Interaction of Several Class II and III RNA Polymerases with Two Families of 7′-Substituted α-Amanitin†

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ABSTRACT: A series of six secondary aminoamatoxins and seven arylazoamatoxins were examined for inhibition of class II RNA polymerases from sea urchin, wheat germ, *Drosophila melanogaster*, and rat liver. These amatoxins were also tested against class III RNA polymerases from sea urchin, *Drosophila melanogaster*, and rat liver. In all cases, the substituents were introduced into α-amanitin at the 7′ carbon of the tryptophan residue and thus lie in the right arm of the crossbar in the T-shaped amatoxin as defined by Kostansek et al. [Kostansek, E. C., Lipscomb, W. N., Yocum, R. R., & Thiessen, W. E. (1978) *Biochemistry 17*, 3790–3795]. The sizes of the substituents ranged from diethylamino, the smallest, to dibenzylamino and 4-benzylpiperidino. Despite the range in size of the substituents, all of the modified amatoxins were effective inhibitors. The six examples of secondary aminoamatoxins showed apparent K_i values with the class II RNA polymerases ranging from 1 to 8 times the K_i found with the parent α-amanitin. The seven examples of arylazoamatoxins showed apparent K_i values with the class II RNA polymerases ranging from 1 to 5 times the K_i found with the parent. With the class III RNA polymerase there was a reduction in the apparent K_i values which in two examples was greater than 10-fold. Manual mixing experiments showed that the two amatoxins with the most bulky substituents inhibited actively transcribing class II RNA polymerase more rapidly than could be determined (<30 s) and in this regard are indistinguishable from α-amanitin. Thus the amatoxin binding site on the class II and III RNA polymerases will accept amatoxins modified in the tryptophan ring with only minimal changes in the K_i and with no apparent steric interaction between the amatoxin binding site and the active site. These two types of chemical modifications yield α-amanitin derivatives suitable as molecular vectors to bind reporter groups into the amatoxin site of wide range of eukaryotic RNA polymerases.

The availability of facile reactions to substitute α-amanitin regiospecifically provides an approach to prepare a collection of amatoxins that, with proper design of the substituent, find use in probing structural, organizational, and functional features of the eukaryotic DNA-dependent RNA polymerase (EC 2.7.7.6). Selective substitutions at the 7 carbon of the 6-hydroxy-2-sulfoxytryptophan residue of α-amanitin has been described in this laboratory (Morris et al., 1978; Morris & Venton, 1982; Falck-Pedersen et al., 1982). While the azo substitution was previously described (Faulstich & Trischmann, 1973; Hencin & Preston, 1979), neither the degree or the positions of substitution nor a satisfactory method of purification was defined. These ambiguities are now removed providing several amatoxin derivatives including the 7′-aminated amatoxins (Morris & Venton, 1982), the 7′-arylazoamatoxins (Falck-Pedersen et al., 1982), and the 7′-iodoamatoxin (Morris et al., 1978). While additional modifications [reviewed in Wieland & Faulstich (1978)] such as the amides and esters at the aspartyl γ-carboxylate of β-amanitin have been described, this line of amatoxin modification is not generally appealing due to chemical inaccessibility and apparent increase in the equilibrium dissociation constant (Wieland & Faulstich, 1978; Faulstich et al., 1981; Wieland & Boehringer, 1960; Kostansek et al., 1978). However, the ethers at the hydroxyl of the tryptophan residue are of particular interest due to the ease of preparation and inhibitory activity against RNA polymerase (Faulstich et al., 1981; this laboratory, unpublished results). These chemical routes in principle provide the researcher with a broad collection of amatoxin derivatives for introduction of, for example, isotopic, fluorescent, and photoreactive moieties into the amatoxin binding sites on eukaryotic RNA polymerases. Several techniques for amatoxin derivatization are now characterized and allow the generation of probes that can initiate a detailed characterization, not only of the amatoxin binding site but also of the structure and function of the entire RNA polymerase. In this paper we examine these restrictions with two families of amatoxin derivatives and class II and III eukaryotic RNA polymerases from a broad range of species. A critical application of amatoxin–reporter group ligands to eukaryotic RNA polymerase studies requires definition of the biochemical restrictions that govern the amatoxin–polymerase interaction.

Experimental Procedures

Materials. Wheat germ was a generous gift of Dixie Portland Mills, Chicago, IL. Other materials used were ethylene glycol and 30% poly(ethylenimine) in H_2O (Aldrich), [^{3}H]piperidine at 51 Ci/mmol and carrier-free ^{125}I (Amersham), and all aryl- and alkylamines (Aldrich). All other materials were analytical reagent grade or as specified below.

RNA Polymerase Preparations. The sea urchin RNA polymerases were purified as previously described (Morris et al., 1978). The *Drosophila melanogaster* adult and K_c cell RNA polymerases were purified by the procedure of L.-T. Lin and P. W. Morris (unpublished results). The wheat germ polymerase was prepared by the procedure of Jendrisak & Burgess (1975) with one modification: the phosphocellulose chromatography was replaced with Bio-Rex 70. The Bio-Rex 70 column (40 mL volume) was equilibrated at 0.05 M $(NH_4)_2SO_4$, and the RNA polymerase II was adsorbed at the same ionic strength and then eluted with 0.35 M $(NH_4)_2SO_4$ in the buffer as described. The wheat germ RNA polymerase was near homogeneity as determined by sodium dodecyl sulfate electrophoresis.

The rat liver RNA polymerases were purified by adaptation of previously used procedures (Lin and Morris, unpublished

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results; Morris et al., 1978). Those fractions from the DEAE-Sephadex A-25 column containing class I or III RNA polymerase were used for assay with the amatoxins; the class II RNA polymerase was obtained from the preceding DE-52 cellulose column.

The amatoxins were introduced into the RNA polymerase assay (Morris & Rutter, 1976) by adding 5 μL of an amatoxin solution to 25 μL of the reaction mixture; the polymerization was initiated by addition of 20 μL of the appropriate RNA polymerase ($<10^{-9}$ M in polymerase as estimated by activity measurements and, in the case of wheat germ RNA polymerase, A_{280}). The polymerization was thermostated at 30 °C and terminated by application of 40 μL to a DE-81 filter disk. Stock solutions of the amatoxins were prepared in water where possible; however, solubility problems in some cases necessitated the use of 50% ethylene glycol in water. In these instances the control assays were adjusted to equal ethylene glycol concentrations since the class II RNA polymerase is quite sensitive to alteration of ethylene glycol concentration (data not presented).

The buffers used for enzyme preparations were the following: Buffer A contained 50 mM Tris-HCl, pH 7.9 at 4 °C, 5 mM $MgCl_2$, 0.1 mM Na_2EDTA, 0.1 mM dithiothreitol, and 25% (v/v) ethylene glycol. This was used for wheat germ and K_c cell polymerases only. Buffer B was buffer A with 25% glycerol rather than ethylene glycol.

Preparation of α-Amanitin Derivatives. The preparation and purification of the nonradioactive derivatives were as described in the preceding papers (Morris & Venton, 1982; Falck-Pedersen et al., 1982).

For the preparation of 7′-[G-^{3}H]piperidino-α-amanitin, 1 mCi of [G-^{3}H]piperidine hydrochloride (Amersham TRK 589; 51 Ci/mmol) was dried under N_2 and dissolved in 0.4 mL of CH_3OH. The reaction was initiated by the sequential addition of 200 nmol of tributylamine, 20 nmol of I_2, and 20 nmol of α-amanitin; each was dissolved in CH_3OH. After 24 h at 23 °C, the CH_3OH was evaporated and the residue taken up in 1-butanol for chromatography over polyamide SC 66 (70 × 0.4 cm column) eluted with 1-butanol/1-octanol (90:10 v/v). Fractions containing coincidental ^{3}H and A_{305} were pooled. The elution profile was similar to the one shown in Figure 1 (Morris & Venton, 1982): yield 58% of the [^{3}H]piperidine and 54% of the α-amanitin as the 7′-piperidino-α-amanitin. The ^{3}H-labeled compound was compared to authentic 7′-piperidino-α-amanitin prepared as described for the morpholine reaction (Morris & Venton, 1982) and was found identical by chromatography over Whatman ODS PSX 5/25 reverse-phase column and by electronic spectra in neutral and alkaline methanol. [^{125}I]Iodoaniline was prepared by the Chloramine T method (Hunter & Greenwood, 1962) from 5 mCi of carrier-free I (Amersham, IMS 300) in 45 nmol of $Na^{127}I$ and purified by high-pressure liquid chromatography over a Whatman ODS PSX 5/25 reverse-phase column equilibrated at 50:50 H_2O/CH_3CN (v/v). The effluent was monitored spectrophotometrically at 280 nm and radiometrically by a low-energy δ detector (Ludlum 44-3) coupled to a rate meter (Ludlum 2). The [^{125}I]iodoaniline eluted identically with authentic *p*-iodoaniline and thus does not contain di- or triiodinated species. The content of *o*-iodoaniline was judged to be low on the same basis: yield 88% of the ^{125}I as *p*-iodoaniline.

The [^{125}I]iodoaniline (41 nmol) was diazotized with excess $NaNO_2$ (5 μmol) in 30 μL of 0.1 M HCl at 0 °C for 5 min. Then α-amanitin in excess (40 μL in CH_3OH, 120 nmol) was added and followed rapidly by 10 μL of 1.0 M NaOH in 80% methanol. The bright orange azoamatoxin was observed, following alkalinization. After 10 min, the alkaline reaction was neutralized with 3.7 M CH_3COOH (5 μL) and partitioned between 1 mL each of 1-butanol and H_2O. The butanol phase was recovered, evaporated to dryness with N_2 gas, dissolved in H_2O/CH_3CN (63:37 v/v), and resolved by reverse-phase chromatography on a Whatman ODS PXS 5/25 column. The effluent was monitored spectrophotometrically at 305 nm and radiometrically (Figure 1): yield 65% of the input [^{125}I]-iodoaniline as 7′-[(4-iodophenyl)azo]-α-amanitin.

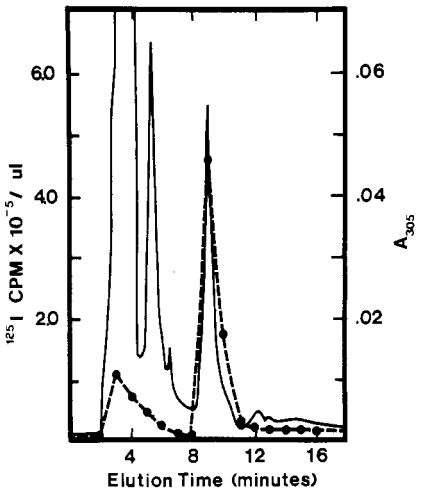

FIGURE 1: Elution profile of 7′-[(4-[^{125}I]iodophenyl)azo]-α-amanitin from ODS PXS 5/25 reverse-phase chromatography. The A_{305} and the ^{125}I content of the effluent stream were monitored continually; the product was collected manually when the A_{305} and ^{125}I count rate both increased.

Alternate methods for checking the course of radiolabeling are suggested from the thin-layer chromatographies routinely used in this laboratory (Morris & Venton, 1982; Falck-Pedersen et al., 1982). The polyamide plates provide a general chromatographic support for all the amatoxins except the azoamatoxins which do not migrate on polyamide. The advantage of the polyamide over cellulose or silica plates lies in the resolution afforded to the aminated and iodinated amatoxins (Morris et al., 1978; Morris & Venton, 1982). Chromatogram development with 1-butanol/acetone/H_2O (89:8:3 v/v) gave $R_f \simeq 0.46$, 0.65, and 0.72 for α-amanitin, 7′-iodo-α-amanitin, and 7′-piperidino-α-amanitin, respectively. The KC-18 reverse-phase silica plate (Whatman) resolves all of the amatoxin derivatives in a manner analogous to the high-pressure liquid chromatography. Development of the KC-18 plate with 0.1 M sodium acetate in H_2O/CH_3CN (6:4 v/v) gave $R_f \simeq 0.22$ for the 7′-[(4-iodophenyl)azo]-α-amanitin, while for α-amanitin, the $R_f > 0.9$.

Results and Discussion

Kinetics of Onset of Inhibition with the Bulky Amatoxins. The amatoxin molecule is an approximate T-shaped structure as deduced from crystallographic studies (Kostansek et al., 1978) with the cysteinyl sulfoxide and the hydroxytryptophan residues forming the right arm, the dihydroxyisoleucyl-hydroxyprolylasparaginyl sequence of the cyclopeptide forming the left arm, and the glycylisoleucylglycyl sequence forming the stem of the T. The solution conformation likely is reasonably close to the crystalline state by reason of the rigid ring system and the highly solvated crystal (Kostansek et al., 1978), as well as the slow-exchange rate for three peptide protons in dimethyl-d_6 sulfoxide/D_2O mixtures at 20 °C (assessed by 220-MHz proton magnetic resonance; D. L. Venton and P. W. Morris, unpublished results). The crystallographic studies

on β-amanitin show three amide protons hydrogen bonded across the interior of the cyclooctapeptide to amide oxygens (Kostansek et al., 1978). These three likely correspond to the slow-exchanging protons observed at 220 MHz. These data picture the solution form of the amatoxin as a T-shaped structure with the indole ring toward the right and the 6 position of the indole facing slightly forward.

Treatment of α-amanitin with the I_2-secondary amine reagent as described (Morris & Venton, 1982) yields the aminated α-amanitin with the substituent at the 6 position in the hydroxytryptophan. The right arm of the approximate T-shaped amatoxin now will have become enlarged by the bulk of the particular secondary amine used. If the RNA polymerase polypeptide is in proximity to this portion of the amatoxin when bound (and this is one possible inference drawn from the decreased K_i of 7′-iodo-α-amanitin relative to α-amanitin; Morris et al., 1978), then one could expect perturbation of the amatoxin–polymerase complexation by a bulky amatoxin. The perturbation could result in a loss of binding to RNA polymerase or in an inability to bind to the RNA·DNA·RNA polymerase ternary complex. α-Amanitin inhibits the transcribing RNA polymerase, apparently noncompetitively with the template (Cochet-Meilhoc & Chambon, 1974; Hencin & Preston, 1979); nonetheless, the distance between the amatoxin and template binding site is unknown. Thus, it is important to determine whether modification of the right arm of the amatoxin selectively perturbs inhibition of the transcribing RNA polymerase.

To examine the ability of bulky amatoxins to inhibit the transcribing RNA polymerase, we chose the derivatives with *N*,*N*-dibenzylamine and 4-benzylpiperidine (Figure 5, amatoxins **4** and **6**, respectively). Maximally, these specific modificatioins extend the amatoxin 0.64 and 1.0 nm, respectively, from the 6′ position. Furthermore, the distal protons of the dibenzylamine moiety can be 1.1 nm apart, and both moieties can sweep a relatively large volume due to rotational mobility. These two amatoxin derivatives, as well as α-amanitin, were examined by manual mixing for any lag in onset of inhibition on transcribing class II RNA polymerase. One typical set of data is presented in Figure 2. Since manual mixing was used with sampling at 30-s intervals beginning 30 s after amatoxin addition, the minimum detectable lag expected is 30 s and, in any case, is limited by the variance of the data. We expect that a lag in inhibition onset would cause a shift in the intersection time of the control portion (t = 1.0–5.0 min) with the inhibited portion (t = 6.0–10.5 min) of a reaction. Analysis of variance about the least-squares regression fit of the data to the equation for a line showed the minimum significant lag > 30 s. In all cases the calculated lag (i.e., the intersection time of the two portions of a reaction) was less than the minimum significant time detectable and was not different for uninhibited vs. amatoxin-inhibited polymerizations. This was true for α-amanitin as well as for the two bulky amatoxin derivatives. We conclude that the bulky amatoxin–polymerase complexation has approached equilibrium in less than 30 s and the $t_{1/2}$ for this pseudo-first-order reaction is less than 10 s. Therefore, there appears to be no steric hindrance to the binding of these modified amatoxins to the ternary complex that occurs during in vitro transcription.

We also examined our data for evidence of irreversible inhibition of the RNA polymerase II in which case one would expect decreasing polymerization rates over some extended period of time. As Figure 2 shows, the polymerization rate changed only at the time of amatoxin addition and not over an extended time period. This was also found for polymerization times up to 25 min following amatoxin inhibition. The question of irreversible inhibition of RNA polymerase by amatoxins is of some interest because, although the amatoxin binding was shown to be reversible (Cochet-Meilhoc & Chambon, 1974), the inhibition was not shown to be reversible. In an attempt to show reversible inhibition, Logas (1978) and P. W. Morris followed *Drosophila* RNA polymerase II activity after removal of excess α-amanitin but could rescue no polymerase activity. Recently, we have observed a partial (25–30%), slow recovery of wheat germ RNA polymerase II activity following free ligand removal by gel filtration over Sephadex G-25 or sucrose density gradient sedimentation at high polymerase concentration (>0.1 mg/mL); yet, we have not been able to demonstrate fully reversible inhibition commensurate with the polymerase mass recovered. Lutter (1982) has obtained full recovery of wheat germ RNA polymerase II activity by photodissociation at 314 nm and 0.5 mg/mL at short times after amatoxin addition. Whether there may be an amanitin-induced denaturation that appears irreversible at low polymerase concentration or during the slow process of gel filtration cannot yet be fully answered. There was no clearly apparent enzyme subunit dissociation at high concentration during sedimentation of the polymerase–amatoxin complex as judged by gel electrophoresis of the gradient fractions (E. M. August and P. W. Morris, unpublished results). The kinetics of inhibition at low polymerase concentration (Figure 2) argues against slow, irreversible inhibition, but direct attempts to show full reversibility with similar RNA

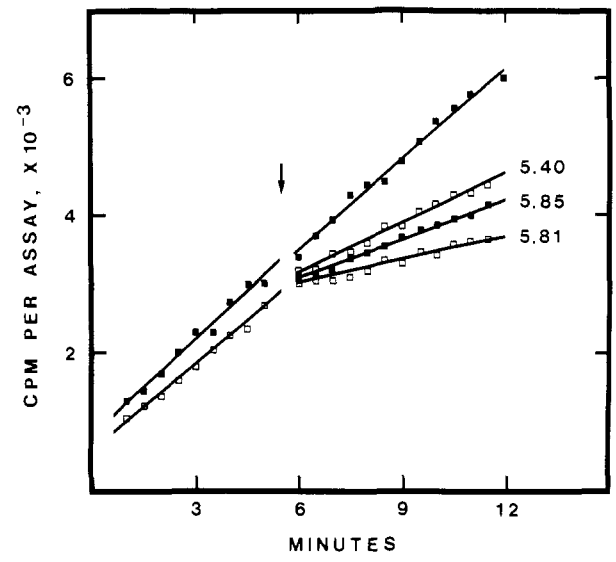

FIGURE 2: Onset of inhibition of *Drosophila* RNA polymerase II with 7′-(*N*,*N*-dibenzylamino)-α-amanitin. RNA polymerase II and denatured calf thymus DNA templated reaction mixture, both prewarmed to 30 °C for 1 min, were combined in four separate assay tubes and thermostated at 30 °C (total assay volume 800 μL/tube). Aliquots of 40 mL were removed to DE-81 filter disks at 30-s intervals beginning 1.0 min after initiation of RNA polymerization. At t = 5.5 min, either H_2O or the above amatoxin was added to the polymerization reaction. The four lines graphed above represent, in sequence from top to bottom, 0, 0.1, 0.3, and 1.0 μM amatoxin. The data were fitted by linear least-squares regression for the two intervals of t = 1.0–5.0 min (n = 9) and t = 6.0–10.5 min (n = 10). The time of intersection for the two portions of each polymerization reaction was calculated and is shown adjacent to each line in the figure above. The four values are not significantly different from each other or from the expected value, 5.5 min, for the control polymerization. Analysis of variance showed that this manual assay method could detect a lag in onset of inhibition >30 for these 4 or 20 similar experiments using both *Drosophila* RNA polymerase II and wheat germ RNA polymerase II. Each polymerase was tested with 7′-(*N*,*N*-dibenzylamino)-α-amanitin and 7′-(4-benzylpiperidino)-α-amanitin.

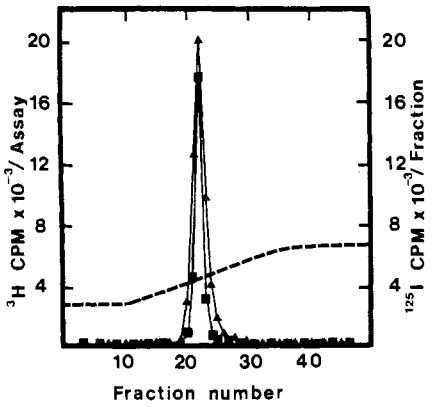

FIGURE 3: Comigration of 7′-[(4-[^{125}I]iodophenyl)azo]-α-amanitin with near homogeneous wheat germ RNA polymerase II (>90% polymerase by protein mass). The ligand (0.05 μM [^{125}I]amatoxin, 100 Ci/mmol) was bound with 0.1 μM class II RNA polymerase in 0.6 mL of buffer A at 0.4 M $(NH_4)_2SO_4$ for 20 min at 0 °C; then 0.5 mL was applied in the gradient sievorptive elution mode (Morris & Rutter, 1976) to a DEAE-Sephadex A-25 column (1.2 × 30 cm bed) equilibrated at 0.15 M $(NH_4)_2SO_4$ in a buffer A and preloaded with a 17-mL gradient from 0.15 to 0.40 M $(NH_4)_2SO_4$ in buffer A. The column was eluted with 0.40 M $(NH_4)_2SO_4$ in buffer A at 13 mL cm^{-2} h^{-1}; fractions of 0.7 mL were collected. Aliquots were assayed for calf thymus DNA directed [^{3}H]UTP polymerization (■) and [^{125}I]amatoxin migration (▲). The $(NH_4)_2SO_4$ gradient profile (0.15–0.40 M) is shown (---). A control experiment using 50 μM α-amanitin saturation of binding sites prior to ligand binding with the [^{125}I]amatoxin was performed. These data are not plotted in the graph above inasmuch as all the values were background level and indistinguishable from the [^{125}I]amatoxin base line shown.

polymerase II concentrations have failed. The remaining question then is whether the bound amatoxin sensitizes RNA polymerase to an irreversible inactivation demonstrable by gel filtration or similar separation methods.

Binding of Radiolabeled Amatoxins to Class II RNA Polymerase. The substitution of α-amanitin with either secondary amines or aryldiazoniums suggests two novel routes for the introduction of isotopic labels into the amatoxins. Unlike the previously described iodine substitution (Morris et al., 1978), the present derivatives potentially allow radioisotope introduction simultaneously with introduction of a photoreactive or other chemical cross-linking moiety. Thus an approach toward a topographical definition of the amatoxin binding site can be facilitated by the present amatoxin derivatives.

Piperidine is isosteric with morpholine and can be expected to yield an amatoxin derivative with physical and biochemical properties close to those of the morpholine derivative (Morris & Venton, 1982). Further, [^{3}H]piperidine is available at ~50 Ci/mmol, corresponding to a theoretical substitution of two ^{3}H atoms out of the ten nonexchanging protons in piperidine. For the arylazo precursors, aniline is readily iodinated and in principle allows one to introduce ^{125}I with the diazonium into α-amanitin. We note that both of the labeling methods examined here use commercially available products that yield specific activities greater than now available through NaB^3H_4 reduction (Wieland & Fahrmeir, 1970) or methylation (Faulstich et al., 1981). At least one commercial company does offer [^{125}I]iodosulfanilic acids, thus affording a radioactive amatoxin related to amatoxin **12** (Figure 5). This latter route to a [^{125}I]amatoxin would remove the necessity for $^{125}I_2$ manipulation with the attendant volatility problems.

Comigration of the present radioactive amatoxins with eukaryotic RNA polymerase II is shown in Figures 3 and 4; each radioactive ligand was prepared as described under Experimental Procedures. The elution profile of 7′-[(4-[^{125}I]iodo-

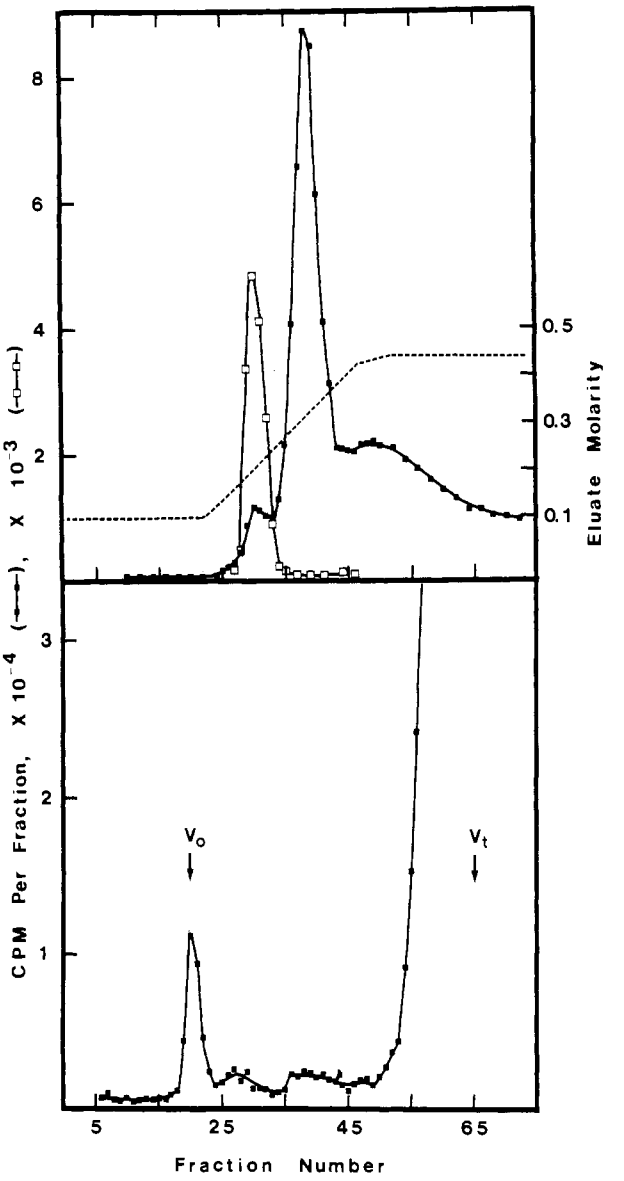

FIGURE 4: Comigration of 7′-[^{3}H]piperidino-α-amanitin with partially purified rat liver RNA polymerase II [specific activty 2.5 pmol of UMP polymerized μg^{-1} (20 min)$^{-1}$; ~0.5% polymerase by protein mass]. The ligand (0.28 μM [^{3}H]amatoxin; 51 Ci/mmol) was bound with class II RNA polymerase [7 nM; estimated from activity measurement and assumed maximal specific activity of 500 pmol of UMP μg^{-1} (20 min)$^{-1}$ at 30 °C with a native calf thymus DNA template] in 0.25 mL of buffer B at 0.30 M $(NH_4)_2SO_4$ for 60 min at 0 °C. The sample was then quantitatively transferred to a chromatography column for separation of bound free ligand. (Top) Gradient sievorptive elution on DEAE-Sephadex A-25 column (1.1 × 20 cm) equilibrated at 0.1 M $(NH_4)_2SO_4$ in buffer B. A preformed gradient sequentially constructed of 1.5 mL each of 0.20, 0.25, 0.30, and 0.35 M $(NH_4)_2SO_4$ in buffer B was followed by the RNA polymerase sample and then eluted with 0.45 M $(NH_4)_2SO_4$ in buffer B. Elution was in an upward flow and controlled to 10 mL h^{-1} cm^{-2}. Fractions of 0.35 mL were collected and assayed for calf thymus DNA directed [^{3}H]UTP polymerization, [^{3}H]amatoxin, and conductivity. [^{3}H]UTP polymerization (□); [^{3}H]amatoxin (■); conductivity (---). (Bottom) Gel filtration on Bio-Gel A 0.5M (1 × 28 cm) equilibrated in buffer B at 0.40 M $(NH_4)_2SO_4$ and eluted with the same solvent at 10 mL cm^{-2} h^{-1}. Fractions of 0.5 mL were collected and assayed as above.

phenyl)azo]-α-amanitin over DEAE-Sephadex A-25 was strongly reminiscent of that observed with 7′-iodo-α-amanitin (Morris et al., 1978). Only a single ^{125}I peak eluted from the column which coeluted with the residual RNA polymerase activity (Figure 3) and the RNA polymerase II subunits as

visualized following denaturing gel electrophoresis of the fractions. Following binding of the ligand, the RNA polymerase II activity was found to equal 85% of the original activity. This extent of activity was also recovered in fractions 19–24 (Figure 3) following DEAE-Sephadex A-25 chromatography. If one assumes that K_D = 170 nM (Table II) and that P + A = PA adequately described the amatoxin–polymerase complexation, then one can calculate [PA] = 16 nM. This is close to the observed inhibition of 15% of the 100 nM RNA polymerase and binding of 30% of the ligand. It is expected for a presumed equilibrium binding that recovery of polymerase activity would ensue after separation of the free ligand; however, as discussed above, this lack of recovery is our common finding rather than an exception. We note that the DEAE-Sephadex A-25 column bound the free ligand in the present experiment as well as with 7′-iodo-α-amanitin (Morris et al., 1978) but did not bind the 7′-piperidino-α-amanitin (Figure 4, top). Because of inability to observe the free ligand elution profile, one cannot apply a rigorous treatment to the binding reaction and, in fact, is forced to the implicit assumption that the binding is thermodynamically irreversible in the experimental conditions employed. Despite this limitation, the DEAE-Sephadex A-25 would appear quite effective for removal of the nonbound ligand.

In contrast to the ^{125}I-labeled arylazoamatoxin, the [^{3}H]-piperidino-α-amanitin was incompletely separated from the binary complex with rat liver RNA polymerase II on DEAE-Sephadex A-25 (Figure 4, top) which rendered it impossible to quantify the bound amatoxin. separation by gel filtration (Figure 4, bottom) showed 1.6 pmol of ligand comigrating with the residual RNA polymerase II activity and an additional 1.60 pmol eluting between V_0 and V_t. The applied enzyme was estimated at 1.7 pmol from activity measurement with native calf thymus DNA templated polymerization. The separation of bound and free ligand that was observed with gel filtration would allow demonstration of dynamic dissociation during the separation process. The 1.60 pmol of [^{3}H]amatoxin eluting between V_0 and V_t may have resulted from dissociation of the complex; in this latter case, the summation for total ligand bound equaled 3.2 pmol. Since this experiment involves a partially purified RNA polymerase II preparation, we judge either 3.2 or 1.6 pmol of bound ligand to be in acceptable agreement to the 1.7 pmol of enzyme estimated by assay. Binding of the [^{3}H]amatoxin, like the [^{125}I]amatoxin binding, was competed fully by previous saturation of the RNA polymerase with 50 μM α-amanitin.

Inhibition Constants for Amatoxin Derivatives with Several Class II and III RNA Polymerases. With the means to place a wide range of substituents at the 7′ position in α-amanitin, we thought it important to examine a phylogenetic spectrum of eukaryotic RNA polymerases with respect to amatoxin inhibition. The RNA polymerases appear to be rather conserved in evolution; that is, observations on their physical and catalytic properties [e.g., Mg(II) and Mn(II) optima, ionic strength optimum, elution from ion-exchange matrices, sedimentation coefficients, subunit composition, etc.] suggest that eukaryotic RNA polymerases of a given class are quite related. This impression has been noted previously and these data have been reviewed frequently (Roeder, 1976; Chambon, 1975). These similarities are, however, related to in vivo transcriptive function and undoubtedly are constrained to be similar throughout the phylogenetic spectrum. In some contrast, the amatoxin binding site shows extensive variability. Mammalian RNA polymerases II exhibit some of the lowest K_i values at $\leq 10^{-8}$ M (Chambon, 1975; Roeder, 1976) as does the sea urchin (Morris et al., 1978), chicken (Wittig & Wittig, 1978), and *Acanthamoeba castellanii* (Detke & Paule, 1978). Progressively higher K_i values are observed for *Drosophila melanogaster* (10^{-7} M; Greenleaf & Bautz, 1975), wheat germ (10^{-7} M; Jendrisak & Guilfoyle, 1978), yeast (10^{-6} M; Valenzuela et al., 1976; Schultz & Hall, 1976), the edible, amatoxin-biosynthesizing mushroom *Agaricus bisporus* (10^{-5} M; Vaisius & Horgen, 1979), and the amatoxin-accumulating mushroom *Amanita hygroscopica* (10^{-3} M; Johnson & Preston, 1980). Thus, phylogenetic change is apparent at the amatoxin binding site of the class II RNA polymerases. A similar argument may be advanced for the class III RNA polymerase with the difference that the K_i values range from 10^{-5} M (mammalian; Roeder, 1975) to 10^{-4} M (sea urchin; Morris et al., 1978) to 10^{-3} M (*Drosophila melanogaster*; Lin and Morris, unpublished results) to unobservable (yeast; Valenzuela et al., 1976; Schultz & Hall, 1976). Moreover, we find a $K_i = 10^{-3}$ M for the *Drosophila melanogaster* adult but a $K_i = 10^{-4}$ M for the RNA polymerase III of the *Drosophila melanogaster* Kc cell line (Lin and Morris, unpublished results; R. Chigurupati and P. W. Morris, unpublished results).

The phylogenetic variation at the amatoxin binding site, the presence of an experimentally accessible amatoxin site on two classes of RNA polymerase, and the ability to selectively substitute the amatoxin raise three questions of interest: (1) What degree of structural relationship exists between the class II and III amatoxin sites? (2) What degree of structural relationship exists within the class II amatoxin sites of equal K_i? (3) What extent of experimental usefulness can be realized from a given amatoxin derivative (e.g., a photoactivated cross-linking reagent)? Data to begin answering the above questions are shown in Tables I–III. All of the amatoxin derivatives were found to inhibit the class II and III RNA polymerases tested. There were no overriding steric hindrances between the 7′-substituted amatoxins and the RNA polymerases in any case.

Regarding the class II and III amatoxin binding sites, the available data argue for structural differences in addition to those implied by the K_i values for α-amanitin. Examples include a significantly decreased K_i with 7′-iodo-α-amanitin relative to α-amanitin for the class II (Table I) but not class III enzymes (Table III). The arylazoamatoxins as a group tend to decrease K_is for the class III enzymes (Table III) and unchanged K_is for the class II enzymes (Table II). The observed decrease was 10-fold or greater in some cases. Likewise, the secondary amine derivatives of α-amanitin (Table I) tend to equal or increase K_i for the class II enzyme, while one example showed a decreased K_i for class III (e.g., amatoxin **6**, K_i = 7 μM for rat liver RNA polymerase III; α-amanitin, K_i = 39 μM; Table III).

Conclusion

Consideration of the structure for these amatoxin derivatives does not lead to a simple rationalization of the inhibition data or a simple picture of the amatoxin binding site. The collected data in Tables I–III argue convincingly that, although the 7′ position of α-amanitin is not immediately juxtaposed to the polymerase topographically, it nonetheless is quite close. For the 7′-C derivatives, the observed changes in inhibition of the RNA polymerase cannot be explained on solvent repulsion alone since the observed changes were in opposite directions for class II and class III polymerases and, even in some cases, for two different class II polymerases [e.g., amatoxin **10** (Figure 5) and rat liver vs. *Drosophila*; Table II]. The enhanced inhibition which we have observed will need to be attributed to some combination of effects from the substituent

Table I: Inhibition Constants for Secondary Aminoamatoxins with Class II RNA Polymerases

secondary aminoamatoxin[b]	K_i (nm)[a] rat liver	sea urchin	*Droso-phila*	wheat germ
α-amanitin	10	15	30[d]	90
diethylamino (1)	49	31	100	790
morpholino (2)	11	20	24	650
N-benzyl-*N*-methylamino (3)	13	11	28	280
N,*N*-dibenzylamino (4)	14	22	30	300
4-phenylpiperidino (5)	26	44	34	480
4-benzylpiperidino (6)	11	41	51	560
7-iodo-α-amanitin		2[c]	1[d]	55

[a] Average K_i values (nM) determined for each amatoxin after a minimum of two experiments with duplicate assays of at least four amatoxin concentrations where P ranged from $0.1P_0$ to $0.9P_0$. P_0 = RNA polymerase activity when unhibited; P = RNA polymerase activity when inhibited. RNA polymerase activity determined with calf thymus DNA templated polymerization of [^{3}H]UTP (10^3 cpm/pmol); RNA polymerase concentrations $\leq 10^{-9}$ M in the assay as estimated from the assumption that the maximal specific activity is 500 pmol of UMP (μg of protein)$^{-1}$ (20-min assay)$^{-1}$ at 30 °C. [b] The 7′-amatoxin substituent is named and numbered to correspond with Figure 5. [c] Morris et al. (1978). [d] L.-T. Lin and P. W. Morris, unpublished results.

Table II: Inhibition Constants for Azoamatoxins with Class II RNA Polymerases

azoamatoxins[b]	K_i (nM)[a] rat liver	sea urchin	*Droso-phila*	wheat germ
α-amanitin	10	15	30	90
phenylazo (7)	12	14	70	80
(4-iodophenyl)azo (8)	70	32	20	170
(3,4-dichlorophenyl)-azo (9)	65	25	220	170
1-naphthylazo (10)	170	31	70	66
(4-carboxyphenyl)azo (11)	16	12	32	80
(4-sulfoxyphenyl)azo (12)	16	6	32	50
[4-(2-aminoethyl)-phenyl]azo (13)	38	63	100	100

[a] Average K_i values (nM) determined for each amatoxin from a minimum of two experiments with at least four amatoxin concentrations where P ranged from $0.1P_0$ to $0.9P_0$ (triplicate determinations at each concentration). Maximum range of experimental K_i determinations: ±35%. Enzyme activities determined and data calculated as in Table I. [b] The 7′-amatoxin substituent is named and numbered to correspond with Figure 5.

Table III: Inhibition Constants for Azoamatoxins with Class III RNA Polymerases

amatoxin[b]	K_i (μM)[a] rat liver	sea urchin	*Droso-phila*
amanitin	39	150	1000
phenylazo (7)	16	13	300
(4-iodophenyl)azo (8)	10		350
(3,4-dichlorophenyl)azo (9)	16		310
(4-carboxyphenyl)azo (11)	13		31
(4-sulfoxyphenyl)azo (12)	15		400
[4-(2-aminoethyl)phenyl]azo (13)	13		100
7′-iodo-α-amanitin		10	1000

[a] See footnote *a* to Table II. [b] The 7′-amatoxin substituent is named and numbered to correspond with Figure 5. Amatoxin 10 is not reported due to insufficient sets of data.

alone and the effects of the substituent on the amatoxin indole. An acceptable rationalization of our data and the alkylation

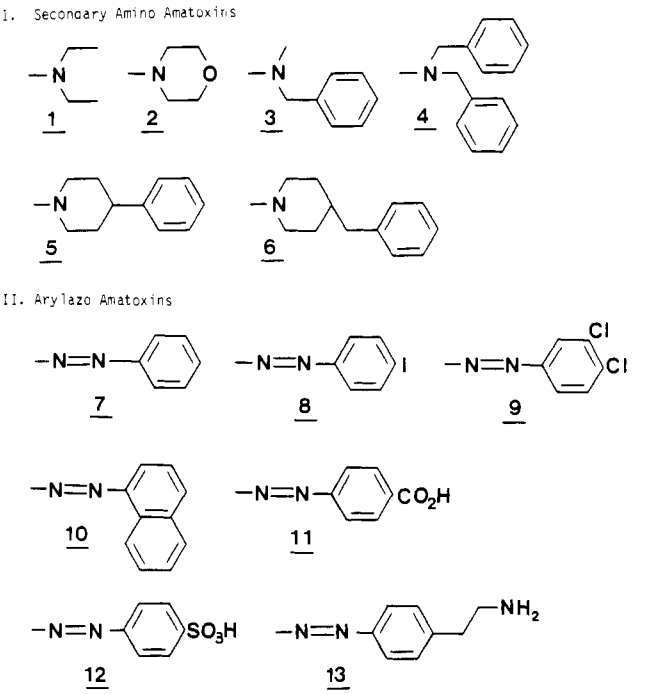

FIGURE 5: Substituents at the 7 position of tryptophan in α-amatoxins.

data (Faulstich et al., 1981) requires the assumption that the amatoxin indole is quite close to the RNA polymerase and may contribute to stabilization of the amatoxin–polymerase binary complex. In this respect we differ from the view expressed by Faulstich et al. (1981) that the indole nucleus is not part of the amatoxins binding site to the polymerase receptor. In any case the present range of modifications cover only three positions on the indole: the heterocyclic N atom (position 1) and the two neighboring carbocylic C atoms (positions 6 and 7). Modifications at position 4 or 5 are not yet reported, and these will have to be considered in order to obtain full information about the amatoxin binding site around the indole.

Despite the uncertainty in the question of amatoxin indole–polymerase proximity, we now have a rationale to follow in amatoxin modification for devising site-specific probes of the eukaryotic RNA polymerase. In addition to substitutions at the indole C-7 position which we have prepared and the alkylation at the indole C-6 hydroxyl (Faulstich et al., 1981), there is yet another site amenable to productive modification. Wieland & Fahrmeir (1970) have shown that IO_4^- cleavage of the vicinal glycol in the dihydroxyisoleucine residue yields a nontoxic amatoxin aldehyde (white mouse assay). The increased K_i was commensurate with the lack of toxicity. Reduction of the aldehyde with $NaBH_4$ yielded the alcohol with an apparent K_i 10-fold larger than that of the parent α-amanitin. We have confirmed these K_i observations by using wheat germ RNA polymerase and have shown, moreover, that the 2,4-dinitrophenylhydrazone of the amatoxin aldehyde exhibits an apparent K_i equal to that of the parent α-amanitin (R. McSwine and P. W. Morris, unpublished results). Reference to the crossbar of the T-shaped amatoxin conformation (Kostansek et al., 1978; vide supra) shows that the hydrazone is on the opposite side of the opposite arm relative to the indole C-7 position and, therefore, can probe a different region of the RNA polymerase.

One now has a reasonable expectation in the preparation of amatoxin based probes that will sample separate regions of the RNA polymerase. These probes, once prepared, can be expected to bind with useful affinity to a variety of class II RNA polymerases. If probes are based upon the arylazo

modifications, these probes may be useful not only for the class II RNA polymerase but also for the class III RNA polymerase.

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