# *AZOTOBACTER VINELANDII RNA POLYMERASE, VII. ENZYME TRANSITIONS DURING UNPRIMED r[I-C] SYNTHESIS**

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*Abstract and Summary.*—Transitions in the state of RNA polymerase were demonstrated during the unprimed synthesis of the r[I-C] copolymer. No detectable change in the usual dimer-monomer pattern was noted during the lag phase (0–25 min at 37°) after analysis of the reaction mixture by acrylamide gel electrophoresis. At the end of the lag phase, a major alteration in the electrophoretic pattern occurred, marked by the disappearance of the dimer-monomer bands and the concomitant appearance of a series of monomer-r[I-C] copolymer complexes. As these complexes of r[I-C] copolymer with one or more polymerase monomer were formed, an enzymatically inactive component ($\gamma$ protein) of the polymerase was displaced. During the phase of rapid r[I-C] copolymer synthesis, the active form of the *A. vinelandii* RNA polymerase was the r[I-C] monomer lacking the $\gamma$ protein.

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RNA polymerase catalyzes two types of *in vitro* reactions, template-directed[1–5] and unprimed.[6–10] Unprimed reactions are characterized by a lag phase that varies in length according to the polymer being synthesized; unprimed synthesis of the r[I-C] copolymer begins after a lag period of about 30 minutes at 37°.[9] We have used acrylamide gel electrophoresis to look for transitions in the state of RNA polymerase during the course of the unprimed synthesis of the r[I-C] copolymer. At the end of the lag phase, the normal dimer–monomer[11] pattern shifts to a series of monomer–r[I-C] complexes. As the enzyme binds the newly formed polyribonucleotide, the $\gamma$ protein component of the polymerase is displaced. During the phase of active r[I-C] synthesis, the active form of the enzyme appears to be the r[I-C]–monomer complex which lacks the $\gamma$ protein.

*Materials and Methods.*—The $T_1$ RNase and ethidium bromide were obtained from Calbiochem; the ITP and CTP came from P. L. Biochemicals and Boehringer, Inc., respectively.

*A. vinelandii* RNA polymerase was prepared with the modification previously published,[11] and had a specific activity of 120. Acrylamide gel electrophoresis and the *in situ* polymerase assay were carried out by the method of Krakow *et al.*[11]

The unprimed synthesis of r[I-C] copolymer was carried out in the following reaction mixture: 0.05 *M* Tris buffer, pH 7.8; 0.01 *M* mercaptoethylamine; 2 m*M* $MnSO_4$; 0.5 m*M* CTP; 0.5 m*M* ITP; and 300 $\mu$g RNA polymerase per ml. The reactions were incubated at 37° for the times indicated, suitable aliquots were removed, and EDTA was added (final conc. 3 m*M*) to stop the reaction. The samples were placed in ice until needed for electrophoresis. 50-$\mu$l samples were analyzed by acrylamide gel electrophoresis (5% gels) at 3 ma per gel for 60 min with the system at about 10°.

The r[I-C] copolymer was prepared in an r[I-C]-directed reaction with the use of essentially the conditions listed above with the addition of 0.3 $OD_{253}$ units of r[I-C] copolymer in a 20-ml reaction volume. After 120 min at 37°, the reaction was terminated with the addition of 3 m*M* EDTA and then deproteinized with silicic acid by the method of Sueoka and Hardy.[12] The r[I-C] was precipitated with ethanol and further purified

by passage through a column of Sephadex G-75 equilibrated with 0.02 *M* Tris, pH 7.8, 0.001 *M* EDTA.

Synthesis of r[I-C] copolymer was followed fluorometrically by a minor modification of the method of LePecq and Paoletti:[13] 0.05 ml of the reaction mixture added to 0.95 ml of a solution containing 5 $\mu$g/ml ethidium bromide in 0.01 *M* Tris, pH 7.8, 0.05 *M* NaCl, 1 m*M* EDTA. The fluorescence of the r[I-C] ethidium bromide was measured in an Aminco-Bowman spectrofluorometer (excitation at 270 m$\mu$; emission measured at 570 m$\mu$).

*Results.*—The time course for r[I-C] synthesis is shown in Figure 1. The fluorometric assay is very sensitive and is especially useful in unprimed reactions, since there is no polymer present at the start of the reaction. There is a slight indication of r[I-C] synthesis at 25 minutes with synthesis apparent at 30 minutes; thereafter rapid polymerization takes place.

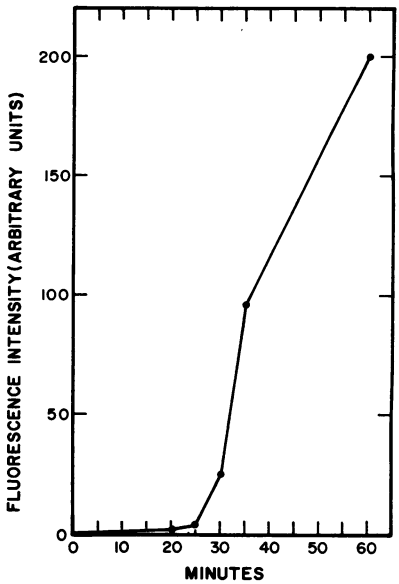


FIG. 1.—Time course of the unprimed synthesis of r[I-C]. At the times indicated, 0.05-ml aliquots were removed and r[I-C] was determined fluorometrically.

When aliquots of the reaction mixture are resolved by acrylamide gel electrophoresis, there is no obvious change in the dimer-monomer pattern up to 20 minutes' incubation (Fig. 2). At 25 minutes, a small band appears with a slower mobility than the dimer, and at 30 minutes a pronounced change in the pattern is seen. The original dimer–monomer pattern is lost and is replaced by a series of r[I-C]–monomer complexes. As RNA polymerase binds to the newly formed polyribonucleotide, a protein component is displaced and can be seen near the bottom of the gel (Fig. 2*d*). The $\gamma$ protein is displaced when the *A. vinelandii* RNA polymerase binds to a variety of polyribonucleotides or to denatured DNA.[14] The appearance of the $\gamma$ protein indicates that the protein bands which appear at the end of the lag phase are r[I-C]–monomer complexes. The simplest interpretation is that the fastest enzyme band is the one-monomer–r[I-C] complex and, in sequence, two-monomer–r[I-C], three-monomer–r[I-C], four-monomer–r[I-C], followed by a blurred enzyme–r[I-C] population. At the 30-minute interval, there is an excess of enzyme over r[I-C] copolymer; the number of mono-

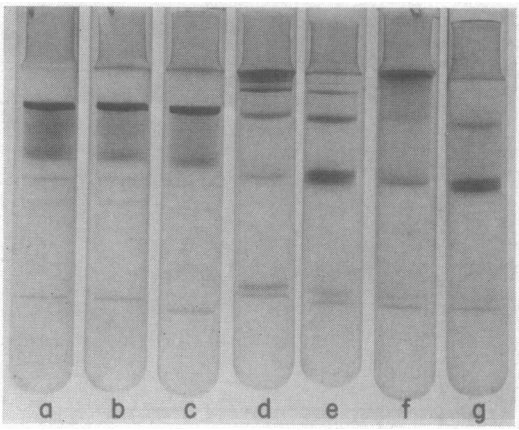


FIG. 2.—Polymerase transitions during unprimed r[I-C] synthesis. Each gel represents 0.05 ml taken from an unprimed reaction (as given in *Materials and Methods*) at the time indicated (see Fig. 1) and resolved electrophoretically. (*a*) 0 min; (*b*) 20 min; (*c*) 25 min; (*d*) 30 min; (*e*) 30-min sample plus 0.2 unit of $T_1$ RNase incubated an additional 5 min at 37°; (*f*) 5 min; and (*g*) 5-min sample treated with $T_1$ RNase as in (*e*).

mers bound to an r[I-C] molecule is limited by the size of the r[I-C] copolymer formed at this time, and the polymerase must be closely packed on the copolymer. When the 30-minute reaction mixture is treated with $T_1$ RNase prior to electrophoresis (Fig. 2*e*), the diffuse enzyme complexes at the top of the running gel disappear and are found predominantly in the monomer-r[I-C] region. The complexes of r[I-C] with two, three, and four monomer units are relatively resistant to hydrolysis by the nuclease, presumably because the tight packing of the enzyme-r[I-C] complex protects the bound r[I-C]. The sharply delineated appearance of these bands suggests a very homogenous size for the complexes. During the phase of active r[I-C] synthesis, the electrophoretic pattern (Fig. 2*f*) is smeared with the lower boundary at the one-monomer–r[I-C] position. The system can be considered one in which the r[I-C] is no longer limiting and no discrete three-monomer–r[I-C] or four-monomer–r[I-C] bands are seen. When this sample is treated with $T_1$ RNase prior to electrophoretic resolution (Fig. 2*g*), the resulting pattern represents monomer–r[I-C] and two-monomer–r[I-C] complexes. Under the conditions of saturating template, the monomers have distributed along the r[I-C] and the nuclease is able to cause extensive breakdown of the complexes.

To demonstrate that the various protein bands seen after electrophoresis actually represent enzymatically active RNA polymerase, a 30-minute sample was resolved and assayed for activity with the use of the *in situ* synthesis of r[I-C] copolymer.[11] Gels *a* and *c* (Fig. 3) were stained for protein; gels *b* and *d* show the pattern for synthesis of r[I-C] in the gels. The patterns shown in gels *c* and *d* were obtained after treatment with $T_1$ RNase. The patterns for enzyme activity are well matched with those of the stained protein bands. In addition, the $\gamma$-protein band is very easily seen and is not required for r[I-C] synthesis in the gel assay. After nuclease treatment, most of the protein and enzyme activity shifts to the one-monomer–r[I-C] position. No enzyme activity is associated with the $\gamma$-protein band.

When the system is assayed electrophoretically during the phase of active r[I-C] synthesis, a smeared pattern with the lower boundary representing polymerase-monomer–r[I-C] complexes is obtained. At this stage in the reaction,

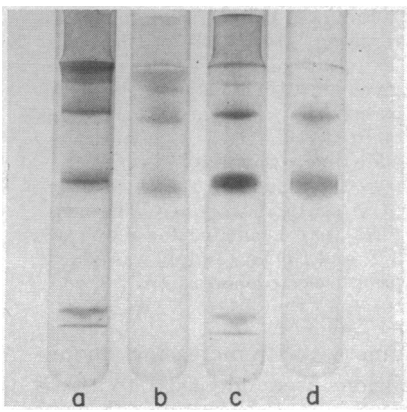


FIG. 3.—Correspondence between protein pattern and enzyme activity. Each gel represents 0.05 ml taken from an unprimed r[I-C] reaction after 30 min at 37°. (*a*) Stained for protein; (*b*) *in situ* assay for r[I-C] synthesis; (*c*) stained for protein after treatment with $T_1$ RNase as in Fig. 2, gel (*e*); and (*d*) *in situ* assay for r[I-C] synthesis after $T_1$ RNase treatment.

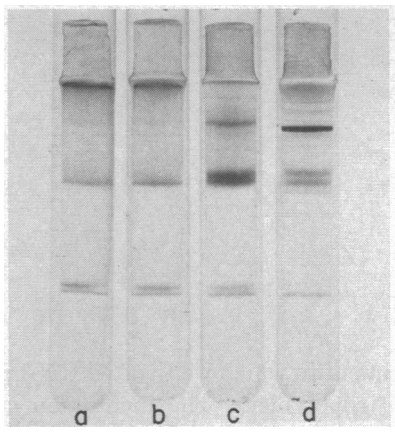


FIG. 4.—Re-formation of polymerase dimer after $T_1$ RNase treatment. Each gel represents 0.05 ml taken from an unprimed r[I-C] reaction after 60 min at 37°. All gels are stained for protein. (*a*) No further incubation; (*b*) incubated 20 min at 37°; (*c*) additional 0.5 unit $T_1$ RNase incubated 5 min at 37°; and (*d*) additional 0.5 unit $T_1$ RNase incubated 20 min at 37°.

the system is template-directed by the r[I-C] synthesized during the unprimed phase. The smeared patterns shown in Figure 4 represent polymerase monomer lacking the $\gamma$ protein, bound to r[I-C] of varying size, and also that may be considered "polypolymerase"—where more than one monomer is bound to r[I-C] during synthesis. By treatment with $T_1$ RNase for longer periods, it is possible to re-form the polymerase dimer. Gel *a* in Figure 4 is the gel obtained from the 45-minute reaction without nuclease treatment. Gel *b* was incubated for 20 minutes at 37° in the absence of nuclease, and there was no major change in the pattern. If $T_1$ RNase is added and the samples are incubated for 5 minutes (gel *c*), the pattern shifts to predominantly the one-monomer–r[I-C] complex; at 20 minutes (gel *d*), the polymerase dimer is re-formed. Concomitant with the reconstitution of the dimer band is the disappearance of the $\gamma$-protein band from its position at the bottom of the gel, presumably by its reinsertion into the dimer complex.

We have interpreted the results shown in Figure 2 as the consequence of an "autotitration" where the newly synthesized r[I-C] copolymer is the limiting component at the end of the lag phase. The high ratio of polymerase to copolymer results in extensive packing of enzyme monomers onto the available r[I-C] copolymer. This shows up as discrete bands of polymerase–r[I-C] after electrophoretic resolution and represents the transition from an unprimed reaction to a template-directed reaction. We have found that the r[I-C] copolymer isolated from unprimed reactions will serve as a template for the synthesis of r[I-C] copolymer or [GrC] copolymer.[15] In Figure 5 are shown the results of an

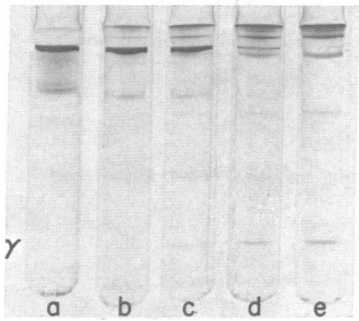


FIG. 5.—Polymerase transitions resulting from r[I-C] titration. The conditions were similar to those given in *Materials and Methods*, except that ITP and CTP were omitted and r[I-C] copolymer was added per 0.05 ml as follows: (*a*) None added; (*b*) 1 μg; (*c*) 2 μg; (*d*) 3 μg; and (*e*) 4 μg. The mixtures were incubated for 2 min at 37°, and EDTA (3 mM) was added prior to electrophoresis.

experiment in which we titrated RNA polymerase with increasing amounts of the r[I-C] copolymer. The incubation conditions were identical to those of the unprimed reaction, except that ITP and CTP were omitted. The electrophoretic analysis shows a transition from the normal dimer-monomer pattern to a series of monomer-r[I-C] complexes similar to those which appear during r[I-C] synthesis in the unprimed reaction. The release of the γ protein as the monomer-template complexes are formed is very readily demonstrated and parallels the loss of the polymerase dimer band during the titration. The pattern shown in Figure 5*d* is of interest in that one can see the residual dimer band with the two-monomer–r[I-C] band just below it.

*Discussion.*—During the course of the unprimed synthesis of the r[I-C] copolymer, a marked alteration in the state of RNA polymerase occurs at the time corresponding to the end of the lag phase. During the period up to 20 minutes' incubation, the usual dimer-monomer pattern is seen, with no obvious difference in pattern detectable by acrylamide gel electrophoresis. At the 30-minute interval, a radical shift in the pattern is evinced with a decrease in the dimer and monomer bands; concomitant with this is the appearance of enzyme-r[I-C] complexes. At this time, another protein (γ protein) appears, which is characterized by a high mobility on electrophoresis and a lack of enzyme activity. The γ protein is also displaced[14] when the *A. vinelandii* RNA polymerase binds poly A, poly C, poly U, and denatured DNA or tRNA; but not native DNA, dAT copoly mer, or d[I-C][16] copolymer. When the template r[I-C] is removed by $T_1$ RNase digestion, the free γ-protein band disappears as the enzyme dimer is re-formed. During the template-directed phase, the active form of the enzyme appears to be the monomer-r[I-C] complex. This is in keeping with the conclusion reached by Smith *et al.*,[17] who showed that *E. coli* RNA polymerase-oligodeoxynucleotide complexes existed primarily as the monomer form of the enzyme.

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