Identification of Structural Features Involved in Binding of α-Amanitin to a Monoclonal Antibody†

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ABSTRACT: Twenty-four derivatives of the cyclic octapeptide α-amanitin were assayed for their affinities to the monoclonal antibody βA1/1. The derivatives were of natural, semisynthetic, and synthetic origin and had K_D values ranging from 2 nM to >70 μM. In the majority of the derivatives the chemical modifications had no detectable influence on the overall shape of the double-ring peptide. Given this condition, binding factors could be calculated from K_D values of the amatoxin derivatives, which were valid for all amatoxins for estimating the contribution made by single structures to complex formation. The complex between α-amanitin and the immunoglobulin involves at least eight sites of contact. Four of them are responsible for strong interactions: (1) the OH group of hydroxyproline[2] (binding factor 413), (2) the lipophilic side chain of isoleucine[6] (binding factor 131), (3) the $-CH_2-$ moiety of the adjacent glycine[5] or the absence of a side chain in this position (binding factor 361), and (4) the proton at the indole nitrogen of hydroxytryptophan[4] (binding factor 140). The residual four interactions are hydrogen bonds of lower strength corresponding to binding factors of 1.5–8. The key role of the unique conformation of the amatoxins in determining their binding properties was shown by two amatoxin derivatives in which changes in the conformation were associated with virtually complete loss of affinity. For all amatoxin derivatives with conformations similar or identical to that of α-amanitin, we found empirical evidence that those structures of the peptide involved in binding make their contributions virtually independent of each other. It is a consequence of this rule that structural features that cooperate in binding could be characterized by the numerical product of their binding factors.

Amatoxins are a family of bicyclic peptides of MW ca. 900 produced by mushrooms of the genera *Amanita*, *Galerina*, and *Lepiota*. These mushrooms are frequent in Europe and North America, where they account for more than 90% of all fatal cases of human mushroom poisoning. Amatoxins inhibit transcription of hnRNA (mRNA) in eukaryotic cells by binding to RNA polymerase II (or B) (Cochet-Meilhac & Chambon, 1974; Vaisius & Wieland, 1982). Their toxicity is thought to be via the blocking of protein synthesis at the transcription level [for review see Faulstich (1980)].

One of the natural amatoxins, β-amanitin, has been crystallized and subjected to X-ray analysis (Kostansek et al., 1978). This structural analysis led to the conclusion that the structure of this peptide must be rigid because of its double-ring nature and the presence of several intramolecular hydrogen bonds. Accordingly, when bound to amatoxin-binding proteins, amatoxins are assumed not to change their conformation as linear peptides may do. This potential stability prompted us to use amatoxins as a model for studying structural aspects of peptide–protein complexes. Among the amatoxin-binding proteins, RNA polymerase II is unstable and composed of numerous subunits. A simpler and more suitable tool for such studies is provided by amatoxin-specific monoclonal antibodies, which can be produced in large amounts and handled very easily.

Polyclonal antibodies against amatoxins have been prepared by several laboratories including our own (Kirchner & Faulstich, 1986). They have been used mostly in immunological assays established for the detection of amatoxins in biological fluids of patients (Faulstich et al., 1975, 1982; Fiume et al., 1975; Andres & Frei, 1987). Monoclonal antibodies and Fab fragments have been obtained from rat/mouse hybridoma cells (Faulstich & Kirchner, 1986) and employed hitherto only for study their potential use in immunotherapy of amatoxin poisoning (Faulstich et al., 1988). One of the monoclonal antibodies, βA1/1, which showed an affinity for α-amanitin comparable to that of RNA polymerase II (K_D = 2.6 nM), was regarded as a suitable tool for the present investigation.

EXPERIMENTAL PROCEDURES

Materials

Preparation of the protein conjugates of amatoxins, such as β-amanitin–fetuin and β-amanitin–BSA[1], was described elsewhere (Faulstich & Fiume, 1985). 6′-*O*-[^{3}H]Methyl-α-amanitin, 6′-*O*-methyl-[^{3}H]dehydroxy-α-amanitin, and 1′,6′-*O*-[^{3}H]dimethyl-α-amanitin were prepared in our laboratory (Faulstich et al., 1975, 1981). All other reagents were of analytical grade.

Methods

Production and Purification of Monoclonal Antibodies. Three-month-old Wistar rats (ca. 200 g body weight) were

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[1] Abbreviations: BSA, bovine serum albumin; CD, circular dichroism; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; FPLC, fast protein liquid chromatography; HPLC, high-performance liquid chromatography; mAb, monoclonal antibody; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFA, trifluoracetic acid.

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Table I: ^{1}H NMR Data of the Methylation Products of α-Amanitin

compd	amino acid	structural feature	chemical shift (ppm)	assignment
6′-*O*-methyl-α-amanitin	(OH)Trp[4]	6′-OCH_3	3.75	s, 3 H
		1′-NH	11.48	s, 1 H
1′,6′-*N*,*O*-dimethyl-α-amanitin	(OH)Trp[4]	6′-OCH_3	3.80	s, 3 H
		1′-NCH_3	11.48	s, 3 H

immunized intraperitoneally with β-amanitin–fetuin in PBS corresponding to amounts of 30 μg of β-amanitin, mixed with two volumes of Freund's complete adjuvant. Four weeks after priming, a booster injection was administered containing the same amount of amatoxin but mixed with Freund's incomplete adjuvant. Three days prior to fusion, a second booster injection without adjuvant was given. Spleen cells were isolated from these animals and fused with mouse myeloma cells (P3-X63-Ag8-653) as described by Hämmerling et al. (1981). Growing hybridoma clones were screened by the ELISA technique according to Engvall and Perlman (1971), using β-amanitin–BSA for coating the wells. The presence of amatoxin-specific antibodies was detected with a goat anti-rat IgG conjugated to horseradish peroxidase (Dianova, Hamburg, Germany). Several clones were adapted to serum-free medium as described by Peters et al. (1985). Using the spinner culture technique, several liters of cell culture supernatant containing the monoclonal antibody could be harvested within 3–4 weeks. Cell culture supernatant was concentrated 25-fold using a Minitan tangential flow (Millipore, Eschborn, Germany). The concentrated solution (up to 40 mL) was applied to a gel-filtration column (250 × 4 cm) with Sephacryl S-300 (Pharmacia, Freiburg, Germany) and eluted with 0.5 M NaCl containing 0.02% NaN_3. The antibody-containing fractions were pooled, dialyzed against 0.1 M PBS, pH 7.0, for 12 h at 4 °C, and concentrated to a protein concentration of 10 mg/mL. Pure monoclonal antibody was obtained after FPLC (Pharmacia) using an alkyl-Sepharose column (Pharmacia). Purity was checked by SDS–PAGE according to Laemmli (1970). Immunoglobulin subclass determination was carried out by ELISA using isotype-specific antibodies (Serotec, Oxford, England).

Preparation and Purification of Amatoxin Derivatives. The natural amatoxins such as α-amanitin, β-amanitin, γ-amanitin, ε-amanitin, amanullin, amanullic acid, amaninamide, and amanin were isolated from the green death cap (*Amanita phalloides*) as described previously [for reviews see Wieland and Faulstich (1983) and Wieland (1986)]. All amatoxins and derivatives were purified by HPLC (Waters, Eschborn, Germany) using a RP 18 column (Merck, Darmstadt, Germany) running a linear gradient from 10% acetonitrile/0.1% TFA to 50% acetonitrile/0.1% TFA.

Methylation of α-amanitin, yielding 6′-*O*-methyl-α-amanitin and 1′,6′-*N*,*O*-dimethyl-α-amanitin, was achieved as followed: 60 mg of α-amanitin was dissolved in 20 mL of methanol. Diazomethane in ethyl ether (25 mL) was added and the mixture was incubated overnight at 25 °C. After evaporation of the solvent, the sample was purified by HPLC as described above. The two products were identified by ^{1}H NMR spectroscopy in deuterated DMSO at 500 MHz (Table I).

Acetylation of 6′-*O*-methyl-γ-amanitin was performed as follows: 15 mg of 6′-*O*-methyl-γ-amanitin was dissolved in 1.5 mL of pyridine containing 0.13 mL of acetic anhydride and incubated for 3.5 h at 25 °C. The solvent was evaporated and the sample dried over $CaCl_2$ under vaccuum overnight. Acetylation was monitored by analytical thin-layer chromatography on silica gel with methanol/chloroform/H_2O (65:25:4). Three acetylation products were isolated by HPLC using a RP 18 column, running a linear gradient of acetonitrile (15–50%) in 25 mM ammonium acetate. Identification of the products was achieved by ^{1}H NMR spectroscopy (Table II).

Table II: ^{1}H NMR Data of the Acetylation Products of 6′-*O*-Methyl-γ-Amanitin

compd	amino acid	structural feature	chemical shift (ppm)	assignment
6′-*O*-methyl-γ-amanitin	(OH)Ile[3]	γ-CH_3	0.81	d, 3 H
		γ-H	2.24	m, 1 H
3-acetyl-6′-*O*-methyl-γ-amanitin	(OH)Ile[3]	γ-CH_3	1.06	d, 3 H
		γ-$OCOCH_3$	1.97	s, 3 H
		γ-H	2.56	m, 1 H
2-acetyl-6′-*O*-methyl-γ-amanitin	(OH)Ile[3]	γ-CH_3	0.81	d, 3 H
		γ-H	2.24	m, 1 H
	(OH)Pro[2]	γ-$OCOCH_3$	1.97	s, 3 H
2,3-diacetyl-6′-*O*-methyl-γ-amanitin	(OH)Ile[3]	γ-CH_3	1.06	d, 3 H
		γ-H	2.57	m, 1 H
		γ-$OCOCH_3$	1.97	s, 3 H
	(OH)Pro[2]	γ-$OCOCH_3$	1.97	s, 3 H

Table III: ^{1}H NMR Data of the Aromatic Protons of α-Amanitin and 7′-Iodo-α-amanitin

compd	amino acid	proton	chemical shift (ppm)	assignment	coupling constant (Hz)
α-amanitin	(OH)Trp[4]	H_4	7.595	d, 1 H	8.80[a]
		H_5	6.690	dd, 1 H	2.16[b]
		H_7	6.760	d, 1 H	
7′-iodo-α-amanitin	(OH)Trp[4]	H_4	7.55	d, 1 H	8.80[a]
		H_5	6.82	dd, 1 H	

[a] $^3J_{HH}$ coupling constant. [b] $^4J_{HH}$ coupling constant.

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7′-Iodo-α-amanitin was obtained by incubating 5 mg of α-amanitin with an equimolar amount of fresh iodine chloride (Aldrich) in methanolic solution for 3 h at 25 °C. The reaction products were separated by HPLC using a RP 18 column (linear gradient of acetonitrile from 10 to 40%) and identified by ^{1}H NMR spectroscopy (Table III).

The monocyclic 6′-*O*-methyl-dethio-α-amanitin was obtained by treating a methanolic solution of 6′-*O*-methyl-α-amanitin with Raney nickel under reflux, as described by Wieland et al. (1952). The product was identified by the typical UV spectrum of 6′-methyltryptophan. Preparation of 6′-*O*-methyl-seco-α-amanitin was carried out by incubation of 5 mg of 6′-*O*-methyl-α-amanitin in 1 mL of 50% TFA for 3 h at 25 °C. The solvent was evaporated and isolation of the reaction product was obtained by HPLC as described above. Identification was achieved by R_f values (TLC, HPLC) as well as by the reaction with ninhydrin.

The synthetic amatoxin derivatives were kindly provided by G. Zanotti, Rome, Italy (Zanotti et al., 1987, 1989).

Affinity (K_A) Determination. Binding capacities and equilibrium dissociation constants (K_D) of the monoclonal antibody were measured by precipitation of the 6′-*O*-[^{3}H]-methyl-α-amanitin complex with polyethylene glycol 6000 (Roth, Karlsruhe, Germany). This assay was performed according to Stenman et al. (1981) with the following modifications: 50-μL samples of purified mAb (ca. 2 nM) in PBS were mixed with 200 μL of 6′-*O*-[^{3}H]methyl-α-amanitin (2–160 nM) in PBS and incubated for 3 h at 25 °C. After addition of 50 μL of 1% bovine serum albumin in PBS and 1 mL of 25% (w/v) PEG 6000 in 0.15 mM NaCl, 50 mM

NaH_2PO_4, and 10 mM EDTA, the samples were cooled on ice and centrifuged for 20 min at 4 °C in a microfuge at 12 000 rpm. The supernatant was discarded, and the pellets were redissolved in PBS and counted in a β-counter (Beckmann, München, Germany). Unspecific binding was measured by performing the same assay without added mAb and subtracting these values from the binding values. Picomoles of 6′-*O*-[^{3}H]methyl-α-amanitin bound were plotted against picomoles of 6′-*O*-[^{3}H]methyl-α-amanitin free, and the resulting curve was fitted by a computer program (Grafit).

Competitive Binding Assay. This assay was performed similarly to the procedure described above: A constant amount of 6′-*O*-[^{3}H]methyl-α-amanitin (80 nM) and mAb (ca. 2 nM) was incubated with the amatoxin derivative to be measured (1 nM–100 μM) under the same conditions as described above. Controls contained no amatoxin derivative (0% substitution) or no mAb (unspecific binding). Percent substitution was obtained from

$$\% \text{ sub} = (\text{cpm}_B - \text{cpm}_0)/(\text{cpm}_T - \text{cpm}_0) \times 100$$

where cpm_B = cpm value bound, measured in the presence of amatoxin derivative, cpm_T = cpm value bound of tracer without the amatoxin derivative, and cpm_0 = cpm value of unspecific binding. Each concentration of the amatoxin derivative was plotted against the percentage of substitution. From the resulting curve, which was fitted by a computer program (Grafit), we determined the concentration of the amatoxin derivative necessary to displace 50% of the labeled amatoxin from the complex. Since these values were obtained with a large excess of ligand (minimum 80 nM; antibody 2 nM), the K_D value of all amatoxin derivatives could be determined from

$$K_D(\text{Ama}) \approx K_D(\text{T})[\text{Ama}_0]/[\text{T}_0]$$

where K_D(Ama) = dissociation constant of amatoxin derivative, K_D(T) = dissociation constant of the tracer used as determined by Scatchard analysis, [Ama_0] = concentration of amatoxin derivative required to displace 50% of tracer from the antibody binding site, and [T_0] = initial concentration of tracer. All experiments were carried out in duplicate.

RESULTS

New Amatoxin Derivatives. Most of the amatoxin derivatives used in this study were previously made for studying the structure–activity relationship of amatoxins with RNA polymerase II. Since proamanullin, a potential precursor of the amatoxins and a key compound for studying the contribution to binding made by the OH group of (OH)proline in position 2, was no longer available, we studied amatoxin derivatives in which this OH group was acetylated. As starting material, 6′-*O*-methyl-γ-amanitin (**6**, Table V) was chosen, a derivative that possesses only two hydroxy groups, at (OH)-pro^2 and in the γ-position of (OH)ile^3. The three acetylation products expected (**17**, **18**, and **19**, Table V) were isolated and identified on the basis of their ^{1}H NMR spectra (Table II).

The double-ring nature of the amatoxins (see α-amanitin, Figure 1) is thought to be of great importance for maintaining the conformation that can be recognized by amatoxin-binding proteins. Therefore, it was of great interest to study the binding properties of monocyclic derivatives of amanitin. Two monocyclic derivatives of α-amanitin have been known since the time of the structural elucidation. One of them is the compound possessing only the peptide ring, 6′-*O*-methyldethio-α-amanitin; the other one is a compound with one of the small rings intact, 6′-*O*-methylseco-α-amanitin.

In order to obtain information on the effects caused by substitutions located at various positions of the indole nucleus,

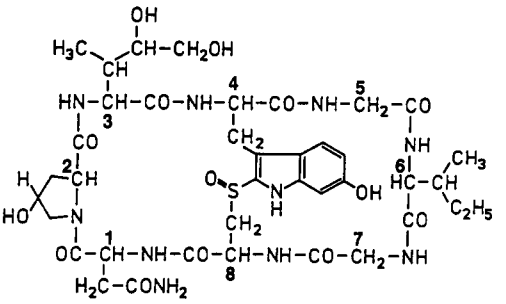

FIGURE 1: Structure of α-amanitin.

Table IV: Structures, Specific Activities, and Apparent Equilibrium Dissociation Constants of Three Tritium-Labeled Amatoxin Tracers

tracer	formula	sp act. (Ci/mmol)	K_D (M)
I	6′-*O*-methyl-[^{3}H]dehydroxymethyl-α-amanitin (**13**)[a]	2.1	1.2×10^{-8}
II	6′-O-[^{3}H]methyl-α-amanitin (**5**)	7.6	5.5×10^{-9}
III	1′,6′-*N*,*O*-[^{3}H]dimethyl-α-amanitin (**20**)	13.8	$>1.0 \times 10^{-7}$

[a] Numbers in parentheses represent the unlabeled analogues in Table V.

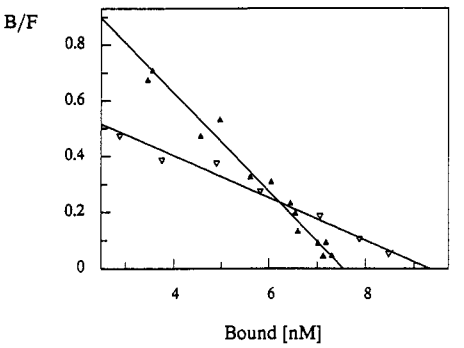

FIGURE 2: Scatchard plots of two tritium-labeled amatoxin derivatives, tracer I (▽) and tracer II (▲), (see Table I). Each point is the mean of duplicate determinations. The affinity of tracer II is higher by a factor of ca. 2.2.

we worked out new preparations of 1′,6′-*N*,*O*-dimethyl-α-amanitin (Faulstich et al., 1981) and 7′-iodo-α-amanitin (Morris et al., 1978; Andres & Frei, 1987). Together with the natural and semisynthetic amatoxins available in our laboratory (Wieland, 1986) and the synthetic peptides prepared by G. Zanotti (Zanotti et al., 1987, 1989), we had 24 derivatives at our disposal, covering nearly all functional groups and side chains of α-amanitin.

Selection of an Appropriate Amatoxin Tracer. Three tritium-labeled amatoxin derivatives, 6′-O-methyl-[^{3}H]dehydroxymethyl-α-amanitin (I), 6′-*O*-[^{3}H]methyl-α-amanitin (II), and 1′,6′-*N*,*O*-[^{3}H]dimethyl-α-amanitin (III) were subjected to Scatchard analysis in order to select the tracer with the highest affinity (Table IV). Tracer II seemed to be the most suitable one and was used in this study. It was particularly superior to tracer I (Figure 2), which until now was most frequently used in radioimmunoassays (Faulstich et al., 1982) and pharmacokinetic studies (Jahn et al., 1980). To make sure that the affinities determined for the various amatoxins did not depend on the kind of tracer used, some of the affinity values shown below were determined in parallel using tracer I. The affinities found with tracer I were very close to those found with tracer II (see Table V, fourth column). It appears that affinity studies of this kind are widely independent of the kind of tracer used.

As Table IV illustrates, the K_D values of the labeled compounds I and II, as determined by Scatchard analysis,

Table V: Structures of All Amatoxin Derivatives Used in This Study, Together with Concentrations Required for 50% Substitution of Tracer II and the Calculated K_D Values

no.	formula	remarks	$[Ama_0]^a$ (nM)	K_D (nM)
1	see Figure 1	α-amanitin	37 (25)[b]	2.6
2	[Asp[1]]-**1**	β-amanitin	78	5.4
3	[γ-hydroxy-Ile[3]]-**1**	γ-amanitin	55	3.8
4	[γ-hydroxy-Ile[3], Asp[1]]-**1**	ε-amanitin	130	9.1
5	[6′-methyl-Trp[4]]-**1**	methyl-α-amanitin, corr. to tracer II	71 (58)[b]	5.0
6	[6′-methyl-Trp[4], γ-hydroxy-Ile[3]]-**1**	methyl-γ-amanitin	115	8.0
7	[methyl-Asp[1]]-**1**	β-amanitin methyl ester	104	7.3
8	[6′-methyl-Trp[4], methyl-Asp[1]]-**1**	methyl-β-amanitin methyl ester	216	15.1
9	[Ile[3]]-**1**	amanullin	465	32.5
10	[Ile[3], Asp[1]]-**1**	amanullic acid	826	57.7
11	[6′-dehydroxy-Trp[4]]-**1**	amaninamide (from *Amanita virosa*)	65	4.5
12	[6′-dehydroxy-Trp[4], Asp[1]]-**1**	amanin	145	10.1
13	[6′-methyl-Trp[4], γ-hydroxy-Val[3]]-**1**	corr. to tracer I	134 (104)[b]	9.4
14	[Ile[3], deoxo-Cys[8], 6′-dehydroxy-Trp[4]]-**1**	totally synthetic	1103	77.1
15	[Ile[3], Ala[6], deoxo-Cys[8], 6′-dehydroxy-Trp[4]]-**1**	totally synthetic	1.5×10^5	1.0×10^4
16	[Ile[3], Ala[5], deoxo-Cys[8], 6′-dehydroxy-Trp[4]]-**1**	totally synthetic	4.0×10^5	2.8×10^4
17	[γ-acetyloxy-Ile[3], 6′-methyl-Trp[4], acetyl-Hyp[2]]-**1**	2,3-diacetyl-6′-*O*-methyl-γ-amanitin	3.0×10^5	2.1×10^4
18	[γ-acetyloxy-Ile[3], 6′-methyl-Trp[4]]-**1**	3-acetyl-6′-*O*-methyl-γ-amanitin	656	45
19	[γ-hydroxy-Ile[3], 6′-methyl-Trp[4], acetyl-Hyp[2]]-**1**	2-acetyl-6′-*O*-methyl-γ-amanitin	4.8×10^4	3.3×10^3
20	[1′,6′-dimethyl-Trp[4]]-**1**	corr. to tracer III	1×10^3	700
21	[6′-methyl-Trp[4], γ-oxo-Val[3]]-**1**	methylaldoamanitin	7.1×10^4	4.9×10^3
22	[Ala[8], 6′-methyl-Trp[4]]-**1**	dethiomethyl-α-amanitin (monocyclic)	$>1 \times 10^6$	$>7.0 \times 10^4$
23	[6′-methyl-Trp[4]]-seco-**1**	peptide ring opened (monocyclic)	89	6.2
24	[7′-iodo-Trp[4]]-**1**	tracer for X-ray analysis	26	1.8

[a] Concentration of tracer II (6′-*O*-[^{3}H]methyl-α-amanitin) in the competitive binding assay was 80 nM. [b] Values in parentheses were obtained by using tracer I (6′-*O*-[^{3}H]dehydroxymethyl-α-amanitin) at a concentration of 90 nM.

Table VI: Binding Factors Identifying the Contribution That Various Structural Features of α-Amanitin Make to mAb Binding[a]

amino acid	structural feature: original	structural feature: modified	binding factors	calcd from amatoxin derivatives[b]
Asn[1]	$CONH_2$	COO^-	2.1, 2.4, 2.2	**1/2, 3/4, 11/12**
(OH)Trp[4]	6′-OH	OCH_3	1.9 2.1, 2.1	**1/5, 3/6, 7/8**
	6′-OH	H	1.8, 1.9	**1/11, 2/12**
	1′-H	CH_3	140	**5/20**
	7′-H	I	0.7	**1/24**
$(OH)_2$Ile[3]	δ-OH	H	1.5, 1.7, 1.6	**1/3, 2/4, 5/6**
	γ-OH	H	6.4, 8.5	**4/10, 3/9**
	γ-OH	$OCOCH_3$	5.7	**6/18**
Ile[6]	$CH(CH_3)C_2H_5$	CH_3	131	**14/15**
Gly[5]	H	CH_3	361	**14/16**
(OH)Pro[2]	γ-OH	$OCOCH_3$	413	**6/19**
Cys[8]/Trp[4]	$-CH_2-SO-C-$	$-CH_3$ H–C–	>10000	**5/22**
$(OH)_2$Ile[3]/ Trp[4]	–CO–NH–	$-COO^-$ $^+NH_3-$	1.3	**5/23**

[a] The amino acid denotes the position. [b] Numbered as in Table V.

differ by a factor of 2.2. For the corresponding unlabeled compounds (**5** and **13**, Table V) we found that the affinity values as determined by the competitive binding assay (see Methods) differed by a factor of 1.9. From the similarity of these numbers we conclude that K_D values of the compounds can be calculated from the data of the competitive binding assay simply by proportionality. The K_D values of all compounds assayed as calculated from data of the competitive binding assay are listed in Table V.

Contribution of Single Side Chains or Functional Groups: (*A*) *Asp/Asn in Position 1.* The mAb βA1/1 was derived from rat cells primed with a protein conjugate of β-amanitin in which the COO^- of β-amanitin was used for coupling. Given the disappearance of the negative charge in position 1 as a consequence of the coupling reaction, antibodies induced by this immunogen were expected to be able to discriminate between neutral (Asn in position 1) and acidic amatoxins (Asp in position 1). We found that, compared to neutral α-amanitin, the affinity for the mAb to the acidic β-amanitin was indeed reduced by a factor of ca. 2.

A similar factor of reduction (Table VI) was found for other pairs of neutral and acidic amatoxins like γ-amanitin/ε-amanitin and amaninamide/amanin (Buku et al., 1980). Although both pairs showed per se reduced affinities for the mAb due to the lack of structural elements in position 3 and 4 (Figure 3A), it appears that fairly the same factor describes the change in affinity occurring in all amatoxins when $CONH_2$ in amino acid position 1 is replaced with COO^-.

(*B*) *The 6′-OH Group of (OH)Tryptophan[4].* Another group expected to be involved in the formation of the amatoxin/antibody complex is the 6′-hydroxy group of (OH)Trp[4]. Indeed, the affinity for the antibody dropped when this OH was replaced with H. Two natural compounds in which this OH group is replaced with H, amaninamide and amanin (**11** and **12**, Table V), could be compared to their hydroxylated counterparts, α- and β-amanitin (**1** and **2**, Table V), respectively. The factors determined were 1.8 and 1.9 (Table VI). In three amatoxins the 6′-hydroxy group of (OH)Trp[4] was methylated, yielding 6′-*O*-methyl-α-amanitin, 6′-*O*-methyl-γ-amanitin, and 6′-*O*-methyl-β-amanitin methyl ester (**5**, **6**, and **8**, Table V). When these compounds were related to their unmethylated compounds (**1**, **3**, and **7**, Table V; Figure 3B), we found that methylation of the OH reduced the affinities for the antibody to fairly the same extent (factors of 1.9, 2.1, and 2.1, respectively; Table VI) as does the replacement of the OH by H.

(*C*) *The δ- and γ-OH of (OH)$_2$Isoleucine[3].* One of the reasons for the existence of a whole family of amatoxins is the variable degree of hydroxylation at the side chain of $(OH)_2$Ile[3]. While the most abundant toxins, α- and β-amanitin, possess hydroxy groups in γ- and δ-positions, the rare members of the family, γ- and ε-amanitin, are hydroxylated only in the γ-position. In order to determine the contribution of the OH in δ-position, three pairs of amatoxins containing either OH or H in δ-position were assayed (comparison of amatoxins **1** vs **3**, **2** vs **4**, and **5** vs **6**; Table VI). The binding factors found for the δ-positioned hydroxy group were 1.5, 1.7, and 1.6, respectively.

When the γ-positioned OH group of this side chain was replaced by H the effect was distinctly larger, as seen from the pairs amanullin vs γ-amanitin or amanullic acid vs ε-amanitin (Table V, Figure 3C). Here we found factors of

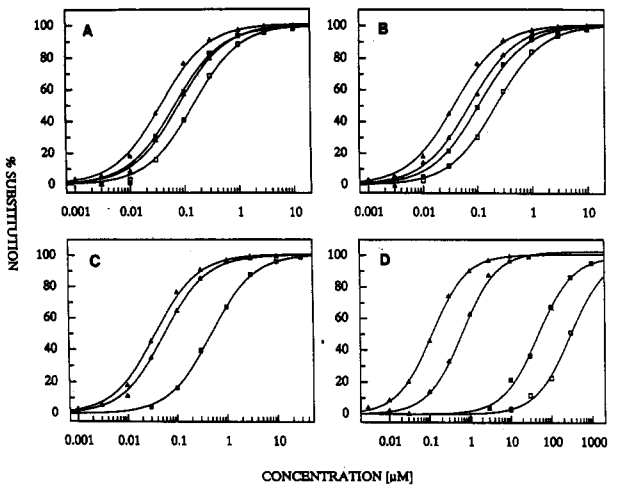

FIGURE 3: (A) Displacement curves of tracer II by increasing concentrations of α-amanitin (▲), β-amanitin (△), amaninamide (■), and amanin (□). By substitution of carboxamide (in α-amanitin, ▲) for a carboxylate (in β-amanitin, △), the affinity was reduced by fairly the same factor as obtained if carboxamide in amaninamide (■) is turned to carboxylate, yielding the acidic amanin (□). (B) Displacement curves of tracer II by increasing concentrations of α-amanitin (▲), 6′-*O*-methyl-α-amanitin (■), γ-amanitin (△), and 6′-*O*-methyl-γ-amanitin (□). This plot shows that by methylation of the indole OH the affinity of α-amanitin is reduced by nearly the same factor as it is by methylation of γ-amanitin. (C) Displacement curves of tracer II by increasing concentrations of α-amanitin (▲), γ-amanitin (△), and amanullin (■). This plot shows the contribution to binding made by the OH groups in position 3 (α-amanitin, 2 OH; γ-amanitin, 1 OH; amanullin, no OH). Loss of the δ-positioned OH group has a minor effect on binding, while loss of the γ-positioned OH group has a significantly larger effect. (D) Displacement curves of tracer II by increasing concentrations of 6′-*O*-methyl-γ-amanitin (▲), 3-acetyl-6′-*O*-methyl-γ-amanitin (△), 2-acetyl-6′-*O*-methyl-γ-amanitin (■), and 2,3-diacetyl-6′-*O*-methyl-γ-amanitin (□). This plot shows the effect on binding produced by acetylation of γ-OH in position 3 and/or the OH group of (OH)proline[2]. Acetylation in position 3 has a moderate effect on binding as compared to the strong effect observed after acetylation in position 2. Interestingly, the decrease of binding for the diacetylated toxin (□) corresponds to the product of the factors measured for the two monoacetylated toxins (△, ■). Each point is the mean of duplicate determinations.

8.5 and 6.4 (Table VI), which compare fairly well to the factor of 5.7 determined for a derivative where the γ-OH group was acetylated (**18**, Table V).

(*D*) *The OH Group of (OH)Proline*[2]. One of the modifications found to be most deleterious to the toxicity of amatoxins against RNA polymerase II is the removal of the OH group at (OH)proline[2] as found, for example, in proamanullin (or [Ile[3], Pro[2]]-α-amanitin). We modified this OH group at (OH)pro[2] by acetylation of 6′-*O*-methyl-γ-amanitin (**6**, Table V). The reaction resulted in a product (**19**, Table V) with an affinity decreased by a factor of 413, indicating that the OH at proline in position 2 is one of the most crucial features also for binding the toxin to the antibody (Figure 3D).

(*E*) *Modifications of the Indole NH of (OH)Tryptophan*[4]. Methylation of the indole nitrogen (**20**, Table V) reduced the affinity by a factor of 140. Iodination of α-amanitin (**24**, Table V), which takes place in the 7′-position, is the only modification which enhances the affinity for the antibody, by a factor of 1.4 (Table VI).

(*F*) *Exchange of Isoleucine*[6] *or Glycine*[5] *for Alanine.* Exchange of an amino acid for another is not the kind of chemical modification to which natural products can be submitted. Therefore, the change in affinity that occurs when the lipophilic side chain of Ile[6] is replaced by a shorter one like that of alanine had to be studied with amatoxins synthesized from the building blocks. G. Zanotti prepared

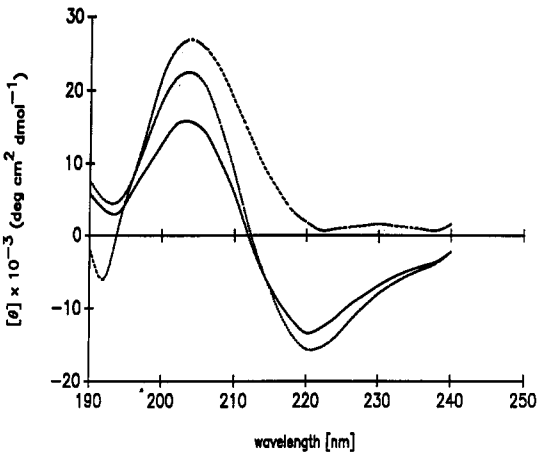

FIGURE 4: Circular dichroism spectra of an active double-ring amatoxin (6′-*O*-methyl-α-amanitin, solid line), an active monocyclic amatoxin (6′-*O*-methylseco-α-amanitin, dotted line), and an inactive double-ring amatoxin (6′-*O*-methylaldomanitin, dashed line). Independent of their chemical nature (bicyclic, monocyclic) the spectra of the two active compounds (solid line, dotted line) are similar to each other but dissimilar from the bicyclic but inactive compound (dashed line).

several synthetic amatoxin peptides, in which for simplification of synthesis several amino acids were truncated (Zanotti et al., 1987, 1989). For this reason the prototype of this family of peptides, [Ile[3], deoxo-Cys[8], 6′-dehydroxy-Trp[4]]-α-amanitin (**14**, Table V), had an affinity for the antibody that was ca. 30 times lower than that of α-amanitin.

We measured the effect on binding when in this prototype Ile[6] was replaced by alanine (**15**, Table V). Encouraged by the observation that binding factors attributed to modified structural features are conserved throughout the amatoxin family provided the conformation of the peptides remains unchanged, we supposed that the binding factors found for the truncated compounds would apply to the natural toxin α-amanitin as well (see Discussion). We found that by this replacement affinity was decreased by a factor of 131. An even higher decrease of affinity was noted when one of the adjacent glycine residues, that in position 5, was replaced with alanine. This synthetic peptide, [Ile[3], Ala[5], deoxo-Cys[8], 6′-dehydroxy-Trp[4]]-α-amanitin (**16**, Table V), had an affinity 361 times lower than that of the reference compound (**14**, Table V).

(*G*) *Two Monocyclic Amatoxins, One of Them with Changed Conformation.* A structural feature that appears to be of great importance for the binding of amatoxins to proteins is the intrachain link between Cys[8] and (OH)Trp[4]. This was shown by removal of the sulfoxide moiety in 6′-*O*-methyl-α-amanitin through treatment with Raney nickel. The resulting monocyclic peptide (**22**, Table V) showed an affinity more than 10 000 times lower than that of 6′-*O*-methyl-α-amanitin. On the other hand, opening of the peptide ring between position 3 and 4, yielding the monocyclic 6′-*O*-methylseco-α-amanitin (**23**, Table V), seems to maintain the major part of the original conformation. As shown in Figure 4, opening of the peptide ring between position 3 and 4 is not reflected by the CD spectra of the two compounds. In line with this the decrease in affinity was very low, corresponding to a factor of only 1.3.

(*H*) *A Conformational Change Induced by a Modified Side Chain.* While in most amatoxin derivatives presented in this study modifications of side chains apparently did not affect the overall shape of the molecule, this was not true for methylaldoamanitin (**21**, Table V). As has long been known, the presence of the aldehyde moiety in this compound induces

a conformational change as seen from the CD spectrum (Figure 4). As a consequence of this conformational change the affinity for the antibody dropped by a factor of ca. 1000. Removal of the aldehyde by hydrogenation (**13**, Table V) almost completely restored the binding capacity as shown by an increase in affinity by a factor of 527. As documented by CD, the reaction product of hydrogenation had a conformation virtually identical to that of α-amanitin (Faulstich et al., 1973).

DISCUSSION

The set of 24 amatoxin derivatives investigated in this study includes amatoxins with modified amino acid side chains in all positions of the octapeptide, except for glycine in position 7. In addition to amatoxin derivatives with modified side chains, two peptides of monocyclic structure and two others with amino acids exchanged were included. From the large number of modified structures investigated we hoped to gain comprehensive information not only on the contact points of the peptide but also on the kinds of interactions through which complex formation with the protein is achieved. α-Amanitin, the main natural toxin, turned out to have the highest affinity for the antibody, apart from the iodinated compound. Accordingly, all deletions and substitutions made were referred to α-amanitin and understood as modifications of this molecule. By correlating the actual decrease of affinity with each of these modifications, we were able to determine what a given structural feature of α-amanitin contributes to complex formation. Moreover, by considering the nature of the binding features we were able to predict structural details of the complementary binding site in the protein.

Conformation Is Crucial for Binding of Amatoxins to the Monoclonal Antibody. The direct correlation of a structural modification with a corresponding decrease of binding capacity was possible only in cases where the modification had little or no influence on the backbone conformation of the amatoxins. Although in cyclic peptides some parts of the peptide backbone may exhibit a considerable flexibility (Bönzli & Gerig, 1990), there seems to be agreement that at least those parts of the molecule which are essential for binding to proteins maintain their relative orientation. Such "rigidity" existing at least in major parts of the peptide was of importance in the present study and seemed to be given for all natural amatoxins as well as for most of the chemically modified derivatives.

Among the few amatoxin derivatives for which the CD spectrum indicated that chemical modifications had induced a conformational change, was 6′-*O*-methylaldoamanitin (**21**, Table V). This derivative has a positive Cotton effect at 220–230 nm, which for other amatoxins is negative (Figure 4). It was speculated that the conformational change that leads to loss of toxicity against RNA polymerase II is produced by an interaction of the strong dipole of the aldehyde moiety with other parts of the molecule (Faulstich et al., 1973). Indeed, recent work from Preston's laboratory has shown that the interaction most probably represents an addition of the aldehyde group to the adjacent amide group under reversible formation of a cyclic acylaminol derivative (Mullersmann et al., 1991). In the present study we found that, similar to the enzyme, the antibody is unable to recognize the changed conformation: 6′-*O*-methylaldoamanitin showed an affinity for the immunoglobulin that was nearly 1000 times lower than that of 6′-*O*-methyl-α-amanitin. This large decrease in affinity cannot be explained by the absence of the hydroxymethyl group in side chain 3 (which was removed by IO_4^- oxidation), because reduction of the aldehyde moiety by $NaBH_4$ to the corresponding alcohol yielded a product (**13**, Table V), which likewise lacks the hydroxymethyl group but whose affinity for the antibody is almost restored. In line with this, the alcohol derivative has a conformation similar to that of α-amanitin on the basis of the CD spectrum [not shown; see Faulstich et al., 1973)].

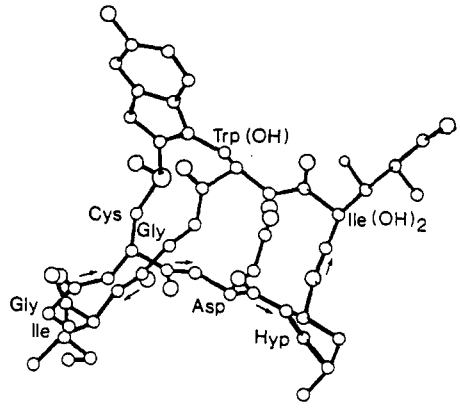

FIGURE 5: Spatial structure of β-amanitin redrawn according to Kostansek et al. (1978).

A departure from the typical amatoxin conformation must also be expected for one of the monocyclic amatoxin derivatives, the 6′-*O*-methyldethio-α-amanitin (**22**, Table V). The conformation of this compound is unknown, because its chromophore is of a different spectral type and its CD spectrum cannot be directly compared with those of amatoxins. Nevertheless, we assume that the preferred conformation this monocyclic peptide adopts does not resemble that of α-amanitin, since no affinity for the antibody could be detected (<0.01% that of the corresponding derivative with the sulfur moiety intact (**5**, Table V).

The situation is completely different for another monocyclic amatoxin, 6′-*O*-methylseco-α-amanitin (**23**, Table V), in which the sulfoxide bridge and, correspondingly, one of the heterodetic peptide rings is intact, while the other one has been cleaved between amino acids 3 and 4. Since the chromophoric system is the same as for the mother compound, the CD spectrum of 6′-*O*-methylseco-α-amanitin (Figure 4) could be compared to that of α-amanitin. We found that the spectra were nearly identical. In another approach we compared the ^1H NMR spectrum of this monocyclic compound with that of its two-ring precursor (not shown), indicating a high degree of coincidence. We conclude from these data that the presence of the intact sulfoxide bridge, together with a hydrogen bond between the carboxamide of asparagine[1] and the amide protons of the residues $(OH)_2$ile[3] and (OH)trp[4], suffices to maintain the original, active conformation. The existence of such a hydrogen bond was suggested by structural work on amatoxins performed in several laboratories (Kostansek et al., 1978; Tonelli et al., 1978; Shoham et al., 1984, 1989).

Evaluation of these three examples leads to the conclusion that the antibody can recognize amatoxin derivatives only if they adopt—at least in those parts being essential for binding—a shape that resembles that of native toxins, which were used for stimulating the immune response. In maintaining this shape the sulfoxide bridge seems to be of great importance. If the bridge is removed, the peptide obviously loses the typical arrangement of functional groups which cooperate in establishing the contact to the protein. However, even with the sulfoxide bridge intact, interactions between side chains may be strong enough to disturb the typical shape and lead to substantial loss of affinity, as shown, for example, by 6′-*O*-methylaldoamanitin.

From reasons discussed below it is likely that the structure most strongly affected by conformational changes is the loop Gly–Ile–Gly which, according to the spatial structure (Figure 5), is exposed in the peptide in a distinct way. From the

Table VII: Comparison of Calculated and Measured Binding Factors of Amatoxin Derivatives Containing Modified Structural Features at Two Positions

	structural feature		binding factor, single modification	binding factor, two modifications	
amino acid	original	modified		calcd	measd
$(OH)_2Ile^3$	δ-OH	H	1.6[a]	3.2	3.1
$(OH)Trp^4$	6′-OH	OCH_3	2.0[a]		
$(OH)_2Ile^3$	δ-OH	H	1.6[a]	3.5	3.5
Asn^1	$CONH_2$	COO^-	2.2[a]		
$(OH)Trp^4$	6′-OH	H	1.8[a]	4.0	3.9
Asn^1	$CONH_2$	COO^-	2.2[a]		
$(OH)_2Ile^3$	γ-OH	$OCOCH_3$	5.7	2355	2600
$(OH)Pro^2$	γ-OH	$OCOCH_3$	413		
$(OH)_2Ile^3$	γ-OH	H	7.4[a]	16	15
Asn^1	$CONH_2$	COO^-	2.2[a]		

[a] Mean values.

absence of any binding of the monocyclic 6′-*O*-methyldethio-α-amanitin, a peptide that contains all functional elements necessary for binding except the bridge between Cys^8 and $(OH)Trp^4$, we further conclude that the correct conformation of the peptide cannot be formed "in situ". Failure of such an "induced fit" may be understood by the absence of a preformed binding region in the peptide that would allow the formation of a transitional complex with the protein long-lived enough to allow folding of other regions of the peptide in a way able to establish further contact sites.

Structural Elements Involved in Binding Act Independently from Each Other. Except for the two examples discussed above, 6′-*O*-methylaldoamanitin and 6′-*O*-methyldethio-α-amanitin, all chemical modifications studied so far had no detectable influence on the overall conformation of the peptide. This may explain why chemical modifications made at one site did not influence the binding properties of others. On the basis of this finding we were able to define binding factors [K_D(modified amatoxin)/K_D(unmodified amatoxin)] which could be attributed to single structural elements and which were shown to be independent of the K_D value of a given amatoxin. The evidence collected for this rule is summarized in Table VI. The table shows that mean values of factors attributable to a given structural element, when calculated from two or sometimes three pairs of amatoxins with variation only in the structure concerned, scattered around the mean value by 3–6%. This was true in all cases with the exception of γ-OH in position 3, where the binding factors scattered in a wider range (ca. 23%) for a reason unknown so far.

The constancy of the binding factors was taken as empirical evidence that complex formation is achieved by cooperation of independent contact sites, each of them contributing a constant amount of binding energy. In accordance with this, the decrease of affinity produced in an amatoxin derivative in which two chemical modifications were made had a value that corresponded to the numerical product of the two binding factors involved. For example, acetylation of the γ-OH in $(OH)_2Ile^3$ reduced affinity by a factor of 5.7. Acetylation of OH in $(OH)Pro^2$ reduced the affinity by a factor of 413. Acetylation of both OH groups would be expected to reduce the affinity by a factor of 5.7 × 413 = ca. 2400. The reduction of affinity actually measured for the bisacetylated derivative (**17**, Table V) was ca. 2600, a number that is close to the calculated value. Some further examples confirming this empirical rule are compiled in Table VII.

The empirical rule detected here encouraged us to apply it to the synthetic amatoxin analogs prepared by Zanotti (Zanotti et al., 1987, 1989) as well. These derivatives are truncated at three sites: by the absence of the two OH groups in $(OH)_2ile^3$, by the absence of the 6′-OH in $(OH)Trp^4$, and by the absence of the sulfoxide oxygen at the thioether bridge. Particularly the latter modification might have a strong influence on binding because the sulfoxide is in close proximity to the proton at the indole nitrogen, which was shown to be one of the major contact points.

While the influence of the SO/S modification on binding could not be examined directly, the effects caused by the three others were measured (see Table VI): replacement of the δ-OH and γ-OH in $(OH)_2Ile^3$ by H decreased binding by average factors of 1.6 and 7.4, respectively, while replacement of the 6′-OH of $(OH)Trp^4$ by H decreased binding by a factor of 1.8. Applying the product rule, all modifications together would make up for a factor of 1.6 × 7.4 × 1.8 = 21. The actual decrease of the truncated peptide was shown to be described by a factor of 30, indicating that the absence of the sulfoxide oxygen is characterized by a factor of only ca. 1.4. Removal of the oxygen at the sulfoxide is thus of minor influence on complex formation. This is confirmed by X-ray analysis (Shoham et al., 1989) of amatoxin sulfide and sulfoxide, showing that the absence of the sulfoxide oxygen has very little influence on the overall shape of the molecule.

On the basis of these lines of evidence we suggest that binding factors, even when derived from the affinities of highly truncated synthetic peptides, may be taken as valid for native amatoxins, provided the peptides correspond in conformation. When in compound **14** (Table V) isoleucine[6] was replaced by alanine, the K_D value rose by a factor of 131. We expect that the same factor would apply if this replacement was made in α-amanitin itself. Similarly we expect that the absence of a side chain in the adjacent glycine residue, $glycine^5$, is of crucial importance for α-amanitin, too. This amino acid is part of the loop Gly–Ile–Gly, which for the amatoxins seems to be of outstanding importance for protein recognition.

The Peptide–Protein Complex Has at Least Eight Sites of Contact. Besides the very important role that conformation plays in complex formation, at least four structural elements in the amatoxin molecule were identified which strongly contribute to binding of the antibody: the OH group of $hydroxyproline^2$, the indole nitrogen of $(OH)tryptophan^4$, the side chain of $isoleucine^6$, and the glycine residue in position 5. Removal or modification of any of these structures resulted in a reduction of affinity by a factor of more than 100, emphasizing their importance for the formation of a tight contact with the protein. Besides these major contact points, four others, most probably hydrogen bonds, were identifed, which seem to be involved in less strong interactions with the antibody.

The pronounced decrease in affinity that was seen when the hydroxy group of $(OH)pro^2$ was acetylated (binding factor 413) is comparable in size to the more than 1000-fold reduction in capacity to inhibit RNA polymerase II that was reported for proamanullin, an amatoxin in which the OH group of $(OH)proline^2$ is absent (Buku & Wieland, 1974). We conclude that hydrogen bonds in which $hydroxyproline^2$ plays the donor part are of crucial importance for binding amatoxins to the enzyme as well as the mAb βA1/1. A hydrogen bond of comparable strength is proposed for the proton at the indole nitrogen, because methylation reduced the affinity by a factor of 141. In the complex of amatoxins with RNA polymerase II the indole part plays a minor role because methylation reduced the inhibition capacity only by a factor of ca. 2 (Faulstich et al., 1981).

The most prominent binding feature, the sequence Gly–Ile–Gly, must be seen as an entity. It appears that the basis

for its binding function is the hydrophobic interaction of the isoleucine side chain. Glycine probably takes part in an intramolecular hydrogen bond, thus stabilizing the C10 β-turn (Shoham et al., 1989). According to the X-ray structure (Figure 5) the β-turn Gly–Ile–Gly protrudes from the peptide in a distinct way and may thus represent that part of the amatoxin molecule that is most sensitive to conformational changes. The two amino acids, isoleucine[6] and glycine[5], are of similar importance for the interaction of amatoxins with RNA polymerase II since replacement of each of the two by alanine reduced the inhibition capacity of amatoxins by factors of 746 and 24, respectively (Zanotti et al., 1987).

From knowledge of the structural features involved in the binding process as well as from their spatial arrangement in the peptide, information can be obtained on the complementary part of the complex, the binding site of the antibody. Given the apparent importance of the motif Gly–Ile–Gly for binding amatoxins to proteins, the central part of the binding region seems to be a hydrophobic pocket for the side chain of isoleucine[6]. Located at nearly equal distances apart from this pocket we presume two strong hydrogen bonds, originating from the indole nitrogen and the OH group of hydroxyproline[2]. To judge from the strength of these hydrogen bonds, the receiving groups may well be carbonyls of the antibody backbone.

CONCLUSIONS

Binding of amatoxins to the monoclonal antibody βA1/1 is mainly achieved by a strong hydrophobic interaction in combination with several hydrogen bonds. There are at least eight points of contact, four of them strong. The number of contact sites is large considering that α-amanitin is a peptide of roughly the size of an epitope. Even so, because the limited number of feasable chemical modifications, the points of contact identified in this study may still not represent all of the interactions that really exist between the amatoxins and the monoclonal antibody. In particular, there may be a large number of weak van der Waals interactions as suggested by recent work on three-dimensional structures of antigen–antibody complexes (Davies et al., 1990; Kabat, 1980).

We hope to identify all the interactions between amatoxins and antibodies by X-ray analysis. Work is in progress to elucidate the three-dimensional structure of the complex between 7′-iodo-α-amanitin and the Fab fragment of the monoclonal antibody βA1/1, which has been crystallized recently. We regard the present investigation as more than merely preliminary work for crystallography; rather, it is a significant complement to the X-ray analysis, since it allows quantification of the contribution made by various contact sites of the peptide to the binding energy.

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