The Reversible Denaturation of Deoxyribonucleic Acid-dependent Ribonucleic Acid Polymerase of *Escherichia coli* *\( \textbf{SUMMARY} \)

RNA polymerase core and holoenzyme isolated from *Escherichia coli* have been denatured and reconstituted in high yield (70 to 100%). Ultracentrifugation studies and measurement of tryptophan fluorescence indicate that the reconstituted enzyme has a structure similar to that of the native enzyme. The reconstituted holoenzyme transcribes T7 DNA as efficiently as the native holoenzyme. RNA synthesized by the reconstituted holoenzyme from T7 DNA is transcribed from the biologically correct heavy strand and is similar if not identical with that synthesized by the native holoenzyme in terms of size and ratio of ATP/GTP termini of RNA chains. Under low salt conditions, the reconstituted holoenzyme is capable of interacting with factor \( \rho \) leading to chain termination.

**MATERIALS AND METHODS**

Tritium-labeled nucleotides were obtained from New England Nuclear Corp. Unlabeled ribonucleoside triphosphates and ultrapure guanidine hydrochloride, urea, Tris, and succrose were obtained from Sigma Chemical Co. and Miles Laboratories, respectively. Purified \( \rho \) factor, \( \alpha \text{32P} \text{ATP} \) and \( \beta \text{32P} \text{CTP} \) were kind gifts of Mr. Probir Sarkar and Dr. Umadas Maitra of this Department. T7 DNA was prepared from purified T7 phage essentially as described by Sadowski (28).

RNA polymerase activity was measured in reaction mixtures (0.25 ml) containing 0.05 M Tris, pH 7.8, 0.01 M MgCl\(_2\), 10\(^{-4}\) M di-

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thioguanosine, 4000 mU/thioguanosine per mg of protein in the presence of 0.1 M NaCl. The fractions containing enzyme were pooled and precipitated by ethanol. The enzyme was then chromatographed on a column of agarose A-5m in 1 M Tris, pH 8, 10% EDTA, 1 M KCl, 50% glycerol, and 0.5 M MgCl2. The fractions containing enzyme of high specific activity were pooled, precipitated with saturated neutralized (NH4)2SO4, and dialyzed against 0.05 M Tris, pH 8, 10% EDTA, 1 M KCl, 50% glycerol, and 0.5 M MgCl2. The fractions containing enzyme of high specific activity were then stored at -20°C in 0.05 M Tris, pH 8, 10% dithiothreitol, 10% EDTA, and 0.2 M KCl for at least 96 h before use.

RESULTS

Dialysis of a solution of E. coli RNA polymerase against concentrated solutions of urea or guanidine hydrochloride results in the complete loss of ribonucleotide-incorporating activity as measured with either poly[d(A-T)] or T7 DNA as template. When denatured holoenzyme was diluted (100-fold) into renaturation buffer at 23°C, a time-dependent reactivation of both T7 and poly[d(A-T)]-directed activities was observed (Fig. 1). Both activities were restored at roughly the same rate and to approximately the same extent (50 to 60%). However, when denatured holoenzyme was diluted into renaturation buffer at 23°C and immediately placed in ice, no T7 DNA-directed activity was observed, even after prolonged incubation (12 h). There was, however, a slow return of poly[d(A-T)]-directed activity under these conditions. When the enzyme incubated at 3-4°C was further incubated at 23°C there was a rapid increase.

The abbreviations used are: SSC buffer, 0.15 M sodium chloride-0.015 M sodium citrate; H and L strands, heavy and light strands, respectively.
in both T7 and poly[d(A-T)]-directed activity. After 2 to 3 hours at 23° the activity regained was equivalent to that of the sample incubated only at 23°.

Enzymatic activity was also recovered by dialysis against reconstitution buffer. Dialysis of either core or holoenzyme for 2 hours against 8 M urea followed by dialysis against reconstitution buffer for 3 hours at 23° and subsequent dialysis for 2 to 12 hours at 3-4° resulted in the regaining of 50 to 100% of the initial enzymatic activity (Table I). The recovery of activity was not markedly dependent upon protein concentration, although recovery at protein concentrations greater than 1 mg per ml was lower.

When core or holoenzyme, denatured by urea, was stored in the denaturant (3-4° or higher) for periods of more than 6 to 8 hours there was a considerable decrease in the activity recovered (Table I). This loss occurred both in solutions of urea and guanidine (data not shown). After 7 days, only 1 to 3% of the initial activity was recovered. Dialysis against freshly prepared urea solutions containing dithiothreitol prior to reconstitution had no effect on recovery nor did the addition of 10^{-4} M ZnSO₄ to the reconstitution buffer. This irreversible loss of activity could be prevented by reaction of denatured enzyme (immediately following denaturation) with potassium tetrathionate (Table I). Such "blocked" enzyme could be stored for as long as 2 weeks with no loss in its ability to undergo reconstitution; recoveries usually ranged from 35 to 50% of the initial activity.

The hydrodynamic properties of reconstituted holoenzyme are similar to those of the native holoenzyme. Sedimentation coefficients were found in 0.2 M KCl for reconstituted and native holoenzyme preparations, respectively. There did not appear to be significant material (<20%) sedimenting at higher or lower s values than the native enzyme.

Fluorescence spectra are known to be sensitive indicators of protein conformation (18, 32, 33). The fluorescence emission spectrum of core enzyme in 8 M urea is shown in Fig. 2, Curve A. A maximum (λₘₐₓ) was observed at approximately 352 nm. When denatured enzyme was diluted into reconstitution buffer (100-fold dilution) and the spectrum again determined (within 1 min of mixing), a shift of λₘₐₓ from 352 to 338 nm was observed (Curve B). Further small changes occurred in the intensity (2 to 3%) which were complete by 5 min, after which no further changes were observed (Curve C). The spectrum of the reconstituted enzyme after 90 min is almost identical to that of native core enzyme (Curve D). Circular dichroism studies also indicated that major chain reordering, as measured by spectral changes in the region 200 to 290 nm, were essentially complete within 1 min (data not shown). It should be noted that although changes in fluorescence and circular dichroism were complete by 5 min, very little enzymatic activity was observed at this time.

Native core enzyme transcribes T7 DNA inefficiently (2-4, 34). Addition of the σ subunit leads to a 5- to 20-fold increase in the efficiency of synthesis (2-4, 34). The effects of the σ subunit on transcription of T7 DNA by native and reconstituted core enzyme are shown in Fig. 3. Addition of the σ subunit resulted in an 8- to 10-fold stimulation of activity with both

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**Table I**

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Activity with T7 DNA at time in 8 M urea (hr)</th>
<th>Activity with poly[d(A-T)] at time in 8 M urea (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td>24</td>
</tr>
<tr>
<td>A. Holoenzyme (μg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>140</td>
<td>90</td>
<td>63</td>
</tr>
<tr>
<td>500</td>
<td>80</td>
<td>47</td>
</tr>
<tr>
<td>600*</td>
<td>53</td>
<td>31</td>
</tr>
<tr>
<td>1400</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| B. Core enzyme (μg/ml) |    |    |    |    |    |    |
| 28                   | 61  |    |    |    |    |    |
| 70                   | 72  |    |    |    |    |    |
| 140                  | 66  | 21 | 15 |    |    |    |
| 280                  | 102 | 35 | 12 |    |    |    |
| 1400                 | 50  | 35 | 15 |    |    |    |

* Enzyme preparations treated with potassium tetrathionate.

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native and reconstituted core enzyme. Thus, the core enzyme is capable of regaining the conformation necessary to interact with the σ subunit in the absence of appreciable quantities of this subunit during reconstitution.

The termination factor ρ described by Roberts (35) has been shown to decrease both the extent of synthesis and the size of RNA products (35-38) synthesized by RNA polymerase with a variety of DNA templates if synthesis is performed at low ionic strength (T/2 < 0.05). Goldberg and Hurwitz (38) have provided evidence which indicates that ρ, acting catalytically, exerts its effect through interaction with the polymerase-template complex. The effects of increasing concentration of ρ on RNA synthesis with T7 DNA as primer catalyzed by native and reconstituted holoenzyme are shown in Fig. 4. Both native and reconstituted enzyme were inhibited approximately 50% by 0.05 μg of ρ factor. No further inhibition was observed with higher concentrations of ρ.

Translation of T7 DNA by native holoenzyme in 0.05 or 0.2 M KCl yields large RNA species (1.5 to 2.2 X 10^4 daltons) (51, 59). If translation is performed at low ionic strength (0.05 M KCl) and in the presence of saturating amounts of ρ, RNA produced is considerably smaller (38). T7 DNA-directed RNA, synthesized by native and reconstituted holoenzyme in 0.2 M KCl, was examined by sucrose density gradient centrifugation in sodium dodecyl sulfate-formaldehyde (Fig. 5A). The products were almost identical. When transcription was performed in the presence of 0.01 M KCl, and saturating amounts of ρ, the product size was considerably reduced (Fig. 5B).
Table II

Initiation of RNA chains directed by T7 DNA with native and reconstituted holoenzyme

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Nucleotide incorporation</th>
<th>Nucleotide incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native holoenzyme</td>
<td>2.18</td>
<td>1.03</td>
</tr>
<tr>
<td>Reconstituted holoenzyme</td>
<td>1.71</td>
<td>0.93</td>
</tr>
</tbody>
</table>

Synthesis was performed in 0.01 M KCl in the absence of factor ρ, the products were comparable in size to those formed in 0.2 M KCl. Thus ρ caused chain termination with both native and reconstituted holoenzyme.

RNA chains formed in the presence of T7 DNA by native holoenzyme are initiated with ATP and GTP in the ratio of 2:1 (34). In contrast, RNA chains formed with core enzyme contain predominantly GTP termini (34). The incorporation of [γ-32P]ATP and [γ-32P]GTP into RNA chains synthesized by native and reconstituted holoenzyme with T7 DNA is shown in Table II. Both enzyme preparations incorporated [γ-32P]ATP and [γ-32P]GTP in the ratio 2:1 as observed by Chamberlin and Ring (34). The reconstituted holoenzyme was approximately 80% as efficient as the native holoenzyme in terms of both [3H]-AMP incorporation and incorporation of γ-labeled nucleoside triphosphates. The relative size of RNA products calculated from total termini and nucleotide incorporated into RNA with each enzyme preparation was similar (4400 to 4500 nucleotides).

During in vivo transcription of T7 DNA in infected E. coli, more than 99% of the RNA is synthesized from the H strand (30). Some workers have reported that 90 to 98% of the RNA made in vitro by E. coli holoenzyme is also transcribed from the H strand (34, 40, 41). Dunn et al. (42), however, have reported considerable transcription from the L strand. They also reported that factor p increased the asymmetry of transcription of T3 and T7 DNA (42). The hybridization of RNA products made from native T7 DNA by native and reconstituted holoenzyme to separated strands of T7 DNA is shown in Table III. Less than 2% of the RNA made with either enzyme preparation hybridized to the L strand, while over 96% hybridized with the H strand. Considerable asymmetry of transcription was also observed with denatured T7 DNA as template.

Table III

Hybridization of RNA synthesized upon T7 DNA by native and reconstituted holoenzyme

<table>
<thead>
<tr>
<th>RNA product formed with</th>
<th>Input RNA hybridized to</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>H strand</td>
</tr>
<tr>
<td>Native holoenzyme</td>
<td>96</td>
</tr>
<tr>
<td>Reconstituted holoenzyme</td>
<td>98</td>
</tr>
<tr>
<td>Native holoenzyme (heat-denatured T7 DNA)</td>
<td>61</td>
</tr>
<tr>
<td>Native holoenzyme (heat- + alkali-denatured T7 DNA)</td>
<td>62</td>
</tr>
</tbody>
</table>

Over 60% of the RNA transcribed from denatured T7 DNA by native holoenzyme hybridized to the H strand while 16 to 17% hybridized to the L strand.

Discussion

Time-dependent inactivation of one or more of the subunits of RNA polymerase stored in concentrated urea solutions has been previously reported (26). Ito and Ishihama (43) attributed the inactivation to reaction of the B′ subunit with cyanate in urea solutions and have shown that the reconstitution of the enzyme can be inhibited by cyanate. The inactivation which we have observed is apparently of a different nature. Since we find that the loss occurs in both urea and guanidine hydrochloride solutions the inactivation we observe most likely cannot be attributed to reaction with cyanate. Moreover, it is an irreversible inactivation since treatment with high concentrations of thiol reagents (prior to reconstitution) does not result in increased yields of reconstituted enzyme. It appears likely that the loss does involve sulfhydryl residues since it can be prevented by treatment of the denatured enzyme with tetrathionate, a reagent which reacts reversibly with sulfhydryl residues (44). It seems possible that the inactivation involves some covalent alteration of one or more of the subunits, perhaps of the type which Tanford (10) noted has been observed by Zahn et al. (45).

It has been suggested that the σ subunit must be present during reconstitution of RNA polymerase if efficient reconstitution is to occur (24, 46). Under the conditions which we have used for reconstitution, we observe no requirement for appreciable quantities of this subunit since the core enzyme is reconstituted in good yield (60 to 100%). Moreover, reconstituted core enzyme is as capable of being stimulated by σ subunit as the native

* Transcription of denatured T3 DNA by the native holoenzyme yields similar asymmetric transcription (P. R. Chakraborty and U. Maitra, personal communication).
core enzyme. Thus the refolding of the subunits (α, β, and β') and their subsequent association shows no requirement for participation of the sigma subunit. It is also apparent that sigma subunit can be denatured and reconstituted since the reconstituted holoenzyme transcribes T7 DNA efficiently. The reconstituted holoenzyme can initiate and terminate RNA chains as well as select the proper DNA strand for transcription. C. B. (1971) J. Mol. Biol. 60, 499

The participation of the sigma subunit. It is also apparent that 12. IXAI, A., FISH, W., AND TANFORD, C. (1973) J. Mol. Biol. 73, 145


It appears likely that the formation of active enzyme from subunits occurs much more rapidly in vivo than in vitro. For example, Kepes (47) has estimated that the synthesis and folding of β-galactosidase does not require more than 3 to 4 min. The rates observed for in vitro refolding of denatured enzymes has ranged from 5 min to several hours (17, 18). In vivo, it would seem likely that folding, especially for large polypeptides would begin before the synthesis of the polypeptide is completed. Certain intermediate conformational states, not on the proper pathway of folding, might thus be excluded resulting in a more rapid activation in vivo than in vitro (12, 18, 48).

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The Reversible Denaturation of Deoxyribonucleic Acid-dependent Ribonucleic Acid Polymerase of *Escherichia coli*
Lynwood R. Yarbrough and Jerard Hurwitz


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