification and nucleotide sequence analysis. The AAG-A fragment was then ligated into pGEX-2T to yield the expression vector pGEX-AAG-A (24).

The transformed cells were grown to a density of ~4 × 10^6 cells/ml and induced with 0.5 mM isopropyl-β-D-thiogalactospyranoside at 30 °C for 4 h. The fusion protein was then purified by glutathione-Sepharose 4B column chromatography and treated with thrombin. The reaction products were purified with a Mono Q fast protein liquid chromatography column (1.6 × 50 mm) to obtain reAAG (25).

Site-directed mutagenesis was carried out as described previously (26). A mutated primer, GSP-8, was synthesized to obtain the site-specific mutant AAG P199N, whereas another mutated primer, GSP-9, was used to obtain ABRa-A N200P (Table I).

Measurement of in Vitro Protein Synthesis Inhibition—The inhibitory effects of the wild-type and mutant proteins on translation in vitro were examined by measuring the incorporation of L-[3H]leucine into protein in a rabbit reticulocyte cell-free system as described previously (25). Various amounts of AAG-A, ABRa-A, or their mutant proteins were mixed with 11.5 μl of rabbit reticulocyte lysate in 20 mM Tris–Cl (pH 7.8) containing 4 μCi/ml L-[3H]leucine, 1.5 mM MgCl2, 5 mM dithiothreitol, and 50 mM KCl, followed by incubation at 30 °C for 90 min. The reaction products were precipitated with 25% trichloroacetic acid and collected on glass microfiber filters, and the radioactivity of the filters was determined with a liquid scintillation counter. Each reported value is the mean of triplicate samples.

Molecular Modeling of AAG and ABRa—Molecular modeling of AAG-A was performed using the crystal structure of ABRa-A obtained from the Protein Data Bank as the template (27). The amino acid sequence of AAG-A was aligned with the sequence of ABRa-A to optimize identities. Amino acid substitutions, insertions, and deletions in AAG-A and ABRa-A were simulated with Protein Quanta on a Silicon Graphics system (28–30).
RESULTS

Purification and Determination of the Amino Acid Sequence of Abrus Agglutinin—Sequential chromatography through Sepharose 6B and Sephadex G-100 columns typically yielded 450 mg of purified AAG from 200 g of seeds. Fractionation of 10 mg of reduced AAG on a Sephadex G-150 column in the presence of 6M urea and 0.13 mM 2-mercaptoethanol yielded roughly 1.5 mg of AAG-A and 2.5 mg of AAG-B. The complete primary structure of AAG was determined by sequencing the peptides generated by Lys-C endoproteinase, S. aureus V8 endoproteinase, or L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin digestion and cyanogen bromide treatment (Fig. 2). The calculated molecular masses of AAG-A and AAG-B were 28,618 and 29,981 Da, respectively.

Alignment of the amino acid sequences of AAG and ABRa showed high homology: AAG-A and ABRa-A had 168 (66.9%) invariant residues and 27 (10.8%) similar residues, whereas AAG-B and ABRa-B had 214 (80.2%) invariant residues and 17 (6.4%) similar residues (Fig. 3). Thirteen amino acid residues at the proposed active site, including two catalytic residues, Glu 163 and Arg 166, have been reported to be highly conserved among type I and II RIPs (31); these 13 residues were completely conserved within AAG-A. Two putative galactose-binding residues, Asn 51 and Asn 260, were present in AAG-B; these residues are also present in ABRa-B at the same sites. ABRa contains two putative N-linked oligosaccharide glycosylation sites (at Asn 100 and Asn 140) in the B chain and none in the A chain (32). Sequence analysis showed that AAG-B contains two glycosylation sites at the same locations as in ABRa-B, whereas AAG-A contains a new glycosylation site, Asn 250, not present in ABRa-A.

Cloning of the Abrus Agglutinin cDNA—RNA extraction from 15 g of A. precatorius seeds yielded 275 mg of total RNA. Sequence analysis showed that the AAG cDNA contains 2047 base pairs, with an open reading frame encoding a preproprotein with 547 amino acid residues: a 20-residue signal peptide, a 258-residue polypeptide (AAG-A), a 2-residue linker peptide, and a 267-residue polypeptide (AAG-B). The complete amino acid sequence of AAG deduced from the nucleotide sequence of AAG cDNA was identical to that determined by the protein sequencing techniques, except that there were two extra residues, Arg 259 and Ser 260, which form the internal linker between AAG-A and AAG-B. These results indicate that the precursor synthesized from the open reading frame of the AAG mRNA is post-translationally cleaved into AAG-A and AAG-B, which are linked by a disulfide bond.

Expression and Function of the Abrus Agglutinin A Chain—The yield of reAAG-A after affinity column purification and thrombin digestion of the glutathione S-transferase-AAG-A fusion protein followed by Mono Q chromatography purification was 1.0 mg/liter of induced culture. reAAG-A was homogeneous upon analysis by 10% SDS-PAGE, with an estimated molecular mass of 29 kDa (Fig. 4). Analysis of the protein synthesis inhibitory activity of reAAG-A showed the IC50 of reAAG-A to be 3.5 nM, which is similar to that of native AAG-A and 70-fold weaker than that of ABRa-A (26).

Molecular modeling was carried out to elucidate the struct-
tural features of AAG-A that might cause its lower inhibitory activity. The model showed that one of the putative substrate-binding sites of ABRa-A, Asn200, which corresponds to Arg213 of ricin (31), was substituted with Pro199 in AAG-A. Expression plasmids carrying AAG-A P199N and ABRa-A N200P were created, and reAAG-A P199N and reABRa-A N200P were purified to examine their inhibitory effects on protein synthesis in the rabbit reticulocyte lysate system. reAAG-A P199N (IC50 = 0.53 nM) was 7-fold more potent than wild-type reAAG-A, whereas reABRa-A N200P (IC50 = 2.3 nM) was 46-fold less potent than wild-type reABRa-A (Fig. 5).

**DISCUSSION**

The results of this study show that AAG is composed of 547 amino acid residues: 258 residues in AAG-A and 267 residues in AAG-B plus a 2-residue linker and a 20-residue signal peptide. Alignment of the amino acid sequence of AAG with those of ABRa, ABRb, and ABRd (32) shows considerable similarity among these four type II RIPs, with 347 invariant amino acid residues (66.1%) (Fig. 3); there are 382, 368, and 380 invariant residues between AAG and ABRa, ABRb, and ABRd, respectively.

The phylogenetic tree constructed from the sequence similarity suggests that the three isoabrin chains are more closely related to each other than to AAG (33). Furthermore, the isoabrin chains have been classified into two subtypes according to their N-terminal amino acid sequences: 258 residues in AAG-A and 267 residues in AAG-B, thus hindering the interactions. The invariant amino acid residues conserved among most type II RIPs have been classified into two subtypes according to their N-terminal sequence; the N-terminal amino acid sequence of type A-1 (ABRa) is Glu-Asp-Arg-Pro-Ile-Lys-Phe, whereas that of type A-2 (ABRb and ABRd) is Glu-Asp-Gln-Val-Ile-Lys-Phe (32). Comparison of the amino acid sequence of AAG-A with those of ABRs indicated that the isoabrin chains of AAG-A are Glu-Asp-Arg-Pro-Ile-Lys-Phe, whereas those of ABRa, ABRb, and ABRd are Glu-Asp-Gln-Val-Ile-Lys-Phe (32).

Comparison of the structures of these two proteins revealed that Asn200 of ABRa-A, corresponding to Arg213 of ricin A chain, is required for the binding of substrate to the active site of AAG-A. The mutant proteins on protein synthesis. The inhibitory effects of reABRa-A and reAAG-A and their mutant proteins on protein biosynthesis were examined in the rabbit reticulocyte cell-free system. reABRa-A, reAAG-A, reABRa-A N200P, reAAG-A P199N.

**Fig. 5. Effects of reABRa-A and reAAG-A and their mutant proteins on protein synthesis.** The inhibitory effects of reABRa-A and reAAG-A and their mutant proteins on protein synthesis were examined in the rabbit reticulocyte cell-free system. reABRa-A, reAAG-A, reABRa-A N200P, reAAG-A P199N.

**Fig. 6. Alignment of amino acid sequences of linker regions of three plant proteins: AAG, ABRa, and A. confusa trypsin inhibitor.** The linkers are underlined. ACTI, A. confusa trypsin inhibitor.

**Fig. 4. SDS-PAGE analysis of native and recombinant proteins.** Samples of purified proteins and recombinant proteins were analyzed by 10% SDS-PAGE and Coomassie Blue staining. Lane 1, AAG; lane 2, molecular mass markers (phosphorylase b, 97.4 kDa; serum albumin, 66.2 kDa; ovalbumin, 45.0 kDa; carbonic anhydrase, 31.0 kDa; trypsin inhibitor, 21.5 kDa; and lysozyme, 14.4 kDa); lane 3, AAG treated with 2-mercaptoethanol; lane 4, AAG-B; lane 5, AAG-A; lane 6, reAAG-A; lane 7, reAAG-A P199N; lane 8, reABRa-A; lane 9, reABRa-A N200P.
between AAG-A and its substrate at the centered GpApGpAp sequence. A similar situation has been reported previously (45) in which Pro¹⁴ of melittin caused 120° bending between segments 1–10 and 14–26 of the α-helix. Replacement of Asn²⁰⁰ of ABRa-A with Pro by site-directed mutagenesis remarkably increased the IC₅₀, whereas substitution of Pro¹⁹⁹ with Asn markedly decreased the IC₅₀ of AAG-A. This suggests that Asn²⁰⁰ of ABRa-A is important for its inhibitory activity.

ABRA and AAG have similar therapeutic indexes for the treatment of experimental mice with tumors (12), but AAG has much lower toxicity (LD₅₀ 5 mg/kg of body weight) compared with ABRa (LD₅₀ 20 μg/kg of body weight). In our recent studies, AAG and ABRa were found to cause apoptosis; the antitumor activity is significantly correlated with apoptosis. Further investigations are needed to study the apoptosis induced by AAG and ABRa with the goal of developing immunotoxins for cancer chemotherapy.

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