Vol. 119, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 29, 1984 Pages 42-48

AFFINITY ISOLATION OF RNA POLYMERASE II ON AMANITIN-SEPHAROSE

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Received December 5, 1983

SUMMARY. We report here the first case of an affinity isolation of eukaryotic RNA polymerase II. The procedure employs an affinity matrix composed of α-amanitin coupled to Sepharose 4B via a ten atom spacer. RNA polymerase II from either calf thymus or wheat germ binds to the amanitin-Sepharose, as indicated by subsequent elution with sodium dodecylsulfate-containing buffer and analysis by polyacrylamide gel electrophoresis. The specificity of binding is demonstrated by the fact that when the enzyme is preincubated with 1 μg/ml of free α-amanitin, subsequent binding to the amanitin-Sepharose is abolished. Elution methods that should permit the recovery of active enzyme from the column are discussed.

A number of methods for the purification of RNA polymerase II (E.C. 2.7.7.6) have been described (for references see 1,2), all of which involve relatively standard ion exchange and gel permeation column chromatography. We report here the first example of an affinity isolation of RNA polymerase II. The affinity adsorbent is composed of Sepharose 4B coupled via a ten atom spacer arm (3) to α-amanitin, a fungal toxin which binds to RNA polymerase II with a high affinity [$K_d \sim 10^{-9}$M](4). We demonstrate that RNA polymerase II from both calf thymus and wheat germ will bind specifically to amanitin-Sepharose, and that the affinity adsorbent can be used to isolate the enzyme from a partially purified fraction.

MATERIALS AND METHODS

Amanitin-Sepharose: O-[5-[[(aminoethyl)-amino]carbonyl]-pent-1-yl]-α-amanitin was synthesized as described (3) and coupled to cyanogen-bromide-activated Sepharose 4B (Faulstich, Zobeley, and Trischmann, in preparation). The resultant affinity adsorbent contained 1 mg amanitin/ml Sepharose.

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ABBREVIATIONS: SDS, sodium dodecylsulfate

0006-291X/84 $1.50

Incubation conditions: Immediately before use the amanitin-Sepharose was washed with 100 volumes H_2O by centrifugation. A final buffer wash was then performed. The binding protocol used for the experimental results shown in Figure 1 is representative of the technique and will therefore be described in detail. Washed amanitin-Sepharose (50 µl packed volume) was added to 25 µg calf thymus RNA polymerase II (5) and 50 µg bovine serum albumin in a total volume of 150 µl of Buffer TA (50 mM Tris-Cl, 100 mM ammonium acetate, pH 7.9). Incubation was for 1 hr at 0°C, after which the resin was washed by centrifugation with two 150 µl aliquots of Buffer TA. The protein bound to the resin was then eluted by three 150 µl washes of 0.2% sodium dodecyl sulfate (SDS). The eluates were pooled and concentrated to about 50 µl by evaporation in a Savant Speed-Vac concentrator and made 10% in glycerol and 1% in 2-mercaptoethanol. The samples were then fractionated by SDS poly- acrylamide gel electrophoresis according to the method described by Laemmli (6). To test for specificity of binding, an identical polymerase sample was incubated (5 minutes, 0°C) with 1 µg/ml α-amanitin (Boehringer) before being added to the amanitin-Sepharose.

Partially purified wheat germ RNA polymerase II fractions were prepared as described (7). The procedure for binding to amanitin-Sepharose was similar to that described above except that the binding was carried out in TEDG buffer (50 mM Tris-HCl, (pH 7.9), 0.1 mM EDTA, 1 mM DTT, 25% (w/v) ethylene glycol) plus 250 mM ammonium sulfate. Also, following incubation the amanitin-Sepharose was washed with Buffer W (50 mM Tris HCl, pH 7.9), 0.1 mM EDTA, 1 mM DTT, 50% (w/v) ethylene glycol, 500 mM ammonium sulfate, 0.1% Brij 35) three times, using twenty times the packed volume of the Sepharose in each wash. The washing was completed with two washes of 1 mM Tris HCl (pH 7.9), after which the bound protein was eluted with 0.2% SDS as above.

RESULTS AND DISCUSSION

Figure 1 shows the results of an experiment in which an aliquot of purified calf thymus RNA polymerase II was gently shaken with an aliquot of amanitin-Sepharose, after which the adsorbent was washed several times with buffer and finally eluted with 0.2% sodium dodecyl sulfate (SDS). The first supernatant (containing the unadsorbed polymerase) as well as the SDS eluate were then electrophoresed in an SDS-containing polyacrylamide gel. In the gel shown in Figure 1 it can be seen that the channel to which was applied the eluate (Figure 1A) contains heavy RNA polymerase II bands, while the channel to which the first supernatant was applied (Figure 1B) contains considerably lighter RNA polymerase bands. Thus the bulk of the polymerase becomes bound to the amanitin-Sepharose, demonstrating that the binding of polymerase to the adsorbent is very efficient.

The results in Figures 1C and 1D establish that the polymerase is bound to the column specifically, i.e. via the attached amanitin. Here an incubation identical to that described above was performed, only this time

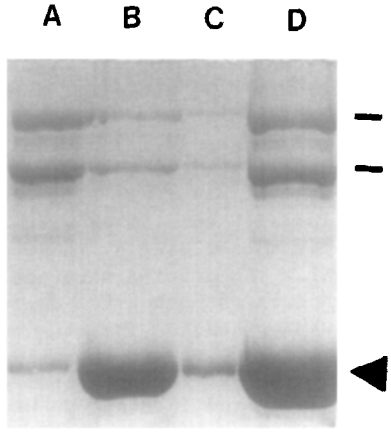

Figure 1. Fractionation of Purified Calf Thymus RNA Polymerase II on Amanitin-Sepharose. Purified calf thymus RNA polymerase II was preincubated with or without 1 μg/ml α-amanitin and then added to amanitin-Sepharose. After a further incubation, the amanitin-Sepharose was washed with Buffer TA. Bound protein was then eluted from the resin by washing with 0.2% SDS (see Materials and Methods for details). The entire first supernatant as well as the entire SDS eluate were concentrated and then subjected to electrophoresis on a 7.5% polyacrylamide gel containing SDS. The samples were: A. SDS eluate, preincubation with buffer only. B. First wash supernatant, preincubation with buffer only. C. SDS eluate, preincubation with 1 μg/ml α-amanitin. D. First wash supernatant, preincubation with 1 μg/ml α-amanitin. The lines at the right indicate the mobilities of the two largest subunits of calf thymus RNA polymerase II, while the triangle at the right indicates the mobility of the BSA added as a carrier.

the enzyme was preincubated with 1 μg/ml free amanitin. Now it can be seen that very little RNA polymerase II is bound to the adsorbent (pellet elution, Figure 1C) and virtually all of the polymerase is found in the supernatant (Figure 1D). In contrast, the BSA carrier protein remains in the supernatant regardless of preincubation conditions. Thus the results in Figure 1 establish that the amanitin-Sepharose can be used to efficiently and specifically isolate RNA polymerase II from a mixture of proteins.

The specificity of binding is further supported by the results in Figures 2 and 3. Figure 2 demonstrates that incubation of a sample of partially purified wheat germ RNA polymerase II (Fraction 4 of Jendrisak and Burgess (7), shown in Figure 2D) to amanitin-Sepharose and subsequent SDS elution of the washed resin results in an enzyme that is substantially purified as indicated by comparison to a reference enzyme preparation which was purified by the standard chromatographic method (Figure 2A). Again,

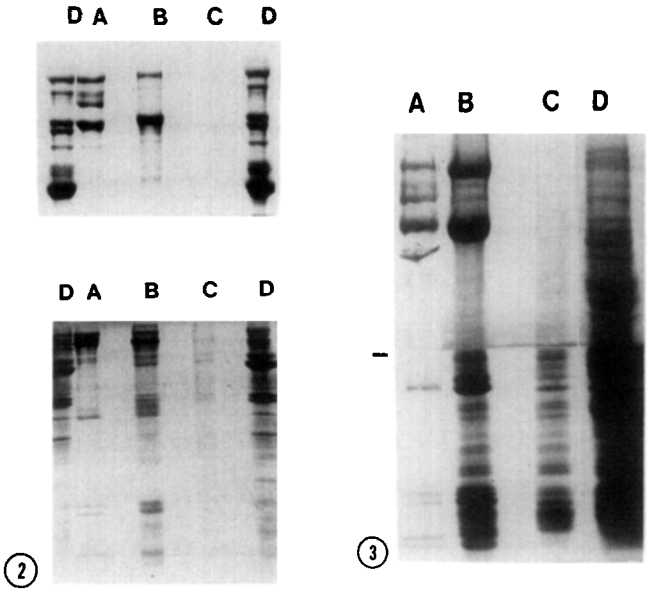

Figure 2. Isolation of Wheat Germ RNA Polymerase II from a Partially Purified Extract by Amanitin-Sepharose. Wheat germ RNA polymerase II was purified through Fraction 4 (Polymin P, $(NH_4)_2SO_4$ precipitation, and DEAE-cellulose chromatography) according to Jendrisak and Burgess (7).

Samples of Fraction 4 were incubated with amanitin-Sepharose with or without a preincubation with 1 µg/ml α-amanitin, and the resin was then washed with buffer and finally eluted with 0.2% SDS (see Materials and Methods). Samples were then subjected to electrophoresis in a 7.5% (upper panel) or 12.5% (lower panel) polyacrylamide gel in the presence of SDS. Samples are A. Wheat germ RNA polymerase II purified according to the method of Jendrisak and Burgess (7) (to serve as purified polymerase reference). B. SDS eluate from amanitin Sepharose where the original Fraction 4 was preincubated with buffer only. C. SDS eluate from amanitin-Sepharose where the original Fraction 4 was preincubated with 1 µg/ml α-amanitin. D. Starting Fraction 4.

Figure 3 Isolation Fractionation of Wheat Germ RNA Polymerase II from Fraction 3. Fraction 3 of Jendrisak and Burgess (7) was used instead of Fraction 4 as in Figure 2. Electrophoresis was carried out in a composite gel, 7.5% acrylamide on top and 12.5% acrylamide below. The bar to the left of the figure indicates the boundary between the two layers. Samples were A. Purified RNA polymerase II reference (c.f. Figure 2A). B. SDS elution from amanitin-Sepharose where the original Fraction 3 was preincubated with buffer only. C. SDS elution from amanitin-Sepharose where the original Fraction 3 was preincubated with 1µg/ml α-amanitin. D. Starting Fraction 3 (0.1% of the amount applied to amanitin-Sepharose).

preincubation of the Fraction 4 aliquot with 1 µg/ml α-amanitin abolishes polymerase bonding (Figure 2C).

Figure 3 shows the results of the use of a less purified fraction of wheat germ as a source of the RNA polymerase for affinity isolation on amanitin-Sepharose. In this experiment Fraction 3 of Jendrisak and Burgess (7) is used as start material. Fraction 3 is a wheat germ crude extract from which has been removed most of the chromatin by a Polymin P/precipitation-elution

step, and its protein content is shown in Figure 3D. Figure 3B shows that after incubating Fraction 3 with amanitin-Sepharose (essentially as described above), the SDS eluate is highly enriched for RNA polymerase. Again, all of the polymerase binding is abolished by preincubation of the Fraction 3 with 1 μg/ml amanitin (Figure 3C). Comparing densitometer traces of lanes B and C of Figure 3, the RNA polymerase II in the eluate can be estimated to be about 60% pure, representing a purification of about 100 fold (cf. reference 7). Figure 3 also shows that while the reference polymerase preparation (Figure 3A) purified by standard chromatographic techniques contains significant amounts of degradation products (8) of the largest subunit (the two bands immediately beneath the highest band in Figure 3A), the affinity-purified enzyme contains by comparison only minor amounts of these components.

The results in Figures 1, 2, and 3 demonstrate that amanitin-Sepharose can be employed as an efficient and specific affinity adsorbent for RNA polymerase II. This is the first description of an affinity chromatographic isolation of this enzyme. However, a significant problem with the use of the amanitin-Sepharose as described is the amount of non-specific binding, which is indicated by the amount of the protein eluted from the resin after incubation with a sample containing excess free amanitin (Figures 1C, 2C and 3C). The presence of this protein in the eluate is probably due to a large extent to the harsh non-specific elution conditions (0.2% SDS) used. It would clearly be preferable to use the classical affinity chromatographic method of specifically eluting with free ligand, i.e. excess α-amanitin. We have tried such a specific elution, but so far our attempts have not been successful. The likely explanation for this failure is the fact that the half time of release of α-amanitin from RNA polymerase II is on the order of days (4).

The search for a specific elution method has led us to investigate the use of photoelution. The plausibility of the photoelution method was

suggested by our earlier observation (9) that amanitin-inhibited RNA polymerase II can be photoreactivated by specifically destroying the amanitin *in situ* via exposure to monochromatic 314 nm irradiation, a wavelength which is absorbed by amanitin but not by polymerase. Preliminary results indicate that 314 nm irradiation of a slurry of amanitin-Sepharose carrying bound polymerase does indeed elute the polymerase. Such a photoelution technique has the following advantages: 1.) the elution method is specific for RNA polymerase II bound to amanitin, which should result in the eluted protein being substantially purer than that eluted by the nonspecific (0.2% SDS) method; 2.) the eluted protein should not only be structurally intact but enzymatically active (9) as well.

There are a number of immediate applications for the amanitin-Sepharose. It can be used to quickly isolate and identify the subunit composition of RNA polymerase II from tissues or cell lines for which the polymerase has not yet been characterized. This should be particularly effective if samples are applied with and without amanitin preincubation and the eluates analyzed by two dimensional electrophoresis (e.g. reference 8). The amanitin-Sepharose should also prove to be especially useful for rapid purification of RNA polymerase II, particularly when the photoelution method described above is perfected. Furthermore, studies which require that endogenous RNA polymerase II be specifically removed from an extract (10,11) so that defined polymerase can be added back (e.g. transcription studies) would benefit from this adsorbent.

Finally, we are employing the amanitin-Sepharose in our studies of transcribing chromatin. We are using it to develop a technique by which transcribing chromatin can be isolated by binding it to amanitin-Sepharose via its RNA polymerase II. Once isolated the transcribing chromatin will be characterized and compared with inactive chromatin. Thus amanitin-Sepharose should prove to be particularly useful in chromatin structure studies as well.

ACKNOWLEDGMENTS

This research was supported by grants from the National Institutes of Health (GM27117) and the American Cancer Society (JRFA-40, MV-185).

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