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Isolation, Primary Sequence Determination, and Disulfide Bond Structure of Cyanovirin-N, an Anti-HIV(Human Immunodeficiency Virus) Protein from the Cyanobacterium *Nostoc ellipsosporum*[1]

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A novel anti-HIV protein, cyanovirin-N (CV-N), was isolated from an aqueous cellular extract of the cultured cyanobacterium (blue-green alga) *Nostoc ellipsosporum*, purified by reverse-phase HPLC, and sequenced by N-terminal Edman degradation of the intact protein and peptide fragments produced by endoproteinase digestions. CV-N consists of a single 101 amino acid chain which exhibits significant internal sequence duplication, but no significant homology to previously described proteins or to the transcription products of known nucleotide sequences. Alignment of residues 1-50 with residues 51-101 reveals 13 conservative amino acid changes as well as direct homology between 16 amino acid residues. CV-N contains four cysteines which form two intrachain disulfide bonds. The positions of the disulfide linkages were established by fast atom bombardment mass spectral studies of peptide fragments generated by a tryptic digestion of the native protein. Reductive cleavage of these crosslinks resulted in loss of anti-HIV activity.

As part of the U.S. National Cancer Institute (NCI)'s effort to discover and develop new classes of compounds which inhibit the infectivity and/or cytopathic effects of the human immunodeficiency virus (HIV), an extensive collection of extracts from terrestrial plants, marine organisms and selected microbial sources has been screened for *in vitro* anti-HIV activity (1). Numerous HIV-inhibitory compounds with diverse viral and cellular targets have been isolated and identified from natural product extracts which were active in the NCI screen. These include novel metabolites which inhibit reverse transcriptase (2-9), block HIV-induced cell fusion (9), disrupt protein kinase-C mediated processes (10, 11), and bind to the cellular receptor CD4 (12).

Cyanobacteria (blue-green algae) were of interest for anti-HIV screening because they are known to produce a wide variety of structurally unique and biologically active non-nitrogenous and amino acid-derived natural products (13-15). These photosynthetic procaryotic organisms are the source of cyclic and linear peptides which often exhibit hepatotoxic (16-20), antimicrobial (21-23), antitumor (24-28), or enzyme inhibitory properties (29-34). Sequencing studies of higher molecular weight cyanobacterial peptides and proteins have generally focused on those associated with primary metabolic processes or ones that can serve as phylogenetic markers (35-39). Proteins with antiviral properties have not generally been associated with cyanobacterial sources.

The discovery, anti-HIV activity profile and other unique biological properties of a novel protein named cyanovirin-N (CV-N) were recently disclosed (40). The protein was produced recombinantly in *Escherichia coli*

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Abbreviations used: HIV, human immunodeficiency virus; HPLC, high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ESI-MS, electrospray ionization mass spectrometry; FAB-MS, fast atom bombardment mass spectrometry; CH_3CN, acetonitrile; TFA, trifluoroacetic acid.

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by expression of a synthetic DNA coding sequence corresponding to the amino acid sequence deduced initially for natural CV-N isolated from the cyanobacterium *Nostoc ellipsosporum* (40). CV-N potently inhibited the *in vitro* cytopathicity of diverse clinical isolates and laboratory strains of HIV type 1, HIV type 2 and simian immunodeficiency virus. CV-N also effectively prevented cell-to-cell fusion and transmission of HIV from infected cells to uninfected host cells. Pretreatment of HIV virions with CV-N irreversibly neutralized virus infectivity, but CV-N was nontoxic to host cells. The unique virucidal effects of CV-N result, at least in part, from its association with the viral envelope glycoprotein gp120; cyanovirin-N apparently interferes with critical interactions between viral gp120 and cell-surface receptors which are required for successful virus fusion and entry into the cell (40). Here we detail the initial anti-HIV bioassay-guided discovery of CV-N from *Nostoc ellipsosporum*, and describe the primary amino acid sequence determination, internal sequence homologies and disulfide bond mapping of the natural protein.

MATERIALS AND METHODS

Cyanobacterial culture, extraction, and workup. The cyanobacterial culture conditions, media and classification have been described previously (41). In brief, the cellular mass from a unialgal strain of *Nostoc ellipsosporum* (culture Q68D170) was harvested by filtration, freeze dried and extracted first with MeOH-CH_2Cl_2 (1:1) followed by H_2O. Individual aliquots of the organic and aqueous extracts were tested for cytoprotective properties in the NCI primary anti-HIV screen (42). Only the H_2O extract showed anti-HIV activity. A solution of the aqueous cyanobacterial extract (30 mg/ml) was precipitated by addition of an equal volume of EtOH. The 1:1 H_2O-EtOH solution was kept at −20°C for 15 hr and then centrifuged to remove precipitated materials. The resulting supernatant was evaporated and then fractionated by step-gradient elution vacuum-liquid chromatography (43, 44) on wide-pore C_4 packing (300 Å, BakerBond WP-C_4). Anti-HIV activity was concentrated in the material which eluted with MeOH-H_2O (2:1). Final purification of the active protein from this fraction was achieved by reverse-phase HPLC.

Protein and peptide purification. Separations were performed by HPLC on a 1.9 × 15 cm 1Bondapak C_{18} (Waters Associates) column eluted with a gradient of increasing concentrations of acetonitrile (CH_3CN) in H_2O. The mobile phase contained 0.05% (v/v) TFA, pH 2. Eluted proteins and peptides were detected by UV absorption at 206, 280 and 294 nm with a rapid spectral detector (Pharmacia LKB model 2140). Individual fractions were collected, pooled based on the UV chromatogram, and lyophilized.

Endoproteinase digestions. Digestions of purified CV-N were carried out with trypsin, chymotrypsin, endoproteinase Glu-C and endoproteinase Asp-N. Protein samples to be digested (22-135 nmoles) were dissolved in a Tris-HCl buffer at pH 8-8.5. Digestions were initiated by addition of the endoproteinase at an enzyme/protein ratio (wt/wt) of 1:100 for trypsin and chymotrypsin, 1:130 for Asp-N and 1:22 for Glu-C. Digestions were carried out at room temperature for 16 hrs with Glu-C, 24 hrs with trypsin and chymotrypsin and 72 hrs with Asp-N.

Reduction of disulfide bonds. Pure protein, peptide digests thereof, and selected HPLC fractions were treated with guanidine hydrochloride (approx. final concentration 8 M) and β-mercaptoethanol (approx. final concentration 2% by vol.) to reductively cleave disulfide linkages. After treatment for 30 min, the mixture was brought to pH 2 by addition of TFA and the resulting products were separated by C_{18} reverse-phase HPLC on μBondapak columns. Proteins and peptide fragments were characterized by amino acid analysis and sequenced by Edman degradation.

Gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using reducing conditions was performed as previously described (45).

Edman degradation. Automatic N-terminal Edman degradations were performed on approximately 1 nmol samples of HPLC purified protein or peptide fragments by use of a pulsed, liquid-phase protein sequencer (Applied Biosystems, Inc.; model 4774A) equipped with an on-line phenylthiohydantoin analyzer (model 120A).

Amino acid analysis. Approximately 5 μg of purified protein or protease-digested peptide was hydrolyzed in 200 μl of 6 M HCl containing 0.1% phenol at 110°C for 24 hr. The samples were dried *in vacuo* and analyzed on a Beckman model 6300 automated amino acid analyzer.

Mass spectrometry. Electrospray ionization mass spectrometry (ESI-MS) was performed with a JEOL SX102 equipped with an Analytica electrospray source. The spectrometer was calibrated using a lysozyme standard (molecular weight = 14305.2) prior to each analysis. Samples were injected into the source in a 1:1 solution of hexafluorosopropanol and 2% acetic acid. The masses reported were averages calculated from the various charged states observed. Fast atom bombardment mass spectrometry (FAB-MS) was performed on the same instrument, with glycerol as the matrix, and masses were measured as the lowest isotope values.

Disulfide determination. To 3.5 nmoles of native, nonreduced CV-N was added 60 μl of 100 mM ammonium bicarbonate (pH = 8), 6 μl acetonitrile and 6 μl of a 40 μM solution of the endoproteinase trypsin in H_2O. The mixture was incubated at 37°C for 16 hrs and then separated by C_{18} HPLC using a linear gradient from 0-100% CH_3CN in H_2O with 0.05% TFA (v/v) in the mobile phase. Eluted peaks corresponding to peptide fragments were analyzed by FAB-MS. Masses reported were $(MH)^+$ average masses. Peptides were then treated with an excess of iodoacetic acid and re-analyzed by FAB-MS.

Sequence comparisons. Amino acid sequences were compared to the Allprot database using the GCG Wisconsin package.

Anti-HIV assay. H_2O-DMSO (3:1) solutions of the chromatographic fractions and pure compounds were tested for anti-HIV activity in a XTT-tetrazolium based assay, as described previously (46).

RESULTS

The present study originated from observations of anti-HIV activity in a crude aqueous extract of cultured *Nostoc ellipsosporum* in the NCI's primary *in vitro* anti-HIV screening assay (42). We used a bioassay-guided fractionation strategy to isolate the anti-HIV active component from the subject extract. Initially, high molecular weight biopolymers were precipitated from an aqueous solution of the extract by addition of an equal volume of EtOH. The resulting precipitate was removed by centrifugation. The HIV-inhibitory supernatant was fractionated by reverse-phase, vacuum-liquid chromatography (43, 44) on wide-pore C_4 packing eluted with increasing concentrations of MeOH in H_2O. Anti-HIV activity was concentrated in the material that eluted with MeOH-H_2O (2:1); SDS-PAGE analysis

of this fraction showed one main protein band with a relative molecular mass (M_r) of approximately 10 kDa. Final purification was achieved by repeated reverse-phase HPLC with a CH_3CN-H_2O gradient containing 0.05% TFA. Pooled HPLC fractions were subjected to SDS-PAGE and amino acid analysis and also tested for their ability to inhibit the cytopathic effects of HIV infection. In the initial HPLC separation, using a linear gradient from 30-50% CH_3CN, the anti-HIV activity coeluted with the principal UV absorbing peak at approximately 33% CH_3CN. Fractions corresponding to the active peak were pooled and split into two aliquots. Reinjection of the first aliquot under similar HPLC conditions, but with a linear gradient from 28-38% CH_3CN, provided an anti-HIV fraction that showed only one protein band (CV-N) by SDS-PAGE. The remaining aliquot from the original HPLC separation was treated with guanidine hydrochloride and β-mercaptoethanol prior to reinjection on the HPLC. Using an identical 28-38% gradient, the reduced material gave one principal peak that eluted later in the run with 36.8% CH_3CN (Fig. 1). The HPLC fractions from the reduced material were essentially devoid of anti-HIV activity, suggesting that disulfide bonds were present in native CV-N and that the intact intramolecular linkages were required for anti-HIV activity.

The amino acid sequence of reduced, HPLC purified CV-N was established by N-terminal Edman degradation of the intact protein and by sequencing of peptide fragments generated by endopeptidase digestion. Cysteine residues were alkylated with vinyl pyridine prior to digestion and sequencing. Cleavage of CV-N with trypsin, chymotrypsin, endoproteinase Asp-N and endoproteinase Glu-C provided sufficient overlapping peptide fragments to unambiguously sequence all 101 amino acids (Fig. 2). CV-N was digested with the individual endopeptidase enzymes, and resulting peptide fragments were purified by HPLC on μBondapak C_{18}. Purified peptides were characterized by amino acid analysis and sequenced by N-terminal Edman degradation. Results from amino acid analyses of each fragment were fully consistent with the sequence derived by Edman degradation.

Electrospray ionization mass spectrometry (ESI-MS) of CV-N showed a molecular ion with *m/z* 11014.2, which was consistent with the calculated value (*m/z* 11013.2) for the deduced amino acid sequence. There was no sign of higher molecular weight ions which would result if CV-N occurred as an oligomer incorporating two or more 101 amino acid subunits. Selected fragments from the endoproteinase Asp-N digestion were also analyzed by ESI-MS. The fragment consisting of residues 1-22 gave a molecular ion at *m/z* 2307.8 (calculated *m/z* 2308.6 for that sequence), residues 1-34 gave *m/z* 3588.7 (calculated *m/z* 3588.9), residues 44-70 gave *m/z* 2953.0 (calculated *m/z* 2953.2), and residues 44-88 gave *m/z* 4987.0 (calculated *m/z* 4987.6). These data fully supported the proposed primary amino acid sequence of CV-N. Amino acid analysis of CV-N was also in good agreement with the deduced primary sequence (Table 1).

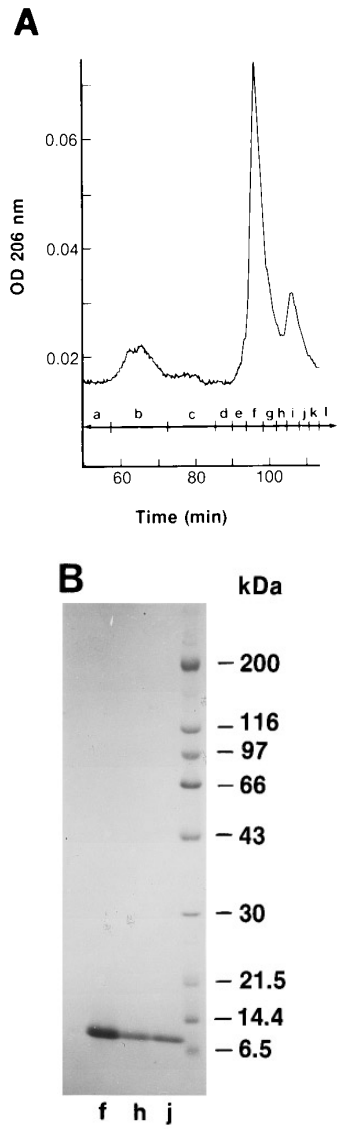

FIG. 1. HPLC separation and SDS-PAGE analysis of CV-N. (A) μBondapak C_{18} HPLC chromatogram of CV-N monitored at 206 nm. The sample was reduced with β-mercaptoethanol and then eluted with a linear CH_3CN/H_2O gradient (buffered with 0.05% TFA) from 28-38% CH_3CN. HPLC fractions were collected as indicated. (B) SDS-PAGE analysis of selected HPLC fractions. Aliquots of the fractions were lyophilized, separated by SDS-PAGE on a 10-20% gradient gel and visualized by staining with Coomassie Brilliant Blue.

To further establish the presence and locations of the intramolecular disulfide crosslinks in CV-N, an aliquot of nonreduced protein was treated with trypsin and the resulting peptides analyzed by FAB-MS after separation by HPLC. The cleavage sites and calculated MH^+ ion *m/z* values for the theoretical peptide fragments generated by complete trypsin digestion are illustrated

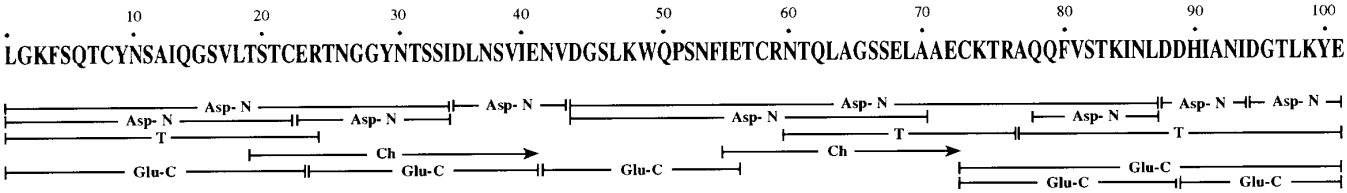

FIG. 2. Primary amino acid sequence of CV-N. The protein was sequenced by N-terminal Edman degradation of intact CV-N and a series of overlapping peptide fragments generated by endoproteinase digestions. Selected peptides isolated by C_{18} HPLC from digests with endoproteinase Asp-N, trypsin (T), chymotrypsin (Ch) or endoproteinase Glu-C are shown. All peptides were characterized by amino acid analysis and Edman degradation. Peptide fragments depicted with brackets were fully sequenced. Fragments designated with arrows were sequenced up to the arrow head, although amino acid analysis indicated this was not the complete sequence.

in Fig. 3. Mass values for cleavage sequences containing cysteine residues were calculated for reduced cysteines with free sulfhydryl (-SH) groups. The peptide fragment for residues 4-24, which contains two cysteines, provided a MH^+ ion at *m/z* 2294.9. This was in good agreement with the calculated value (*m/z* 2294.5) for the fragment with a disulfide link between cysteines at positions 8 and 22, and was approximately 2 daltons less than the calculated value if both cysteines existed as free sulfhydryls. The remaining two cysteines were located in separate digest fragments consisting of residues 49-59 and residues 60-74. A disulfide bond that joined these two fragments was evidenced by a MH^+ ion at *m/z* 2901.0 (calculated *m/z* 2901.2 for the disulfide linked fragments). This ion could not result from incomplete cleavage at arginine 59, as the calculated mass of fragment 49-74 is *m/z* 2885.2. The cysteine-containing peptides from the digest were unaffected by treatment with iodoacetic acid, which confirmed that no free sulfhydryl groups were present. The mass spectral data clearly indicated cysteines 8 and 22 were joined by a disulfide bridge, as were cysteines 58 and 73. No mass spectral evidence for other disulfide linkages was observed during analysis of the tryptic digest.

Reduction of the disulfide bonds by treatment of purified CV-N with guanidine hydrochloride and β-mercaptoethanol, followed immediately by HPLC purification, resulted in a product with essentially no anti-HIV activity. However, solutions of the reduced CV-N kept in prolonged storage at −20° showed full recovery of anti-HIV activity. Furthermore, when CV-N was treated with guanidine hydrochloride and β-mercaptoethanol but not subjected to C_{18} HPLC, and was instead simply desalted on a 3 kDa ultrafilter, reconstituted and assayed, it showed virtually the same anti-HIV activity as the untreated protein.

TABLE 1

Amino Acid Analysis of Reduced, HPLC-Purified Cyanovirin-N

Amino acid	Residues determined from amino acid analysis	Residues determined from sequencing
D & N	13.9	14 (5D & 9N)
T	9.6	10
S	12.3	12
Q & E	12.1	12 (6E & 6Q)
P	1.0	1
G	6.9	7
A	6.1	6
V	3.7[a]	4
M	0	0
I	6.4[a]	7
L	8.0	8
Y	3.0	3
F	3.1	3
H	1.3	1
K	4.9	5
R	3.1	3
W	—[b]	1
C	4.0[c]	4
		101 Total

[a] Presence of V^{39}-I^{40} bond would be expected to give a low yield in a 24 hr acid hydrolysis.

[b] Not determined.

[c] C was analyzed as its performic acid oxidation product with an estimated 80% overall yield.

DISCUSSION

The primary amino acid sequence of cyanovirin-N was demonstrated unambiguously by a combination of

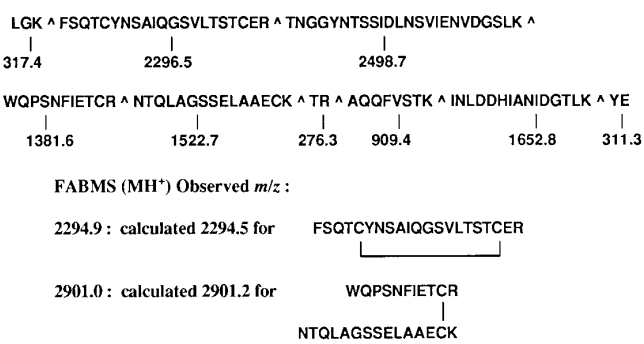

FIG. 3. Disulfide determination by FAB-MS analyses. Cleavage sites (^) and theoretical peptide fragments generated by tryptic digestion of CV-N and their calculated MH^+ ion masses are shown. Mass values for peptides containing cysteine residues were calculated for reduced cysteines with free sulfhydryl groups.

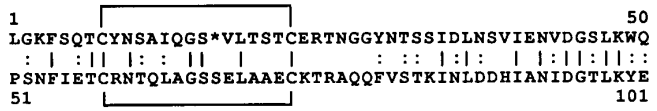

FIG. 4. Comparison of the amino acid sequences of domains 1-50 and 51-101 of CV-N. Sequence homology of the domains was maximized by insertion of one gap (*) following amino acid 16. Identical amino acids (solid bars) and conserved amino acids (dots) are indicated. The disulfide cross links are marked (solid lines) above domain 1-50 and below domain 51-101.

N-terminal Edman degradation, amino acid analysis and mass spectral characterization of the intact 101 amino acid protein and peptide fragments generated by digestion with a series of endoproteinases. There was no sign of post-translational modification of any residues. Mass spectral analyses indicated that the active form of CV-N exists as a single polypeptide chain, not an oligomeric complex. The measured molecular weight of CV-N, as determined by ESI-MS, was within 1.0 atomic mass units of the calculated molecular weight (11013.2) and no higher molecular weight species were observed. The two intact intramolecular disulfide bonds in CV-N appear to be required for anti-HIV activity, since cleavage of these bonds by β-mercaptoethanol treatment essentially abolished the HIV-inhibitory effects. However, when stored in solution phase, reduced CV-N could apparently reform the required disulfide bonds, as evidenced by rapid recovery of anti-HIV activity. These observations, as well as the ability of CV-N to retain activity following treatment with detergent, high salt concentrations, H_2O_2 or extreme heat (40), suggest that the active tertiary conformation of CV-N is remarkably resistant to denaturation, and/or that it can rapidly and spontaneously renature.

There are no more than 8 contiguous amino acids nor more than 20% overall sequence homology shared between CV-N and any known protein or transcription product of any known nucleotide sequence. However, the two "halves" of CV-N (comprising amino acids 1-50 and amino acids 51-101) share a high degree of internal sequence homology; if these two subsequences are aligned with a one amino acid gap inserted after serine 17, then 16 residues (32%) share direct homology and 13 (26%) represent conservative amino acid changes (Fig. 4). The intrastrand loop formed by the C8-C22 disulfide also corresponds very closely with the loop defined by the C58-C73 disulfide link. A high degree of direct sequence homology between the two halves of CV-N is observed in the region of the disulfide-induced loops and in a series of amino acids farthest from the loops (residues 40-49 and 91-100). Duplication of amino acid sequences is well known in other proteins (eg. myoglobins, hemoglobins and ferredoxins), and is believed to result from gene duplication during the evolution of these proteins.

It is interesting to note that CV-N, the product of a procaryotic organism, is extremely effective at blocking *in vitro* infection and replication of HIV, a virus that presumably can only target eukaryotic hosts. Viruses (cyanophages) are known to lethally infect a wide variety of cyanobacteria, including members of the genus *Nostoc* (47-50). However, the physiological role(s) of CV-N within cyanobacteria that produce it, and the evolutionary significance, if any, of CV-N's ability to inactivate mammalian immunodeficiency retroviruses, are unknown, but pose interesting questions for further consideration.

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