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A β-turn in α-amanitin is the most important structural feature for binding to RNA polymerase II and three monoclonal antibodies

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Abstract

Four amatoxin-binding proteins with K_D values in the nanomolar range, 3 monoclonal antibodies and RNA polymerase II, were studied with respect to their affinities to 24 α-amanitin derivatives with modified side chains. From K_D values we estimated the amounts of binding energy that single side chains of the amatoxins contribute to complex formation. Ile^6, previously identified by X-ray analysis to be part of a β-turn (Kostansek EC, Lipscomb WN, Yocum RR, Thiessen WE, 1978, *Biochemistry 17*:3790–3795) proved to be of outstanding importance in all complexes. Replacement of the isoleucine with alanine reduced the affinity to all binding proteins to <1%, suggesting a strong hydrophobic interaction. A strong effect was also seen when Gly^5 was replaced with alanine, suggesting that the absence of a side chain in proximity to the β-turn is likewise important. In addition to the β-turn, each of the proteins showed at least 2 other points of strong contact formed by hydrogen bonds. Donors are the indole NH of 6′-hydroxy-Trp^4 and OH of hydroxy-Pro^2 and dihydroxy-Ile^3. All the antibodies, but not RNA polymerase II, recognized the indole nucleus of 6′-hydroxy-Trp^4. The geometric arrangement of the 4 strongest contact points suggests that the amatoxin binding site is different in each of the 4 proteins, except for the 2 antibodies raised in the same animal. Here, most of the contact points were identical but differed in strength of interaction. The method of structural analysis presented in this study is useful for identifying contact sites in complexes of proteins with peptides of rigid conformation. Furthermore, the method complements X-ray data by providing information on the amount of binding energy contributed by single structural elements.

Keywords: β-turn; dissociation constant; hapten antibody complex; monoclonal antibody; peptide protein complex; RNA polymerase II

In addition to α-helix and β-sheet, β-turns make up a considerable part of nonrandom structures in proteins. In many cases, the loops are sites at which such events as binding, cleavage, or posttranslational modification occur. Particularly in the interaction of peptides with proteins the various types of β-turns (for a classification, see Venkatachalam, 1968; Rose et al., 1985) have frequently been found to be involved, be it for binding peptide immunogens to antibodies (Stanfield et al., 1990; Rini et al., 1992; Scherf et al., 1992) or for binding peptide hormones to receptors (Buku et al., 1987; Nachmann et al., 1991). Because of compactness, β-turns are thought to be important structural elements for constructing peptide mimetics (Veber, 1981; Hirschmann, 1991).

In a previous paper (Baumann et al., 1993), extensive chemical modification of side chains in α-amanitin was successfully used for identifying structural elements of amatoxins that are involved in complex formation with the monoclonal antibody βA1/1. From the dissociation constants (K_D) with each of a total of 24 amanitin derivatives, we were able to estimate the amounts of binding energy contributed to complex formation by distinct structural features. Furthermore, we provided empirical evidence that single binding structures make their contribution independent from others, provided the backbone conformation remains unchanged. We identified at least 8 points of contact between α-amanitin and the antibody βA1/1, four of them being strong, because removal or modification of these structures reduced affinity to <1%. From the geometry of the sites of strong contact we were able to identify that part of the peptide surface that is in contact with the immunoglobulin.

In the present study we applied this method of structural analysis to 2 more monoclonal antibodies and to RNA polymerase II, the natural target protein of the amatoxins. We wanted to elucidate, on the atomic level, what structural elements of the

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amatoxins are involved in the complex formation with the proteins. Given the fact that all 4 proteins bind amatoxins with similar affinity and that an octapeptide like α-amanitin presents only a limited number of structures accessible for chemical modification, it was of interest for us to find out whether the structural features of the amanitin molecule addressed by the 4 different binding proteins were the same in all cases.

Results

Three clones of rat/mouse hybridoma producing amatoxin-specific antibodies with high affinity to amatoxins were selected for the present study. Immune response in mice was induced using fetuin conjugates of α-amanitin with a linker to the asparagine side chain in position 1 (Fig. 1). Affinities were determined by Scatchard analysis (Fig. 2) using 6′-*O*-[^{3}H]methyl-α-amanitin, an amatoxin closely related to α-amanitin (Fig. 1), as tracer. With K_D values of 6 nM (clone βA1/1, from rat 1) and 7 nM (clone βA2/7, from rat 2), the affinities of the tracer to 2 of the selected antibodies were similar to that of calf thymus RNA polymerase II, determined as the inhibition capacity of the unlabeled 6′-*O*-methyl-α-amanitin (K_I = 4 nM). The third antibody (clone βA2/2, from rat 2) differed from the 2 others and the enzyme by a reduced affinity (K_D = 13 nM). Although the methyl-group introduced into α-amanitin as a tracer moiety had virtually no influence on the interaction of the toxin with RNA polymerase II, the modification decreased the affinity to the antibodies ($\Delta\Delta G^0$ = −1.7 to −4.7 kJ/mol), indicating that the indole part is of greater importance for binding the antibodies than for binding RNA polymerase II.

Altogether, we studied 24 amatoxin derivatives with respect to their affinities for 3 monoclonal antibodies as well as their capacities to inhibit the activity of RNA polymerase II. Eight of these amatoxin derivatives were of natural origin, isolated from *Amanita phalloides* or *Amanita virosa* mushrooms. Eight others were methylation or acetylation products of the native peptides. Two others were obtained by reaction with periodate (glycol cleavage) or iodination of the indole nucleus. Two of the amatoxin derivatives were monocyclic compounds, produced either by mild acidic cleavage of a distinct peptide bond, or by hydrogenolytic removal of the central sulfur atom. Finally, but importantly, 3 amatoxin derivatives were prepared by synthesis from the building blocks (Zanotti et al., 1987). In total, we had at our disposal amatoxin derivatives with chemical modifications in each of the amino acid residues of α-amanitin (Fig. 1), except for glycine in position 7. The amatoxin derivatives are listed in Table 1.

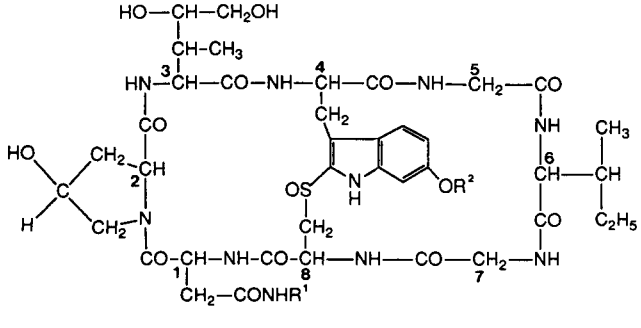

Fig. 1. Structures of α-amanitin (R^1 = H, R^2 = H); 6′-O-[^{3}H]methyl-α-amanitin employed as tracer (R^1 = H, R^2 = [^{3}H]CH_3); and the amanitin-fetuin conjugate used for immunization (R^1 = fetuin, R^2 = H).

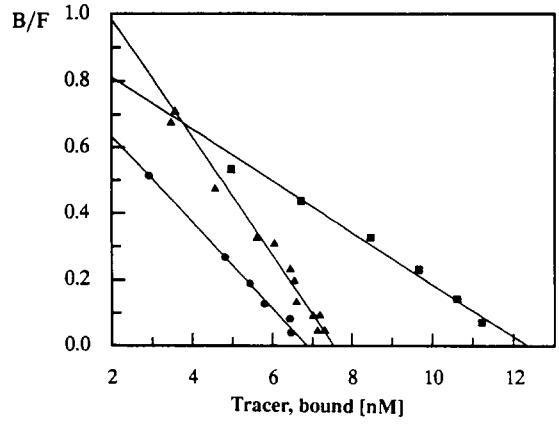

Fig. 2. Scatchard plots of 3 monoclonal antibodies using 6′-*O*-[^{3}H]methyl-α-amanitin as tracer. ▲, βA1/1; ■, βA2/2; ●, βA2/7.

The affinity values listed in Table 1 were obtained from competitive binding assays. We measured the concentration of each derivative required for replacement of 50% of 6′-*O*-[^{3}H]methyl-α-amanitin from the antibody's binding site. Separation of free and bound tracer was in all cases achieved through polyethyleneglycol precipitation according to Stenman et al. (1981). It was assessed that amatoxin binding to the antibodies was not disturbed by the precipitation procedure. In the earlier study we had shown that from these concentration values K_D values can be calculated by correlating the data from binding assays with data from Scatchard analysis (Baumann et al., 1993). All K_D values shown in Table 1 were calculated in this way. The table contains numerous compounds that differ in only 1 structural element. For such pairs of closely related amatoxins we have shown that it is possible to calculate the contribution made to binding by the differing structural element from the ratio of the K_D values of the 2 amatoxins concerned. These changes in K_D values attributed to a distinct structural modification in 2 amatoxin derivatives are expressed in differences of ΔG^0 ($\Delta\Delta G^0$, Table 2).

One of these structural elements is the side chain in position 1, which can be neutral (Asn) or negatively charged (Asp). In total, we had 3 pairs of amatoxins for which asparagine or aspartic acid in position 1 was the only variation: α/β-amanitin (Table 1, nos. 1/2), γ/ε-amanitin (Table 1, nos. 3/4), and amanullin/amanullic acid (Table 1, nos. 9/10). Each of the 4 proteins distinguished this structural detail in an individual way (see Table 2, first line): 2 antibodies with preference for the neutral side chain (average $\Delta\Delta G^0$ value of ca. −2 kJ/mol, and −1 kJ/mol, respectively); 1 antibody with preference for the charged side chain (average $\Delta\Delta G^0$ value of ca. 1 kJ/mol); and RNA polymerase II with almost no preference (average $\Delta\Delta G^0$ close to zero). For a given protein the $\Delta\Delta G^0$ values of the 3 pairs of amatoxin derivatives were fairly constant, confirming our previous finding that single structural elements involved in binding can be characterized by $\Delta\Delta G^0$ values, which apply to other amatoxin derivatives as well, and hence are roughly independent from other binding structures.

The individuality in amatoxin binding became more conspicuous when we compared the way the 3 antibodies recognized dis-

Table 1. *Structures of all amatoxin derivatives used in this study, together with the calculated K_D values to the 3 monoclonal antibodies βA1/1, βA2/2, βA2/7, and the K_I values measured for RNA polymerase II*

No.	Formula	K_D [nM] βA1/1	K_D [nM] βA2/2	K_D [nM] βA2/7	K_I [nM] RNAPII
1	α-Amanitin (=1, see Fig. 1)	2.6	2.2	0.9	3.3
2	(Asp^1)-1	5.4	1.6	1.4	3.4
3	(γ-Hydroxy-Ile^3)-1	3.8	1.7	1.0	5.9
4	(γ-Hydroxy-Ile^3)-(Asp^1)-1	9.1	1.0	1.4	7.1
5	(6′-Methyl-Trp^4)-1	5.0	12	6.0	3.9
6	(6′-Methyl-Trp^4)-(γ-hydroxy-Ile^3)-1	8.0	8.7	6.5	5.9
7	(Methyl-Asp^1)-1	7.3	5.5	1.2	n.d.
8	(6′-Methyl-Trp^4)-(methyl-Asp^1)-1	11	n.d.	n.d.	n.d.
9	(Ile^3)-1	33	1.7	1.0	n.d.
10	(Ile^3)-(Asp^1)-1	58	1.1	1.3	291
11	(6′-Dehydroxy-Trp^4)-1	4.5	25	22	5.6
12	(6′-Dehydroxy-Trp^4)-(Asp^1)-1	10	18	40	n.d.
13	(6′-Methyl-Trp^4)-(γ-hydroxy-Val^3)-1	9.4	8.9	5.7	n.d.
14	(Ile^3)-(deoxo-Cys^8)-(6′-dehydroxy-Trp^4)-1	71	27	49	141
15	(Ile^3)-(Ala^6)-(deoxo-Cys^8)-(6′-dehydroxy-Trp^4)-1	1.0×10^4	2.3×10^4	1.1×10^4	2.8×10^4
16	(Ile^3)-(Ala^5)-(deoxo-Cys^8)-(6′-dehydroxy-Trp^4)-1	2.8×10^4	83	2.2×10^3	1.3×10^4
17	(γ-Acetyloxy-Ile^3)-(6′-methyl-Trp^4)-(acetyloxy-$hypro^2$)-1	2.1×10^4	85	26	7.1×10^3
18	(γ-Acetyloxy-Ile^3)-(6′-methyl-Trp^4)-1	45	6.4	6.0	295
19	(γ-Hydroxy-Ile^3)-(6′-methyl-Trp^4)-(acetyloxy-$hypro^2$)-1	3.3×10^3	65	30	222
20	(1′,6′-Dimethyl-Trp^4)-1	700	159	15	4.1
21	(6′-Methyl-Trp^4)-(γ-oxo-Val^3)-1	4.9×10^3	1.3×10^3	924	1.4×10^3
22	(Ala^8)-(6′-methyl-Trp^4)-1	$>7.0 \times 10^4$	$>1.6 \times 10^5$	$>8.4 \times 10^4$	$>5.0 \times 10^4$
23	(6′-Methyl-Trp^4)-seco-1	6.2	11	6.3	3.5
24	(7′-Iodo-Trp^4)-1	1.8	79	53	4.0

tinct segments of the indole nucleus, namely 1′-NH, 6′-OH, and 7′-H. While methylation of the nitrogen diminished the binding to βA1/1 by a $\Delta\Delta G^0 = -12.2$ kJ/mol, $\Delta\Delta G^0$ values of only −6.4 and −2.3 kJ/mol were found for βA2/2 and βA2/7, respectively. On the contrary, replacement of 6′-OH for H had the strongest effect on antibody βA2/7: in the same sequence this modification decreased the affinities to the 3 antibodies with $\Delta\Delta G^0$ values of −1.5, −6.0, and −8.1 kJ/mol, respectively. This is in line with the finding that modification of the adjacent 7′-position by introducing an iodine atom decreased the binding analogously, namly by $\Delta\Delta G^0 = 0.9$, −8.9, and −10.1 kJ/mol. These data indicate that even a structural element limited in size like the indole nucleus is recognized by the 3 antibodies in different positions; whereas the contact of the aromatic part to βA2/7 must be strongest at the 6′ and 7′ position, the strongest interaction of βA1/1 must be at position 1, i.e., at the 5-membered ring. Large differences between the affinities of the 3 antibodies were also found for amatoxins in which Gly^5 was replaced with alanine. While this modification had only little effect on binding by antibody βA2/2 ($\Delta\Delta G^0 = -2.8$

Table 2. *Differences in binding energy ($\Delta\Delta G^0$) identifying the contribution made by various structural features of α-amanitin to binding of the mAb βA1/1, βA2/2, βA2/7, and RNA polymerase II*

Amino acid	Structural feature Original	Modified	$\Delta\Delta G^0$ [kJ·mol^{-1}] βA1/1	βA2/2	βA2/7	RNA PII
Asn^1	$CONH_2$	COO^-	−1.8, −2.2, −2.0	0.8, 1.3, 0.8	−1.1, −0.8, −1.5	−0.1, −0.5
$(OH)Pro^2$	(γ)-OH	$OCOCH_3$	−14.9	−4.9	−3.8	−9.0
$(OH)_2Ile^3$	(δ)-OH	H	−0.9, −1.3, −1.2	0.6, 1.2, 0.8	−0.3, 0.0, −0.2	−1.4, −1.8, −1.0
	(γ)-OH	H	−5.4, −4.6	0.0, −0.2	0.0, 0.2	−9.2
	(γ)-OH	$OCOCH_3$	−4.3	0.8	0.2	−9.7
$(OH)Trp^4$	6′-OH	OCH_3	−1.6, −1.8, −1.8	−4.2, −4.1	−4.7, −4.6	−0.4, 0.0
	6′-OH	H	−1.4, −1.5	−6.0, −6.0	−7.9, −8.3	−1.3
	1′-H	CH_3	−12.2	−6.4	−2.3	−0.1
	7′-H	I	0.9	−8.9	−10.1	−0.5
Gly^5	H	CH_3	−14.6	−2.8	−9.4	−11.2
Ile^6	$CH(CH_3)C_2H_5$	CH_3	−12.1	−16.7	−13.4	−13.1

kJ/mol), the effect on antibody βA2/7 was much greater ($\Delta\Delta G^0 = -9.4$ kJ/mol) and even more for βA1/1 ($\Delta\Delta G^0 = -14.6$ kJ/mol).

One modification decreased binding to all antibodies most strongly: the replacement of Ile^6 with alanine. Shortening of the side chain in this position reduced the affinity to all antibodies to <1% ($\Delta\Delta G^0 < -12$ kJ/mol). Because a corresponding effect was found also for RNA polymerase II, we conclude that the isoleucine side chain is the structural element of the amatoxins that is of outstanding importance for binding the peptide to all the amatoxin-binding proteins examined. It was exceeded in importance only by 2 modifications of the amatoxins that changed the backbone conformation of the peptide, namely periodate degradation of side chain 3 (Table 1, no. 21) and removal of the bridging sulfur atom (Table 1, no. 22). Affinities of the latter 2 compounds to all 4 proteins were close to 0 (<0.1% for the aldehyde derivative, and <0.01% for the dethio compound). For the aldehyde derivative, a change in conformation was proven by CD (Faulstich et al., 1973); for the dethio analog, the loss of the original shape by removal of the stabilizing sulfur bridge must be assumed.

Discussion

In a previous paper (Baumann et al., 1993) empirical evidence was provided that binding of an amatoxin to a protein occurs via a number of structural elements that cooperate in the binding process. Given that the typical and rigid backbone conformation of the amatoxins was conserved, the contributions made to complex formation by single binding structures were shown to be largely independent from each other. As a consequence of this rule, we found that for all pairs of amatoxins that differed only in one and the same structural element, the ratio of their K_D values and therefore the $\Delta\Delta G^0$ values were constant and could be taken as a measure for characterizing the contribution made by this structural element. Constancy of the $\Delta\Delta G^0$ values was observed even in cases when the affinity levels of the pairs of amatoxins to be compared differed to a large extent.

Based on this rule, and using the K_D values of an Ile^6- and an Ala^6-amatoxin derivative, we calculated that the contribution to binding made by the hydrophobic side chain of Ile^6 must be so important that it is crucial for all 4 protein complexes examined in this study. Because Ile^6 is on top of a β-turn as seen from the X-ray structure of β-amanitin elucidated by Kostansek et al. (1978) (Fig. 3), we anticipate that the Ile^6 side chain finds a hydrophobic homing pocket in each of the 4 proteins. In addition to this, hydrogen bonds between the peptide and the proteins located in close proximity, and possibly including CO and/or NH of the peptide backbone, may contribute to stabilization of this hydrophobic interaction. This suggestion is based on the fact that Ile^6 is flanked by 2 glycine residues (Gly^5, Gly^7) in which at least Gly^5 cannot be replaced by alanine. Hydrogen bonds between the backbones of a protein and a peptide are not rare and were shown to exist, for example, in the complex of cathepsin D and pepstatin (Balwin et al., 1993). As shown in Table 2, presence of a methyl side chain in position 5 leads to strongly reduced binding to 3 of the 4 proteins.

The fit of the β-turn is certainly based on the compactness of this structural moiety. It is known that (4 → 1) type II β-turns are held together by an intramolecular hydrogen bond (Venkatachalam, 1968), which stabilizes the 10-membered loop. In the case of amatoxins, it appears that besides this hydrogen bond another structural element, the sulfoxide bridge, is required, which adds to either stabilization or orientation of the β-turn. This was concluded from the observation that removal of the bridging sulfur atom by reduction with Ni decreased affinity to all proteins to almost 0.

Besides the strong interaction of the β-turn, binding of the amatoxins to proteins depends on several hydrogen bonds, the donor parts being in most cases hydroxy groups of the peptide. Unlike the β-turn, which is important for all protein complexes, the various OH groups of amanitin are recognized by the 4 proteins in a quite individual manner. For example, it has long been known that amanullin, a natural amatoxin peptide, which lacks the hydroxy group in the γ position of Ile^3, is nontoxic in the white mouse (Wieland, 1986) due to a strongly reduced affinity to RNA polymerase II (Zanotti et al., 1989). In the present study we confirmed that through the absence or acetylation of this hydroxy group, binding to RNA polymerase II is indeed reduced by $\Delta\Delta G^0$ values of −9.2 to −9.7 kJ/mol. On the other hand, we showed that this modification has only little or no effect on amatoxin binding to any of the 3 antibodies. Similarly, the hydroxy group in Pro^2 is of great importance for binding amatoxins to one of the antibodies (βA1/1) and likewise contributes to binding of RNA polymerase II, but has only little effect on binding to the 2 other antibodies. With growing flexibility of side chains, the contribution of OH groups to binding becomes 0 for all proteins, as shown for the δ-positioned OH in Ile^3 (Table 2).

Other strong hydrogen bonds of the peptide–protein complexes include the tryptophan part of the amatoxins. Donors are, for example, the indole NH, as in the complexes with mAb βA1/1 and mAb βA2/2, but not in complexes with mAb βA2/7 and RNA polymerase II. The 6′-OH is important for complexation of mAb βA2/2 and mAb βA2/7, but not for binding to the antibody of animal 1 or inhibiting RNA polymerase II. Thus, like the hydrogen bonds originating from OH in Ile^3 and Pro^2, the hydrogen bonds emanating from the indole nucleus reflect the individual ways the 4 proteins recognize structures on the amatoxin surface.

In general, we found that hydrophobic forces, together with strong hydrogen bonds, represent the essential part of amatoxin protein interaction as predicted (Cochet-Meilhac & Chambon, 1974) on the basis of their studies on the amanitin–RNA polymerase II complex.

Besides hydrophobic interaction and hydrogen bonds, a third kind of interaction may play a role in some of the amatoxin protein complexes, namely the intercalation of the aromatic part of 6′-OH-tryptophan. This is suggested from the finding that iodination in 7′ position in 2 cases (mAb βA2/2 and βA2/7) reduced the binding ($\Delta\Delta G^0 = -8.9$ and -10.1). Because it is unlikely that hydrophobicity of the aromatic side chain is significantly changed by introduction of an iodine atom, we believe that it may be through steric effects of the large substituent that the significant decrease of affinity is explained.

As shown in Table 2, amatoxins have at least 6–8 contact points with each of the proteins, probably more, because in an octapeptide the number of feasible chemical modifications is limited. At least some of these contacts in each complex are strong, i.e., the $\Delta\Delta G^0$ values are ≤5.0 kJ/mol. By far the

strongest contact in all 4 protein complexes is the β-turn. Its significance can be estimated from the addition of the $\Delta\Delta G^0$ values attributed to 2 amino acid residues that are part of the β-turn, Gly^5 and Ile^6. These values are ca. 20 kJ/mol in all cases. This means that disturbance of the shape of the β-turn by simultaneous replacement of the 2 amino acids with alanine decreases the affinity to all binding proteins to <0.05%. For comparison, the loss of the 2 strongest hydrogen bonds results in $\Delta\Delta G^0$ values of −12.2 and −14.6 kJ/mol, corresponding to residual binding activities that are ca. 10-fold higher, 0.7 and 0.3%, respectively. The residual hydrogen bonds are weak ($\Delta\Delta G^0 = -0.9$ to −5.0 kJ/mol), which correspond to binding activities of 67 and 13% of α-amanitin, respectively.

It appears that in all proteins the interaction of the β-turn represents the core part of contact, which is stabilized by hydrogen bonds of varying strength located nearby. From the β-turn and the 3 strongest hydrogen bonds, as illustrated in Figure 3, contact areas on the amanitin surface addressed by the 4 pro-

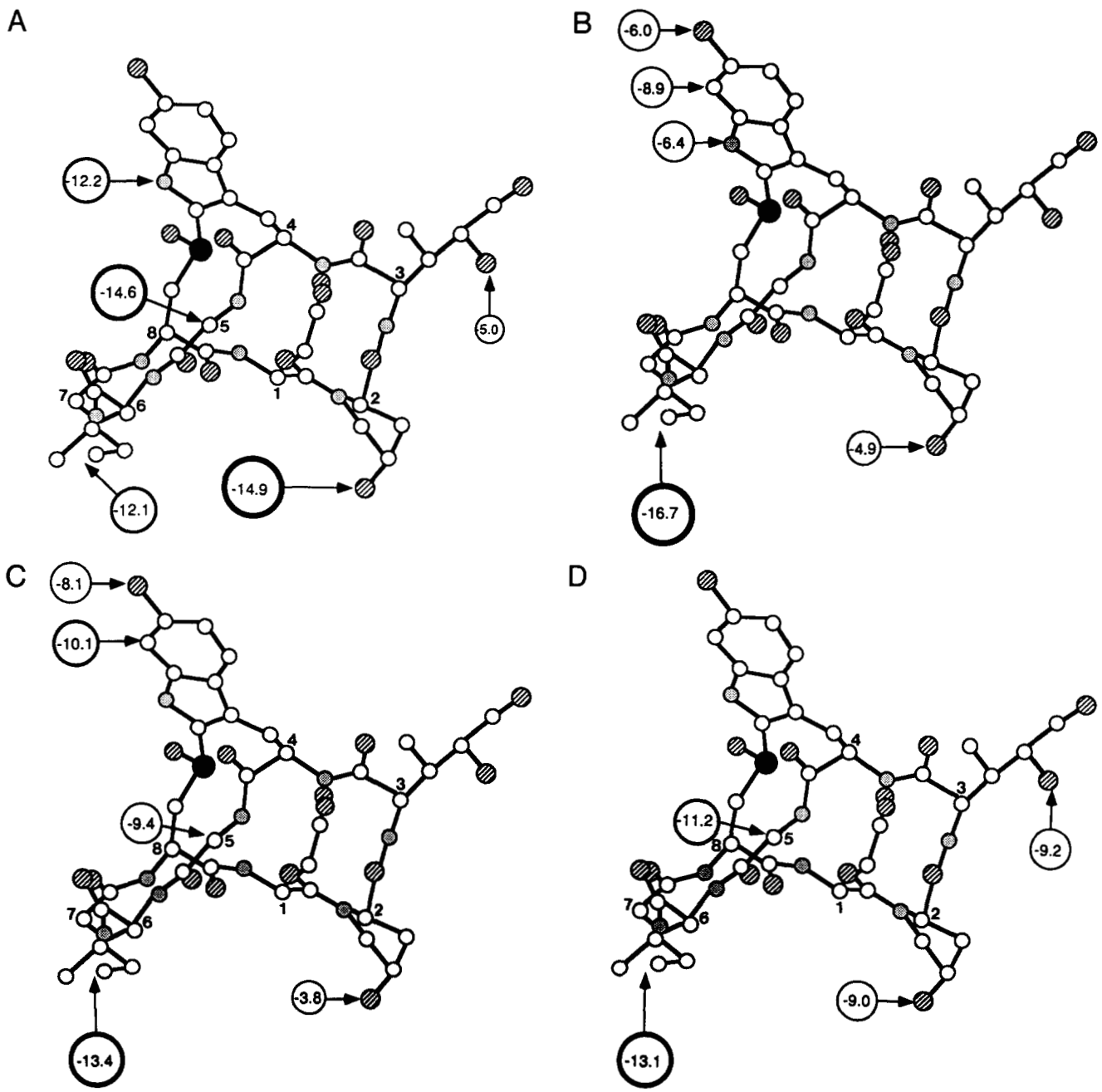

Fig. 3. Spatial structure of amanitin with the sites of contact to the 4 amatoxin-binding proteins indicated (hatched: oxygen; double hatched: sulfur; stippled: nitrogen; the α-carbon atoms of the amino acids are numbered as in Fig. 1). **A:** Antibody βA1/1. **B:** Antibody βA2/2. **C:** Antibody βA2/7. **D:** RNA polymerase II. The encircled numbers denote $\Delta\Delta G^0$ values (kJ/mol), indicating the strength of interaction with the structure specified. The spatial model of amanitin was taken from Kostansek et al. (1978) and modified.

teins can be deduced. All 3 antibodies obviously recognize the aromatic side chain, although at different segments and to different extents. Remarkably, RNA polymerase II has no contact with any part of the aromatic side chain; rather, as appears from Figure 3D, the indole nucleus is juxtaposed to the contact site. These data are in line with our finding that methylation of the OH in the 6′ position affected binding to all antibodies but not to RNA polymerase II (see Fig. 2). It appears that, in the enzyme, the missing interaction with the aromatic part is compensated for by another strong hydrogen bond with the γ-OH of Ile^3, a contact that in the reverse is of no, or only minor, importance for binding to the antibodies. Nonparticipation of the indole part in complexes with RNA polymerase II is also in good agreement with previous results from our laboratory, in which we showed that introduction of even bulky residues into the 6′ position did not seriously impair the inhibitory capacity of amatoxins (Faulstich et al., 1981).

Figure 3 further shows that two of the antibodies (βA2/2 and βA2/7) have several features in common, such as the ability to recognize positions 6′ and 7′ of the indole nucleus, contacts that are absent in the other proteins. Likewise they share the weak to moderate interaction with OH of Pro^2. Such relationships may reflect that the 2 antibodies were raised in one and the same animal (rat 2). On the other hand, the common contacts differ in strength. In addition, the 2 antibodies vary in their ability to recognize Gly^5 and NH of indole. We therefore believe that these 2 antibodies, although obviously related, possess, in the end, individual means for amatoxin binding.

In conclusion, we found that, of the 4 amatoxin protein complexes examined, each amatoxin binding protein developed its own mode of contact by recognizing an individual combination of several prominent structures on the amatoxin surface. Not only do the 4 proteins differ in the combination of such contact points, but these contacts likewise vary in their strength of interaction. This suggests that all proteins, although similar in their K_D values, employ individual structures for forming the amatoxin binding pocket.

Materials and methods

Materials

Preparation of the protein conjugates of amatoxins, such as β-amanitin–fetuin and β-amanitin–bovine serum albumin (BSA), was described elsewhere (Faulstich & Fiume, 1985). 6′-*O*-[^{3}H]methyl-α-amanitin was prepared in our laboratory (Faulstich et al., 1981). All other reagents were of analytical grade. Purified and active RNA polymerase II from calf thymus was a generous gift of C. Kedinger (CNRS, Strasbourg, France).

Production of monoclonal antibodies

Three-month-old Wistar rats (ca. 200 g body weight) were immunized intraperitoneally with β-amanitin–fetuin in phosphate-buffered saline corresponding to amounts of 30 μg of β-amanitin mixed with 2 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 Perlmann (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). Purification of the antibodies has been described (Baumann et al., 1993).

Preparation and purification of amatoxin derivatives

The natural amatoxins α-amanitin, β-amanitin, γ-amanitin, ε-amanitin, amanullin, amanullic acid, amaninamide, and amanin were isolated from the green death cap (*A. phalloides*) as described previously (for reviews, see Wieland & Faulstich, 1983; Wieland, 1986). Chemical modification as well as characterization of the amatoxins by UV and CD spectroscopy and [^{1}H]NMR were carried out as described previously (for details see Baumann et al., 1993).

Determination of dissociation constants and competitive binding assay

Binding capacities and K_D values of the monoclonal antibody were measured by precipitation of the 6′-*O*-[^{3}H]methyl-α-amanitin complex with polyethyleneglycol 6000 (Roth, Karlsruhe). This assay was performed according to Stenman et al. (1981). The same procedure provided the basis for the competitive binding assay (for details, see Baumann et al., 1993).

RNA polymerase inhibition assay

This assay is based on the measurement of the in vitro incorporation of tritium-labeled UTP into mRNA by calf thymus RNA polymerase II. Amatoxin derivatives were added to the assay in increasing amounts in order to determine the concentration that reduced RNA polymerase II activity by 50% (K_I values). The assay was carried out according to Kedinger et al. (1972) as follows: Amatoxin derivatives (0.25 nM–2.5 mM) were incubated with RNA polymerase II (12.8 U/mL) in 64 mM Tris/Cl buffer, pH 7.9, containing 16% glycerol; 0.03 mM EDTA; 1.0 mM dithiothreitol; 3 mM $MnSO_4$; 50 mM $(NH_4)_2SO_4$; 0.5 mM ATP, GTP, CTP; 12.5 μM [^{3}H]UTP (3.2 Ci/μmol); and 0.2 mg/mL calf thymus DNA for 1 h at 37 °C (final volume 25 μL). The reaction was stopped by the addition of 2 μL EDTA (0.2 M). The samples were applied to filter papers (Whatman DE 81), washed 5 times with 10% $Na_2HPO_4 \cdot 12H_2O$, 0.5% sodium pyrophosphate, 0.1% SDS, and twice with 90% ethanol. After drying, the filters were put into a scintillation cocktail and counted in a β-counter.

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