proach of Adam & Delbrück (1968) to interpret our results both in low and high salt medium.

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Virotoxins: Actin-Binding Cyclic Peptides of *Amanita virosa* Mushrooms†

Heinz Faulstich,* Angeliki Buku, Heinz Bodenmüller, and Theodor Wieland

ABSTRACT: Virotoxins are toxic peptides singularly found in *Amanita virosa* mushrooms. After purification and resolution by high-pressure liquid chromatography, the main component, viroisin, was selectively cleaved and submitted to Edman degradation. The structure could be completely elucidated and was in part found to be the same as in phallotoxins. Differing from the phallotoxins, however, virotoxins are monocyclic peptides and contain D-serine instead of L-cysteine. In addition, two amino acids were detected in virotoxins which thus far have not been found in nature: 2,3-*trans*-3,4-dihydroxy-L-proline and 2′-(methylsulfonyl)-L-tryptophan. The biological activity of viroisin is comparable to that of the phallotoxins: e.g., with 2.5 mg of viroisin per kg (white mouse), 50% of the animals die within 2–5 h by hemorrhagia of the liver. Also, on the molecular level, the virotoxins behave similar to the phallotoxins. Thus, viroisin binds to rabbit muscle actin as proved by difference UV spectroscopy. With an apparent equilibrium dissociation constant $K_D \sim 2 \times 10^{-8}$ M, the affinity of viroisin is very similar to that of phalloidin. However, the flexibility of the monocyclic structure and the presence of two additional hydroxy groups in the virotoxins suggest a different mode of interaction with actin. While there is proof that the bicyclic phallotoxins possess a rigid binding site, the virotoxins may adopt the biologically active conformation by an induced-fit mechanism upon contact with actin.

While the toxic cyclic peptides of the green death cap *Amanita phalloides* (Vall. ex. Fr.) Secr. have been extensively investigated during the past 4 decades by Wieland and coworkers [for a review, see Wieland & Faulstich (1978)], intensive research on the toxins of the white species *Amanita virosa* Lam. ex. Secr. was only recently initiated.

α-Amanitin was detected in *A. virosa* by Tyler et al. (1966). In a more rigorous investigation of this species, in two specimens from different locations in Europe, the α-amanitin content was measured to be 1.2–1.4 mg per g dry weight, this being slightly higher than that in the green species (Faulstich et al., 1974). However, in samples of *A. virosa* collected in

† From the Max-Planck-Institute for Medical Research, Department of Chemistry of Natural Products, D-6900 Heidelberg, West Germany. *Received October 22, 1979.*

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the United States (Yocum & Simons, 1977), including also those of the present study, α-amanitin was absent. In these cases a nearly equivalent amount of an amanin-like toxin was found, which in a recent study has been identified as amaninamide (Buku et al., 1980b). Concerning the homologous amatoxins known from the green death cap, neither β- nor γ- nor ε-amanitin was detected in the white mushrooms, be they of European or North American origin. This is in good agreement with the recent analysis of *A. virosa* by Yocum & Simons (1977).

Phallotoxins in *A. virosa* were detected by Faulstich et al. (1974) and confirmed by Malak (1976). Likewise, in the group of the phallotoxins, the broad toxin pattern of the green species was absent in the European sample in favor of high amounts of phalloidin and phallacidin. In North American samples phallacidin may be absent as was the case for the mushrooms investigated in this study.

Uncertain as the presence of some amatoxins and phallotoxins in *A. virosa* may be, one group of toxins was present in all samples of this species investigated so far. These toxins were called virotoxins and represent the subject of this study.

Experimental Procedures

Materials

A. virosa mushrooms were collected by one of us (Th.W.) in West Virginia. All solvents were freshly distilled and all reagents were of analytical grade. The L- and D-amino acid oxidases were from Boehringer (Mannheim). Actin was prepared from rabbit muscle according to Lengsfeld et al. (1974).

Methods

Isolation of Virotoxins. Air-dried *A. virosa* mushrooms (100 g) were soaked in 100 mL of distilled water for 1 h. After addition of 200 mL of distilled methanol, the mixture was homogenized with a mixer and the homogenate was magnetically stirred for 18 h under an N_2 atmosphere. After centrifugation, the pellet was washed 3 times with warm methanol, and the washings were added to the extract. After evaporation of the solvent in vacuo, the residue was twice stirred, magnetically, with dry peroxide-free diethyl ether to remove lipid material, and the ether was decanted. The residue was extracted again 3 times with methanol (60 °C). The extracts were combined, evaporated, dissolved in warm water, centrifuged, applied to a Sephadex LH-20 column (250 × 2.5 cm), and developed with water. Fractionation was in 15-mL portions, and the elution was monitored at 280 nm. The fraction containing the virotoxin mixture was rechromatographed on the same column by using 0.004 M NH_4OH as the solvent.

LC[1] Resolution of the Virotoxin Mixture. The mixture of virotoxins as obtained from the second column was subjected to high-pressure liquid chromatography (LC) on a Du Pont chromatograph using a Zorbax ODS column (200 × 9 mm). The peptides were eluted with a linear gradient of acetonitrile–water at a pressure of 1500 psi and at a flow rate of 2 mL/min. Detection was by optical density at 254 nm.

Thin-Layer Chromatography and Electrophoresis. The resolution of the LC procedure was confirmed by TLC on silica (Merck, Darmstadt, 60F-254) in 2-butanol–ethyl acetate–water, 14:12:5 (v/v), or in chloroform–methanol–water, 65:25:4 (v/v). Detection was by the UV absorption of the toxins under a 256-nm lamp on the fluorescent silica or by reaction with cinnamic aldehyde–HCl. High-voltage paper electrophoresis was performed by using buffers of pH 6.5 [pyridine–acetic acid–water, 20:2:178 (v/v)] and pH 1.9 [acetic acid–formic acid–water, 3:1:16 (v/v)].

UV and CD Spectra and Optical Rotations. UV spectra were performed on a Pye-Unicam SP 1700 ultraviolet spectrophotometer with 1.35×10^{-4} M aqueous solutions. The same solution was used to measure the circular dichroism (CD) on a Dichrograph II (Roussel-Jouan). Optical rotations were measured with a Perkin-Elmer polarimeter. Difference UV spectra were obtained by mixing a 2×10^{-5} M solution of F-actin (in 0.1 M KCl and 1 mM Tris, pH 7.4) with a 2×10^{-5} M solution of the toxin in the same buffer in a tandem cuvette (Wieland et al., 1975).

Amino Acid Analysis. Pure cyclopeptides were hydrolyzed for 6 h in 6 N HCl at 110 °C and dried over solid KOH for several hours. The residues were applied to a Biotronik amino acid analyzer and developed with buffers of pH 3.2 and 4.2 and a NaCl-containing buffer, pH 5.8. The hydroxylated leucine units were identified as amino lactones according to Faulstich et al. (1973).

Preparation of Secoviroisin. Viroisin (30 mg) was stirred for 24 h at room temperature in 20 mL of 80% trifluoroacetic acid. After evaporation in vacuo the residue was applied to a Sephadex LH-20 column (150 × 2.5 cm) and developed with methanol. The seco compound eluted in the fraction at 465–555 mL: yield, 19 mg of pure, linear peptide as shown by electrophoresis at pH 1.9 and 6.5, as well as by TLC.

Edman Degradation (Petersen et al., 1972). The amino acid sequence of the linear peptide was determined either by using the dansyl technique (Gray, 1972) or by analysis of the PTH derivatives. The latter were examined by mass spectrometry (Hagenmaier et al., 1970) or compared to authentic samples of the PTH-amino acids by TLC on silica gel (Merck, Darmstadt, 60F-254) with chloroform–methanol, 9:1 (v/v), as the solvent.

Isolation of the Chromophoric Amino Acid Unit. Viroisin (86 mg, 0.094 mmol) was dissolved in 2 mL of pyridine. After addition of 3 equiv of tosyl chloride, the mixture was allowed to react for 10 h at 0 °C and for another 2 days at 35 °C. The solvent was removed by evaporation in vacuo, and the residue was applied to a Sephadex LH-20 column (250 × 2.5 cm) and developed with methanol. The first eluted fraction, as detected by UV absorption at 280 nm, represented 150 mg of pertosylated viroisin.

The compound was hydrolyzed in 3 mL of propionic acid–12 N hydrochloric acid, 1:1 (v/v), for 0.5 h at 120 °C (Westall & Heser, 1974). After evaporation of the acids in vacuo and drying of the residue over solid KOH, the material was developed on a Sephadex LH-20 column (250 × 2.5 cm) with 1.5% acetic acid. The latest eluting fractions (900–975 mL) were evaporated and applied to a silica thin-layer plate (Merck, Darmstadt, 60 F-254) in 1-butanol–acetic acid–water, 4:1:1 (v/v). The main component (R_f = 0.44) was scraped off, eluted immediately with methanol, and applied to another Sephadex LH-20 column (150 × 1 cm) and developed with methanol. The chromophoric amino acid eluted at 55 mL as a single peak: yield, 5 mg of pure chromophore [2′-(methylsulfonyl)tryptophan].

Synthetic Procedure for PTH-2′-(methylsulfonyl)tryptophan. 2′-(Methylthio)tryptophan (**1**) (*Savige & Fontana, 1976*). 3a-Hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-*b*]-indole-2-carboxylic acid (0.46 g, 2 mmol) was dissolved in 20

[1] Abbreviations used: LC, high-pressure liquid chromatography; TLC, thin-layer chromatography; CD, circular dichroism; PTH, phenylthiohydantoin; MS, mass spectrometry.

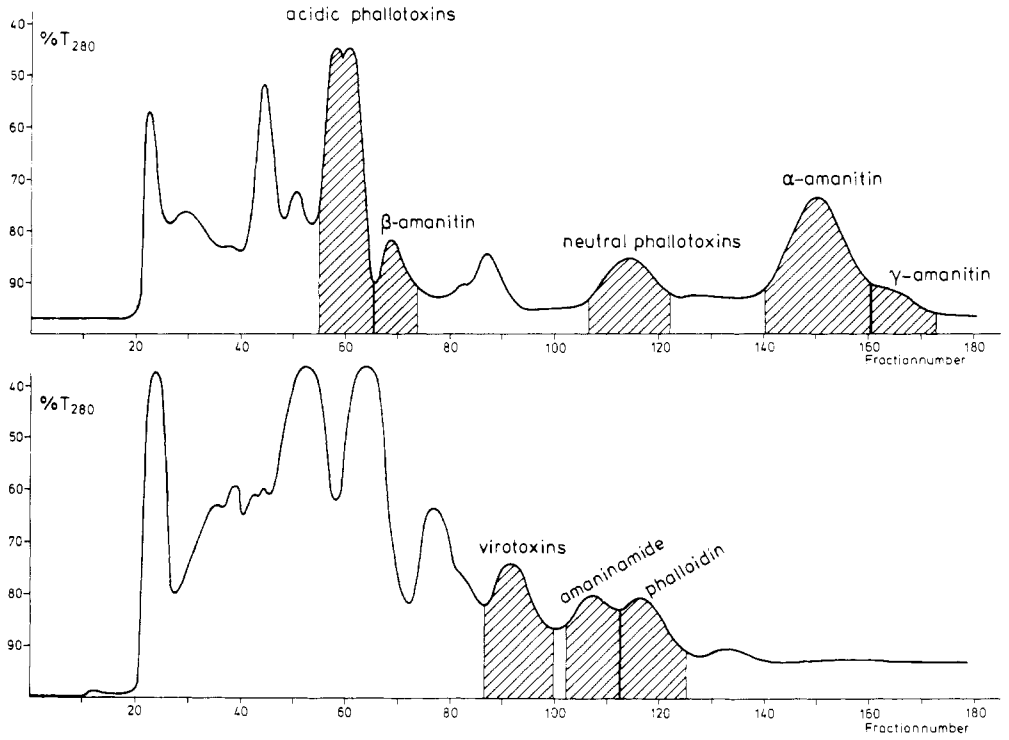

FIGURE 1: Elution diagrams of methanolic extracts of *A. phalloides* (top) and *A. virosa* (bottom) from a Sephadex LH-20 column monitored by optical density at 280 nm.

mL of 20% acetic acid. Methylmercaptan (2.7 equiv) in 3 mL of 20% acetic acid was added and the mixture was stirred for 4 h at 80 °C. After evaporation the residue was crystallized from ethanol by addition of a few drops of water: yield, 250 mg (50%); characterization, UV spectrum as typical for 2'-thioether of tryptophan, blue color after reaction with cinnamic aldehyde–hydrochloric acid, and TLC in ethanol–water, 7:3 (v/v), R_f = 0.75.

2'-(Methylsulfonyl)tryptophan (**2**) (*Wieland et al., 1974*). **1** (100 mg, 0.4 mmol) was dissolved in acetic acid and 20 equiv of 33% H_2O_2 was added. After stirring for 22 h at 22 °C, we evaporated the solvent in vacuo and crystallized the residue from methanol–water: yield, 60 mg (53.2%); TLC, R_f (ethanol–water, 7:3 v/v) = 0.69. Anal. Calcd for $C_{12}H_{14}N_2O_4S$ (M_r 282): C, 51.06; H, 4.96; N, 9.92; S, 11.34. Found: C, 50.55; H, 5.08; N, 9.98; S, 11.35.

PTH-2'-(methylsulfonyl)tryptophan (**3**). **3** was prepared from **2** and phenylisothiocyanate according to Cherbuliez et al. (1963): TLC, R_f (chloroform–methanol, 9:1 v/v) 0.78; MS, 399 (M^+); LC (Zimmermann et al., 1977), retention time = 2.6 min.

Isolation of the Hydroxylated Proline Unit. Procedure 1. Viroisin (100 mg) was submitted to hydrolysis with 5 mL of 70% perchloric acid (Galasinski et al., 1978) at 120 °C for 2 h. The mixture was cooled in an ice bath and neutralized by addition of 1 N KOH, kept 1 h at 0 °C, and then centrifuged. The clear brown supernatant was evaporated in vacuo and dried.

Chromatographic fractionation of the residue was performed on a 40 × 1.1 cm Amberlite IR-120/AS (H^+ form) ion-exchange column (~40 g of resin; Serva, Heidelberg) using pyridine–formate buffer, pH 3.1, at 50 °C according to Nakazime & Volcani (1969). The fractions obtained were tested by TLC on silica (Merck, Darmstadt, 60F-254) in ethanol–water, 7:3 (v/v). The column fraction eluting at 22–34 mL yielded 8.5 mg of crystalline product upon standing at 4 °C: characterization, TLC (ethanol–water, 7:3 v/v), R_f = 0.52; visualization by ninhydrin, yellow color; $[\alpha]^{26}_D$ −45° (*c* 1, H_2O); paper electrophoresis (pH 1.9, 40 mA, 90 min), 9.7 cm toward cathode.

Procedure 2. Viroisin (15 mg) was hydrolyzed with 6 N HCl at 110 °C for 6 h. After evaporation the residue was kept overnight in the presence of solid KOH and submitted to preparative electrophoresis for 4 h on a 1-mm filter sheet at pH 1.9 (formic acid–acetic acid–water, 1:3:16 v/v). That section of the sheet containing the imino acid as identified by ninhydrin reaction was separated and eluted with water–acetic acid, 20:1 (v/v). The eluted material was submitted to another silica TLC with ethanol–water (7:3 v/v). After elution and evaporation, the residue was dansylated according to Pataki (1966) and analyzed by mass spectrometry.

Reaction with L- *and* D-*Amino Acid Oxidases.* The hydrolysate of viroisin was incubated at 37 °C under an O_2 atmosphere with L-amino acid oxidase for 24 h and with D-amino acid oxidase for 48 h, according to Sieber et al. (1970). The nondegraded amino acids were analyzed on a Biotronik amino acid analyzer as described above.

Equilibrium Dialysis with Actin. These experiments were performed according to Faulstich et al. (1977).

Results

Chromatography of Mushroom Extracts. The methanolic extract of the North American sample of *A. virosa* was resolved on a Sephadex LH-20 column with an efficacy comparable to that described for *A. phalloides* (Faulstich et al., 1973, 1974). However, the toxin pattern proved to be completely different from that of *A. phalloides* (Figure 1). In the American sample of *A. virosa* neither acidic toxins nor α-amanitin could be detected. Beside phalloidin, two toxic components, completely absent in *A. phalloides*, were separated. One of them turned out to be amaninamide, which differs from α-amanitin by the lack of the 6'-hydroxy group (Buku et al., 1980b), while the other proved to be a mixture of toxic peptides different from all toxins characterized thus far. From the known toxins they could be definitely distinguished by their serine content, by their nonreactivity with

Table I: Amino Acid Composition of the Five Virotoxin Fractions after LC

LC fraction no.	composition						
	Ala	Val	Ser	Thr	ninhydrin, yellow moiety[a]	$(OH)_3$-Leu lactone	$(OH)_2$-Leu lactone
1	2.00	0.1	0.85	0.92	0.11	0.63	0.21
2	1.00	0.98	0.86	0.91	0.18	0.81	0.1
3	1.00	0.97	0.82	0.95	0.16	0.79	0.1
4	1.00	1.02	0.88	0.93	0.15	0.1	0.89
5	1.00	0.94	0.85	0.89	0.09	0.1	0.69

[a] Calculated on the basis of proline.

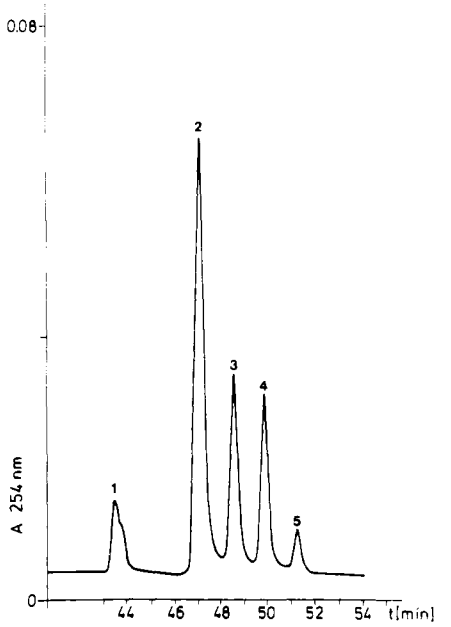

FIGURE 2: Chromatogram of a typical virotoxin mixture obtained by LC on a Zorbax ODS column and monitored by optical density at 254 nm. For the fraction numbers, see Tables I and III.

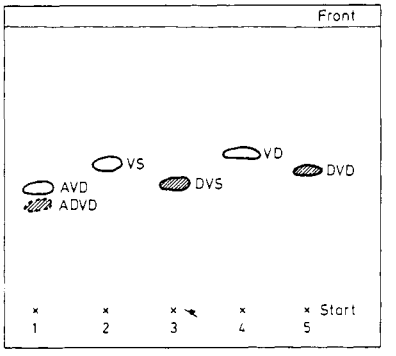

FIGURE 3: TLC in 2-butanol–ethyl acetate–water, 14:12:5 (v/v), of the virotoxin fractions 1–5 as obtained by LC. Detection was by UV absorption at 256 nm (open patches) or by UV absorption as well as by reaction with cinnamic aldehyde–HCl (hatched patches). For abbreviations see Table III.

Table II: Molecular Ions of PTH Derivatives Obtained by Edman Degradation of Viroisin

step no.	M^+	structural variability attributed to
1	234	PTH-valine
2	236	PTH-threonine
3	222	PTH-serine
4	228, 264	PTH-pyrrolecarboxylic acid and PTH-dihydroxyproline
5	206	PTH-alanine
6	399	PTH-2′-(methylsulfonyl)tryptophan

cinnamic aldehyde–hydrochloric acid, and by their UV absorption spectrum which closely resembled that of the semisynthetic phalloidin sulfone.

LC Resolution of the Virotoxin Mixture. Further resolution of the toxin mixture was achieved by LC. In total, six components were differentiated, four of which could be obtained in a pure state (Figure 2). The LC separation revealed that not only was the mixture composed of peptides possessing the 2′-sulfonylindole chromophore but also some minor components with the 2′-sulfoxyindole chromophore were present. This chromophoric system is known from the semisynthetic phalloidin sulfoxides (Faulstich et al., 1968) and likewise from the naturally occurring amanin (Wieland et al., 1967) and the amaninamide. After resolution by LC, the various toxin components could be distinguished by TLC. Although the R_f values were only slightly different, the six components could be easily characterized by their reaction with cinnamic aldehyde–hydrochloric acid, which with sulfoxides gives a grey brownish color but which is negative with all sulfones (Faulstich et al., 1968) (Figure 3). Since the molar extinction coefficients of both chromophoric systems, the sulfoxides and the sulfones, are similar at the wavelength used for detection, the elution diagram of the LC resolution allows a fairly good estimation of the amounts of the components present in the mixture. Peak 2 in Figure 2 represents 49% of the total; i.e., it is by far the main toxin. It received the name viroisin with reference to the nomenclature used for the family of phallotoxins. Here the neutral toxin, which like viroisin contains trihydroxylated L-leucine, received the name phallisin. Correspondingly, the component of peak 4, amounting to 18%, which possesses dihydroxylated L-leucine similar to phalloidin, was named viroidin. All other components were regarded and named as derivatives of the two main toxins. Names and structures of the various components are given in Figure 4 and Table III.

Structure of Virotoxins. The amino acid analysis of the LC fractions is compiled in Table I.

The sequence of the amino acids in the virotoxins was investigated with pure viroisin (LC fraction 2). As with the phallotoxins, the presence of a γ-hydroxylated leucine unit allowed the selective cleavage of the adjacent peptide bond with trifluoroacetic acid at room temperature. The linear seco compound thus formed was submitted to the dansylation procedure of the Edman degradation (Gray, 1972). Identification of the amino acids was achieved by thin-layer chromatography of the dansylated N-terminal amino acids as well as by mass spectrometry of the molecular ions of their PTH-amino acids (Hagenmaier et al., 1970). These data are compiled in Table II. The PTH-pyrrolecarboxylic acid found in step 4 by peak matching most probably represents the degradation product of the dihydroxylated proline unit which, by chemical degradation procedures, NMR analysis, and optical rotation properties, was identified as 2,3-*trans*-3,4-*trans*-3,4-dihydroxy-L-proline. The elucidation of that structure is published elsewhere (Buku et al., 1980a). The moiety isolated in step 6 was found to be identical, with respect to the mass

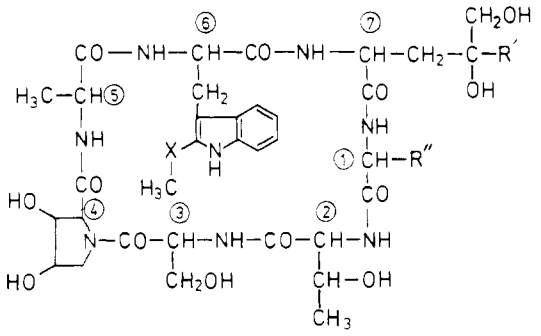

FIGURE 4: General formula of virotoxins.

Table III: Structural Variability in the Side Chinas of Positions 1, 6, and 7 in Virotoxins

peak no. (LC)	compd	X	R′	R″	% of total
4	viroidin (VD)	SO_2	CH_3	$CH(CH_3)_2$	18%
5	desoxoviroidin (DVD)	SO	CH_3	$CH(CH_3)_2$	4%
1	[Ala¹]viroidin (AVD)	SO_2	CH_3	CH_3	10%
1	[Ala¹]desoxo-viroidin (ADVD)	SO	CH_3	CH_3	
2	viroisin (VS)	SO_2	CH_2OH	$CH(CH_3)_2$	49%
3	desoxoviroisin (DVS)	SO	CH_2OH	$CH(CH_3)_2$	19%

of its molecular ion, with PTH-2′-(methylsulfonyl)tryptophan. Further proof for the identity was obtained with the free amino acid as isolated from the peptide hydrolysate which had a specific molar rotation very similar to that of the synthetic 2′-(methylsulfonyl)tryptophan [$[\alpha]^{20}_D$ +22 and +23°, respectively (*c* 0.1, 50% acetic acid)]. In addition, the natural and the synthetic 2′-(methylsulfonyl)tryptophans coincided in their ^1H NMR spectra (data below) and in their stability to β-elimination in alkaline medium: C_4 (d, 1 H) 7.78 ppm; C_5 (t, 1 H) 7.12 ppm; C_6 (t, 1 H) 7.31 ppm; C_7 (d, 1 H) 7.46 ppm; $C_\alpha H$ (m, 1 H) 4.14 ppm; $C_\beta H$ (br d, 2 H) 3.59 ppm; CH_3 (s, 3 H) 3.38 ppm. The hydroxylated leucine units were identified by direct comparison with the corresponding units isolated from the various phallotoxins (see Figure 4 and Table III).

Configuration of the Amino Acids. The configurations of the amino acids forming viroisin were determined by measuring their optical rotation as well as by investigating their stability toward enzymatic degradation (Sieber et al., 1970). For example, incubation of the hydrolysate with D-amino acid oxidase left alanine and valine undegraded, while in the analogous assay with the L-specific enzyme, serine and threonine remained intact, thus indicating their D configuration. The small amount of 3,4-dihydroxyproline was stable against both enzymes. Its L configuration could be established by determining the difference of its specific molar rotation values in acidic vs. neutral solution, making use of the rule of Clough (1918) and Lutz & Jirgensons (1931), which postulates this value as positive in the case of an L configuration. The configuration of the trihydroxylated leucine isolated from viroisin was determined by submitting it to IO_4 oxidation to obtain aspartic acid. L-Amino acid oxidase degraded the aspartic acid formed completely, while with D-amino oxidase 0.85 equiv was recovered (Ala, Val = 1.0). Finally, the L configuration of the tryptophan unit (Figure 5) was determined by its specific molar rotation, being almost identical with that of the corresponding synthetic moiety (see above).

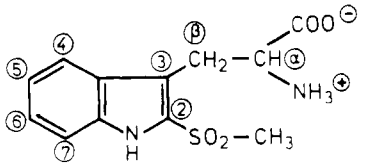

FIGURE 5: Formula of (methylsulfonyl)tryptophan.

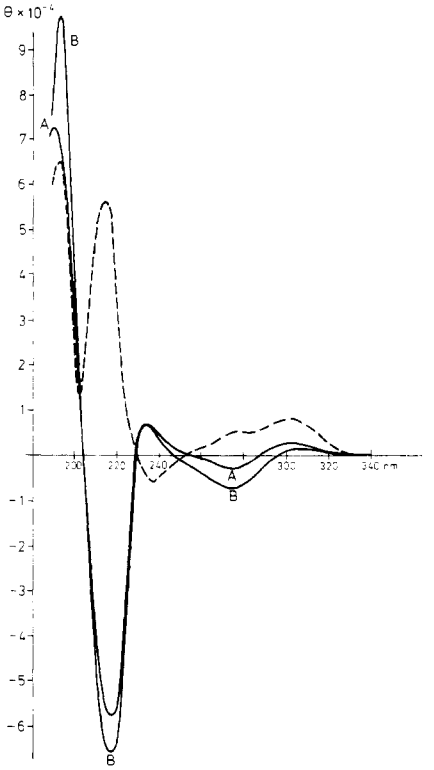

FIGURE 6: CD spectra of viroidin (solid line, curve A), viroisin (solid line, curve B), and phalloidin sulfone (dashed line) (1.4×10^{-4} M, in water).

Circular Dichroism and Specific Molar Rotation Values. Since with the group of phallotoxins chiroptical measurements had revealed substantial conformational data (Faulstich & Wieland, 1971), circular dichroism (CD) was also investigated for the virotoxins. As a bicyclic model compound with a similar chromophoric system suited for direct comparison, the CD of phalloidin sulfone was measured. The curves are presented in Figure 6.

For two virotoxins, viroisin and viroidin, the $[\alpha]^{20}_D$ values were determined to be −22.4 and −13.6°, respectively (for comparison, the values of the bicyclic phalloidin and phalloidin sulfone were +62.8 and +52.7°, respectively) (Faulstich, unpublished experiments).

Biological Activities of Virotoxins. The biological activity of the virotoxins is very similar to that of the phallotoxins. We determined their in vivo toxicity and their interaction with rabbit muscle actin.

Toxicity. Since pure toxins were thus far available in minute amounts only, the toxicity of viroisin has been solely determined so far. After intraperitoneal administration of the toxin into the white mouse, the LD_{50} was determined to be 2.5 mg/kg of body weight. (This value may be taken as representative for any kind of parenteral administration, since in previous experiments with phallotoxins the LD_{50} values of iv, ip, or im administration were very similar or identical.) The LD_{50}, like the course of the intoxication, was found to be indistinguishable from that of phalloidin. The animals died within 2–5 h after administration of the toxin from hemor-

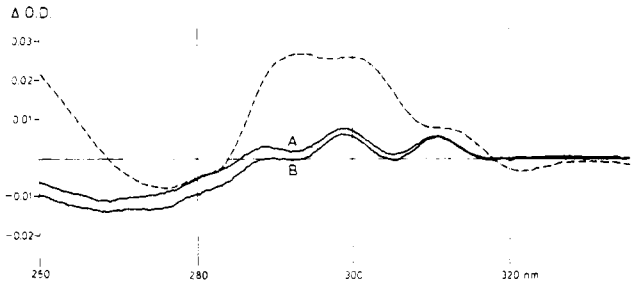

FIGURE 7: Difference UV spectrum obtained by mixing 1×10^{-5} M F-actin (in 0.1 M KCl and 1 mM Tris, pH 7.4) with an equimolar solution of phalloidin sulfone (dashed line) or viroidin (curve A) or viroisin (curve B) in a tandem cuvette.

rhagia of the liver, which appeared enlarged to twice its normal size due to accumulation of blood.

Interaction with Actin. On the molecular level, both viroidin and viroisin were shown to bind to rabbit muscle actin. Like in the case of the phallotoxins, the binding of the peptides could be followed directly by the difference UV spectrum shown in Figure 7. For determination of the apparent equilibrium dissociation constant, the [^{3}H]demethylphalloin–actin complex was prepared, and the labeled toxin was substituted competitively by viroidin (or viroisin) during equilibrium dialysis. As shown in Figure 8, the virotoxins were more effective by a factor of 2 than unlabeled demethylphalloin used in a standard experiment (identical substitution).

Discussion

Purification Procedure. Toxic peptides as obtained by methanolic extraction of some deadly poisonous *Amanita* mushrooms in aqueous medium adsorb to Sephadex LH-20 and can be efficiently separated for analytical purposes or on a small preparative scale (Faulstich et al., 1973, 1974). For example, by development of the extract of the green death cap *A. phalloides*, a single run separates the acidic phallotoxins from the acidic β-amanitin and amanin, as well as from the neutral phallotoxins and the neutral amatoxins, which themselves are partially separated into α-amanitin and γ-amanitin (Figure 1, top). Similarily effective was the procedure for the toxins of *A. virosa* although the pattern is different. Besides two pure toxins, phalloidin and amaninamide, a mixture of six similar toxins, the virotoxins, was isolated and required a further resolution step. This was achieved by analytical LC. Amounts of viroisin large enough for further operations were obtained by pooling analytical batches from the Zorbax column. Most recently, pure viroisin and pure viroidin could be produced at a preparative scale also on analytical TLC plates by using the chloroform–methanol–water solvent as described under Experimental Procedures.

Chemotaxonomy. While the toxin patterns of the green species *A. phalloides* and the closely related white species *A. phalloides* var. verna mushrooms are very similar (Faulstich et al., 1974; Seeger & Stijve, 1979), the toxin distribution in *A. virosa* differs from them both and provides evidence that *A. virosa* is, in fact, an individual species. For chemotaxonomy, the virotoxins described in this study are of highest value among the toxic peptides present. Virotoxins have been detected in all samples of *A. virosa* investigated by us so far despite having been collected at widely differing locations. On the other hand, virotoxins have never been described as being present in any of the other *Amanita* species.

Other toxins in *A. virosa* vary considerably and hence are less suited for chemotaxonomic identification. For example, in the North American sample used in the present study, the acidic phallotoxins are completely absent. However, in two European samples, phallacidin, the main component of acidic phallotoxins, was found in rather high amounts. On the other hand, a neutral amatoxin, probably amaninamide, was present in some of the specimens of North American origin as described by Yocum & Simons (1977) but was not present in others. The same authors report that α-amanitin was present only in some of their *A. virosa* mushrooms, while it was found in large amounts in all European samples.

When compared to the highly constant toxin pattern of *A. phalloides*, the above variations seem to be one characteristic

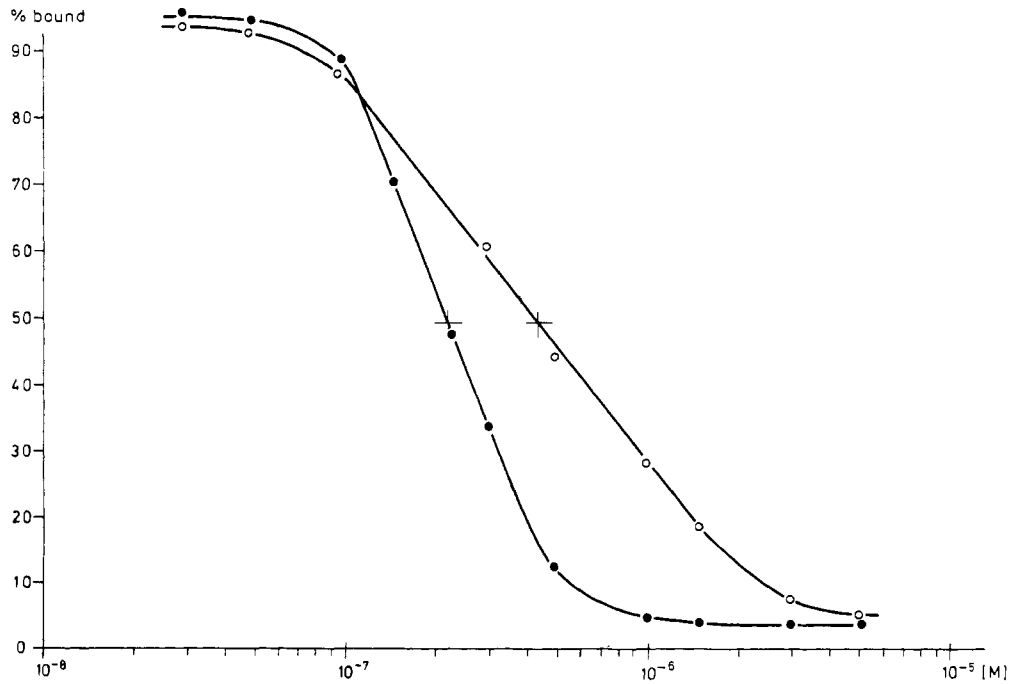

FIGURE 8: Competition of viroidin or viroisin (closed circles) and demethylphalloin (open circles) for the binding site in the [^{3}H]demethylphalloin–actin complex, followed by equilibrium dialysis in 0.1 M KCl and 1 mM Tris, pH 7.4. The percentage of labeled toxin vs. the concentration of unlabeled toxin is plotted.

feature of *A. virosa*, excepting, however, the virotoxins. They seem to be the most reliable marker toxins in *A. virosa*. Therefore, they may be helpful even in fastidious chemotaxonomical problems like the differentiation of *A. virosa* from the very similar *Amanita bisporigera*, which so far has proved to be impossible when based solely on the well-established toxins, such as α-amanitin (Mahoney & Holton, 1980).

Structure. So far, only viroisin has been prepared in amounts sufficient for a complete analysis of its structure. The structure of viroisin, as presented in Figure 4 and Table III, has been proved by UV spectroscopy, amino acid analysis, sequencing, and enzymatic as well as chemical characterization of the amino acid units. In addition, the structure was confirmed by extensive decoupling experiments with ^{1}H NMR (Dabrowski et al., unpublished experiments).

After elucidation of the viroisin structure, the remaining five peptides needed no sequencing and could be characterized by UV spectroscopy and amino acid composition only, by making use of our knowledge in the phallotoxin series. Thus, sulfoxides can be clearly distinguished from either thioethers or sulfones by their typical UV spectra. Second, in the phallotoxin series variations occur in a few discrete positions only, e.g., in position 1 (alanine for valine) and in position 7 (mono-, di-, or trihydroxylated leucine), which are exactly the same positions of variability as found in the virotoxins. Hence, it was possible to identify the unknown compounds by combinding their UV spectra and their amino acid composition with the known structure of viroisin.

Confirmation for the five structures was obtained by TLC, where, as expected from the phallotoxin series, the sulfones were less polar than the sulfoxides and the analogues with valine in position 1 were less polar than those with alanine (Figure 3). The information from the UV spectra and from the different behavior in TLC was even sufficient to characterize the two components remaining unresolved in fraction 1. By the absence of valine in favor of two alanine moieties (Table I), as well as by the TLC data [R_f values and reaction with cinnamic aldehyde–HCl (Figure 3)], they could be assigned as the alanine[1] analogues [(AVD) and (ADVD)] of viroidin (VD) and deoxoviroidin (DVD).

Derivatives of 2′-(Alkylsulfonyl)tryptophan. Two chromophoric systems of this type had been investigated in a previous study (Faulstich et al., 1968), namely, ethyl 2-skatyl sulfone and phalloidin sulfone, obtained by synthesis or by oxidation, respectively, of the natural product. The similarity of the UV spectrum of viroisin (Figure 9, curve B) to those of the mentioned compounds had provided early evidence that viroisin contains an (alkylsulfonyl)tryptophan. However, viroisin differed from the two sulfones by its stability in alkaline solution. While, e.g., phalloidin sulfone is degraded rapidly by β-elimination [for a 1.3×10^{-4} M solution at pH 12 (25 °C), the half-life time $\tau/2 = 4.0$ min], viroisin under these conditions is stable for hours as proved by the UV spectrum of its anion (Figure 9, curve B′). By acidification the spectrum of viroisin (Figure 9, curve B) is completely retained. This indicates the absence of any abstractable β-located protons. Among the two structural possibilities—absence of a β-C atom or presence of a tertiary β-C atom—the first one was most likely. In fact, a methyl group at the sulfonyl moiety could be verified by a singlet signal in the ^{1}H NMR of viroisin.

Additional proof for the presence of 2′-(methylsulfonyl)-tryptophan (Figure 5) in viroisin was obtained when the moiety was isolated from the hydrolysate of the toxin. In parallel, the moiety was synthesized by the method of Savige & Fontana (1976). Direct comparison of the natural and synthetic

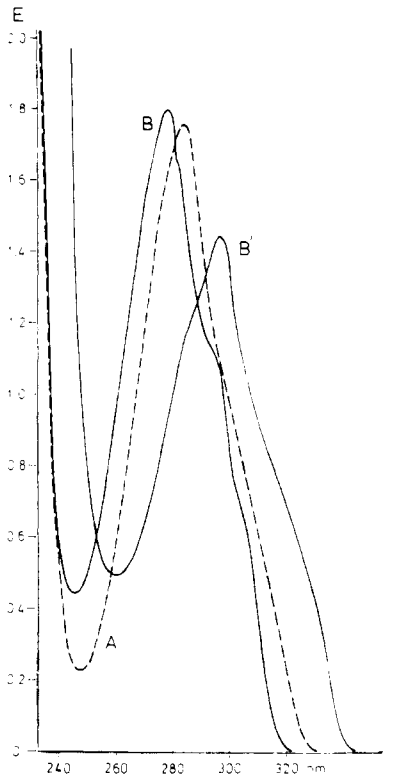

FIGURE 9: UV absorption spectra of phalloidin sulfone (curve A), viroisin at pH 7 (curve B), and viroisin at pH 11 (curve B′) (1.4×10^{-4} M, in water).

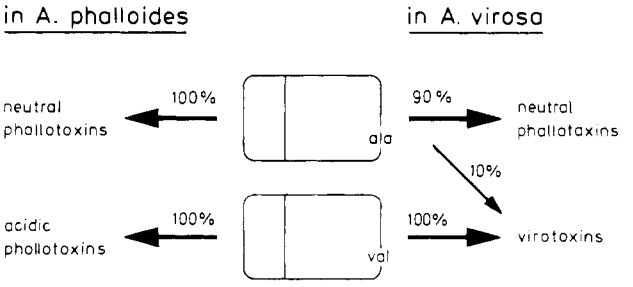

FIGURE 10: Suggested development of virotoxins and neutral or acidic phallotoxins in *A. phalloides* and in the North American sample of *A. virosa*, from possible precursors, as dependent upon the side chain of the amino acid in position 1.

compounds showed identity in several respects. In ^{1}H NMR both exhibited the identical singlet signal in a position similar to that in viroisin. Furthermore, both moieties behaved identically on TLC and with respect to alkaline treatment. Finally, their specific rotations were very similar, +22 and +23°, respectively.

Biosynthesis. Of the six virotoxins separated and identified so far, those containing valine at position 1 prevail (~90%) over those containing alanine (~10%). This is reminiscent of the acidic phallotoxins, which contain valine exclusively. Since all neutral phallotoxins possess an alanine moiety in this position, it may be concluded that in possible precursor molecules, the side chain of the amino acid in position 1 can determine the further processing leading to three types of toxins. In detail, a possible precursor with alanine in position 1 would be 90–100% converted to neutral phallotoxins in *A. phalloides* or in the North American *A. virosa*. In contrast, if valine is in position 1, the isopropyl side chain seems to induce in *A. phalloides* the 100% formation of acidic phallotoxins and in the North American *A. virosa* the exclusive

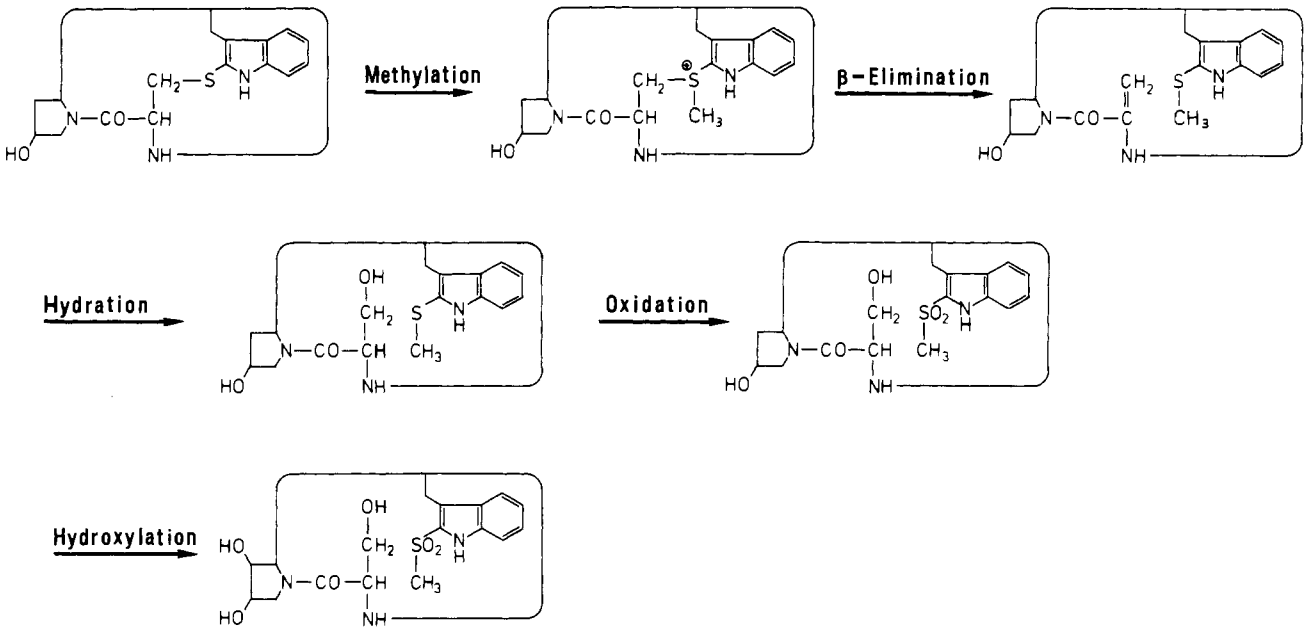

FIGURE 11: Sequence of physiological reactions which possibly converts a phallotoxin-type precursor into a virotoxin, e.g., viroidin.

production of virotoxins (Figure 10). The conditions in the European *A. virosa* may be somewhat different, in that valine in position 1 can induce the development of virotoxins as well as of acidic phallotoxins.

One possible way of processing leading to virotoxins from phallotoxin-like precursors is shown in Figure 11. The sequence of reactions, which need not necessarily proceed in the order suggested here, would include steps of methylation, oxidation, and β-elimination at the chromophoric unit, the addition of water to the intermediate dehydroalanine moiety, and, finally, the hydroxylation in position 3 of the proline unit. All reactions could occur under physiological conditions.

The addition of water to dehydroalanine forming the serine moiety must occur under enzymatic control. Otherwise, DL-serine should be expected instead of D-serine. In fact, there exist two further examples in literature where α-C atoms of the D configuration in amino acids most probably arise from dehydroamino acids. For nisin and subtilin, Gross and coworkers (Gross & Morell, 1970; Gross et al., 1973) reported the presence of *meso*-lanthionine and *meso*-β-methyllanthionine. Since in nisin and subtilin dehydroamino acids are also present, the authors concluded that the *meso*-lanthionine moieties may have been formed during synthesis of the peptide by the addition of the thiol group of L-cysteine to a dehydroalanine moiety. Also here, the addition reaction leading to the D configuration would be under complete steric control of an enzyme. However, such rationalizations lack proof in both cases. Neither for nisin and subtilin nor for the Amanita toxins has any enzyme involved in the synthesis of the peptides thus far been detected.

Structure and Biological Activity. As already mentioned, natural variations in the virotoxins as discussed above occur in exactly the same positions, 1 and 7, as in the phallotoxins. In the phallotoxin family, this part of the molecule had been determined to be of minor importance for biological activity. In contrast, several moieties without natural variability have been established to be essential for the biological activity. The corresponding amino acids have also been found without any variability in the virotoxins. Hence, we believe that these moieties are likewise essential for virotoxin activity.

These structures include the allohydroxy-L-proline, the L-alanine in position 5, and the tryptophan moiety, although in virotoxins the latter is not in a fixed position as part of a bridged peptide ring. By the absence of the covalent link between the amino acids in positions 3 and 6 as present in phallotoxins, the virotoxins become monocyclic peptides.

In the family of phallotoxins all monocyclic derivatives, e.g., dethiophalloidin (Wieland & Schmidt, 1952), have proved to be devoid of biological activity. Surprisingly, this generalization is not valid for the virotoxins. In these peptides, the lack of the sulfur bridge and, hereby, of the bicyclic structure does not affect the biological activity. On the other hand, the monocyclic virotoxins contain some structural features which are absent in phallotoxins and may provide additional and virotoxin-specific interactions. Among these are the two hydroxy groups at the β-C atoms of serine and dihydroxyproline, as well as the inverted configuration of the serine side chain. In fact, a virotoxin analogue lacking these features and prepared from phalloidin by A. Buku (unpublished experiments), [L-Ala[4],L-*a*-Hyp[5]]viroidin, was devoid of biological activity. Conclusively, one of the three structural features, or some combination thereof, must be involved in the interaction of virotoxins with the target protein.

Virotoxins not only represent the first peptides of the *Amanita* species found to contain the 2′-sulfonyltryptophan moiety but also represent the first peptide toxins having been found in two different states of oxidation, i.e., as sulfones and sulfoxides. Since the virotoxin pattern was similar in all samples of *A. virosa* investigated thus far, we conclude that the mixture of sulfoxides and sulfones present in the virotoxins is of native origin. The low amounts of pure virotoxins of the sulfoxide type have so far not allowed the determination of the configuration at the sulfur atom. In the case of phalloidin sulfoxides, the *R* stereoisomer had been found to be toxic, while the *S* stereoisomer was not (Wieland et al., 1974). Correspondingly, the two peptides differed widely in their affinity to muscle actin. By different chromatographic behavior and other criteria, it became obvious that the *S* configuration at the sulfoxide was accompanied by conformational changes in the indole part or in the peptide backbone, resulting in the loss of the biological activity. Such a strong influence of the configuration at the sulfur atom on the backbone conformation cannot be expected for the sulfoxides of the monocyclic virotoxins. The methylsulfoxy group can rotate freely, and,

therefore, both the *R*- and *S*-configurated sulfoxides can be expected to be of similar biological activity.

Circular Dichroism and Conformation. Peptides containing the 2′-sulfonyltryptophan moiety give rise to relatively small Cotton effects in the 250–320-nm range, when compared to thioethers or sulfoxides. This is true for both the bicyclic phalloidin sulfone and the monocyclic viroisin as shown in Figure 6. However, at wavelengths below 250 nm the Cotton effects of phalloidin sulfone and viroisin become distinct and may be used for conformational analysis. For example, the two Cotton effects located at 235 and 217 nm are opposite in sign and indicate that the conformations of virotoxins and phalloidin sulfone are different. Additional proof for the differing shape of the two types of peptides comes from the observation that antibodies raised against phalloidin cross-react to some extent with phalloidin sulfone but show no interaction with viroisin (H. Faulstich et al., unpublished experiments).

Interaction with Actin. Phallotoxins bind to the filamentous form of actin, forming a 1:1 complex with each protomer in the filament [for a review, see Wieland & Faulstich (1979)]. Direct evidence for the formation of such a complex was provided by a difference UV spectrum (Wieland et al., 1975) and by the visualization of actin fibers in mammalian cells by means of a fluorescent phallotoxin (Wulf et al., 1979). The affinity of the phallotoxins to actin is high; by equilibrium dialysis the apparent dissociation constant of [^{3}H]demethylphalloin was measured to be $K_D = 3.8 \times 10^{-8}$ M. The main component of the phallotoxins, phalloidin, has an affinity which is even 1.5 times higher than that of the phalloin derivative. One consequence of phallotoxin binding to actin is the decrease, by a factor of 30, of the actin monomers that are in equilibrium with the polymeric form (Faulstich et al., 1977).

Both components of the virotoxins which have been purified on a preparative scale, viroisin and viroidin, interact with muscle actin, as concluded from the difference spectra in Figure 7. In quantitative assays, for example, in equilibrium dialysis, displacement of [^{3}H]demethylphalloin in a complex with actin begins at 10^{-7} M concentrations of the virotoxins, similar to demethylphalloin (Figure 8). However, the slope of the dose–binding curve of the virotoxins differs from that of demethylphalloin and, in general, from that of all phallotoxins. As a consequence, at a concentration of 4.4×10^{-7} M of unlabeled toxin, where unlabeled demethylphalloin in equilibrium has displaced 50% of the labeled toxin, the virotoxins have substituted ~85% of the labeled toxin from the complex. Hence, for the two types of toxins, the molecular mechanism of interaction with the protein is probably different.

A different mode of interaction with actin may be understood from the fact that virotoxins are monocyclic. For the phallotoxins it has been established that the bicyclic nature stabilizes the rigid conformation of the smaller, 15-membered ring, which is the most important structural detail for their biological activity. Virotoxins lack such rigid conformation and interaction with actin should be weaker. From the greater flexibility of the virotoxins, a lower free energy can be expected for the complexation reaction, resulting in a decrease of their affinity to actin. On the contrary, the interaction is comparably strong, as may be concluded from the apparent dissociation constants of the two virotoxins, $K_D = 2 \times 10^{-8}$ M, being similar to that of the strongest phallotoxin, phalloidin. Evidently, the unfavorable flexibility in the virotoxin conformation is compensated by additional binding opportunities as discussed above.

At the rigid binding site of the phallotoxins, three functional groups, the methyl residue of alanine[5], the allohydroxy group of proline[4], and the indole moiety as part of the thioether bridge of tryptophan[6] and cysteine[3] seem to be favorably arranged for binding to actin (Faulstich & Wieland, 1975). In the virotoxins, the above structural elements also exist but in a certainly less stable arrangement. From the similarities of the biological activities, it is reasonable to assume that in the virotoxins these elements are also involved in the binding process. However, since the conformation of viroisin in solution differs as proved by the absent cross-reaction with antiphalloidin antibodies, the peptide probably adopts the active conformation only upon contact with actin by an induced-fit mechanism.

Toxicity. Since the phallotoxins and the virotoxins, although of different structure, coincide in their binding to actin and in their course of intoxication, they provide evidence that binding of the peptides to liver cell actin most likely represents the mechanism of their poisonous activities. So far, the toxicity of phallotoxins can best be understood by considering the decrease of monomeric actin in liver cells, which, due to the resulting deficiency of the mobile form of actin, may lack some vital functions (Wieland & Faulstich, 1979). For the virotoxins, probably the same mechanism of toxicity is valid. However, it should be noted that thus far only viroisin and viroidin have been investigated in detail. It may well turn out that one or the other of the remaining components of the virotoxins has a diminished or even absent toxicity.

Possible Physiological Role of Virotoxins. A question unanswered thus far, but of interest for all types of toxic peptides in *Amanita* mushrooms, is that of their possible physiological function. It is conceivable that amatoxins may help regulate the activity of RNA polymerases in mushroom cells and that the phallotoxins and the virotoxins may play a role in cellular functions involving the contractile apparatus, i.e., actin. Some support for the latter possibility can be derived from the fact that in the case of phallotoxins and virotoxins, two types of peptides exist with very different structures but with the same biological activity. Apparently, nature has conserved some special function of these peptides rather than their structure, pointing out a possible physiological importance of the actin-binding peptides.

Acknowledgments

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Chromatium vinosum Cytochrome *c*-552. Reduction by Photoreduced Flavins and Intramolecular Electron Transfer†

Michael A. Cusanovich* and Gordon Tollin

ABSTRACT: Cytochrome *c*-552 from *Chromatium vinosum* is an unusual heme protein in that it contains two hemes and one flavin per molecule. To investigate whether intramolecular electron transfer occurs in this protein, we have studied its reduction by external photoreduced flavin by using pulsed-laser excitation. This approach allows us to measure reduction kinetics on the microsecond time scale. Both fully reduced lumiflavin and lumiflavin semiquinone radical reduce cytochrome *c*-552 with second-order rate constants of approximately 1.4×10^6 M^{-1} s^{-1} and 1.9×10^8 M^{-1} s^{-1}, respectively. Kinetic and spectral data and the results of similar studies with riboflavin indicate that both the flavin and heme moieties of cytochrome *c*-552 are reduced simultaneously on a millisecond time scale, with the transient formation of a protein-bound flavin anion radical. This is suggested to be due to rapid intramolecular electron transfer. Further, steric restrictions play an important role in the reduction reaction. Studies were conducted on the redox processes following photolysis of CO–ferrocytochrome *c*-552 in which the flavin was partly oxidized to resolve the kinetics of electron transfer between the heme and flavin of cytochrome *c*-552. Based on these results, we conclude that intramolecular electron transfer from ferrous heme to oxidized flavin occurs with a first-order rate constant of greater than 1.4×10^6 s^{-1}.

Chromatium vinosum cytochrome *c*-552 is a low-potential flavin containing *c*-type cytochrome (Bartsch, 1978). Its molecular weight is 72 000, consisting of a flavin-containing subunit (M_r ~45 000) and two heme-containing subunits (M_r ~15 000 each) (Kennel, 1971; Brown and Cusanovich, unpublished observations). The native protein has an absorption spectrum which is consistent with that of a mixture of flavin and low-spin heme *c*. The flavin moiety has been shown to be FAD (Hendriks & Cronin, 1971), with the 8α-methylene group of the flavin covalently bound to the sulfur of a protein cysteinyl side chain (Walker et al., 1974; Kenney et al., 1974; Kenney & Singer, 1977). *Chromatium vinosum* cytochrome *c*-552 can catalyze the oxidation of sulfide with a variety of electron acceptors (Fukumori & Yamanaka, 1979), suggesting that its physiological function is that of sulfide dehydrogenase.

The oxidation–reduction potential of both the heme and flavin moieties is approximately 30 mV (Vorkink, 1972; Brown and Cusanovich, unpublished observations), and there is no evidence for formation of a flavin semiquinone during redox titrations. The circular dichroism (CD) spectrum of *Chro-*

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