DISSOCIATION AND RECONSTITUTION OF ACTIVE DNA-DEPENDENT RNA-POLYMERASE FROM *E. COLI*

V.SAGAR SETHI and W.ZILLIG
with technical assistance of H.BAUER
Max-Planck-Institut für Biochemie, München, Germany

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1. Introduction

DNA-dependent RNA-polymerase from *E.coli* consists of 4–5 subunits, namely β' (MW ~ 160,000), β (~ 145,000), σ (~ 85,000), α (~ 40,000) and may be ω (~ 12,000) [1, 2]. These have been separated by column chromatography on Sephadex G-200 in 1% SDS and on DEAE-cellulose in 8 M urea [2] as well as by preparative electrophoresis on cellulose acetate blocks in 6 M urea [1]. Attempts to reconstitute active enzyme from these subunits have been unsuccessful so far. Ishihama and Hurwitz [3] reported a partial recovery of enzymatic activity after incubation with DNA of fragments which had been obtained by the dissociation of enzyme at low urea concentrations.

Recently we reported the dissociation of enzyme into its subunits in 4 M lithium chloride and showed that the isolated sigma subunit was fully active in stimulating the activity of the core enzyme [4]. In this paper we report the reconstitution of the active enzyme from isolated subunits as well as the specific binding of the $\beta'\beta$-fraction to ^{3}H-labelled T4-DNA.

2. Materials and methods

The purification and assay of DNA-dependent RNA-polymerase from *E.coli* K12 Hfr (λ) followed procedures described in [5, 6]; the sigma subunit and core enzyme were prepared by cellulose phosphate chromatography [7]. The α-subunit was isolated from the core enzyme by sucrose gradient centrifugation in 3.5–4 M LiCl [4]. ^{14}C-Labelled ATP was from Amersham, Radiochemical Centre, England; other substrates were from C.F.Boehringer u. Söhne, Mannheim; LiCl p.A. from E.Merck, Darmstadt. TMA buffer, pH 7.3, contains (mM) 10 tris-acetate, 10 Mg-acetate, 22 NH_4Cl, 0.25 EDTA and 0.1 mercaptoethanol. Polyacrylamide gel electrophoresis was performed in gels containing 7.5% acrylamide, 0.1% sodium dodecylsulfate (SDS), 6 M urea and 0.4 M tris-HCl pH 9.5. Protein was determined as described by Lowry et al. [8]. The filter binding test of the enzyme and its subunits to ^{3}H-labelled T4-DNA was according to Jones and Berg [9] using MF 14 filters (Sartorius, Göttingen).

3. Results and discussion

3.1. *Reconstitution of enzyme activity from the subunits of RNA-polymerase*

A number of consecutive steps are involved in the synthesis of RNA by DNA-dependent RNA-polymerase. In order to reconstitute active enzyme it appears advantageous to start from native subunits; therefore conditions should be chosen which yield subunits in the native state. For this reason we investigated the kinetics of the loss of enzyme activity of the full and core enzyme at different LiCl concentrations (fig. 1). The diagram clearly shows that there is a sharp decline in the activity between 2.5 and 3 M LiCl. Below 1 M LiCl no dissociation of enzyme was observed; thus LiCl concentrations of 2.75 to 3.0 M was chosen for the separation of subunits used in reconstitution experiments. A further protection of the subunits against inactivation is obtained by the

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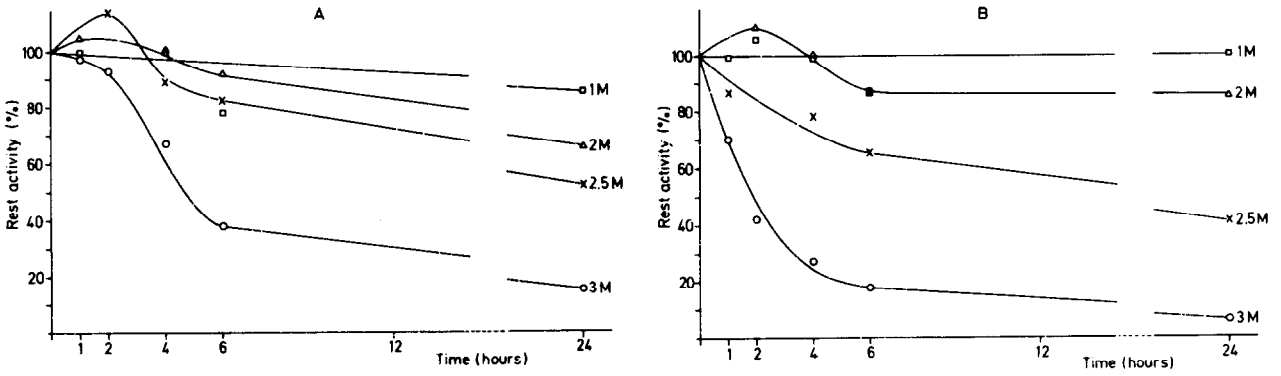

Fig. 1. Kinetics of the loss of enzyme activity of RNA-polymerase (A) and core enzyme (B) at different lithium chloride concentrations at 4°. 1 mg/ml of the enzyme in TMA buffer containing 10% sucrose and 5% glycerol was incubated with varying amounts of lithium chloride. 5 μl of the reaction mixture was taken at different times and the enzyme activity was measured after incubation in the assay mixture (total volume = 0.250 ml) for 15 min at 37°. The activity at 0 min was taken to be 100. In case of core enzyme, 1 μg of σ was added in each test.

addition of glycerol and sucrose to the dissociation mixture.

In order to obtain the subunits in the native state, sucrose gradient centrifugation of the full and core enzyme was carried out in 2.75 to 3 M LiCl for 15 hr at 4° according to [4]. The fractions collected from the heavy side of the protein peak (H) in polyacrylamide gel electrophoresis show β' and β and a low amount of α, whereas the fractions collected from the middle (M) show β' and β with more α; the light side of the peak contains only α. In case of complete enzyme σ distributes between both heavier fractions (fig. 2). Pure α can be better separated from $\beta+\beta'$ on a sucrose gradient containing 3.5–4 M LiCl [4].

The H fraction can be stimulated from AMP incorporation by the addition of pure α (fig. 3(A)). The α-subunit alone is inactive and the activity of $H+\alpha$ is inhibited by actinomycin D. A plateau is reached with an amount of α slightly higher than that stoichiometrically required. The plateau activity corresponds to about 25% of the activity of a comparable amount of full enzyme. Fig. 3(B) shows the kinetics of the RNA synthesis by H and by $H+\alpha$. The fraction M, which contains and excess of α, also strongly stimulates H. An α-free $\beta'\beta$ fraction isolated from a sucrose gradient containing 3 or more molar LiCl, is not activated by the addition of α. Since α and σ subunits isolated in a sucrose gradient even at 4 M LiCl are active in stimulating the

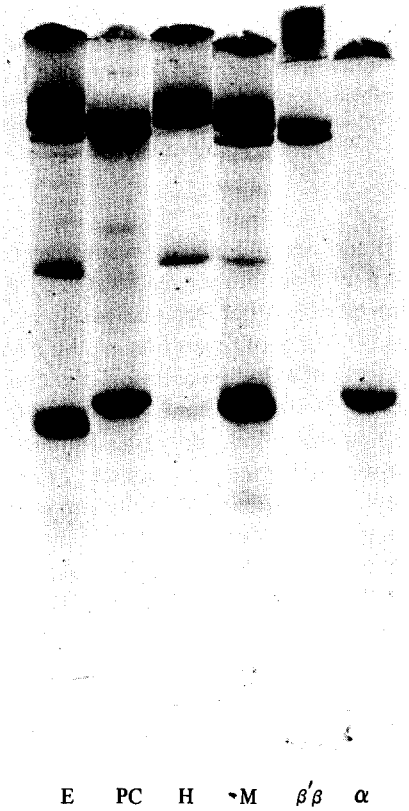

Fig. 2. Polyacrylamide gel electrophoresis of full enzyme (E), core enzyme (PC) and fractions H, M, $\beta\beta'$, α in 6 M urea and 0.1% SDS. H and M are the heavy and middle fractions obtained from sucrose gradient centrifugation run in 2.75 M LiCl of complete enzyme at 50,000 rpm for 15 hr at 4° according to [4]

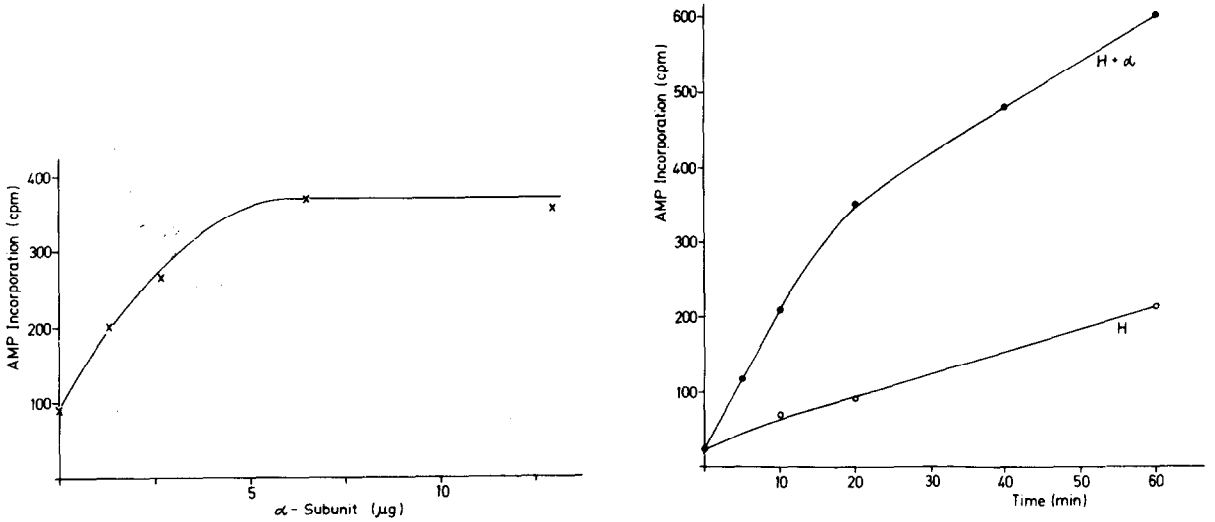

Fig. 3. (A). *Activation of fractions* H *by* α. 9 μg of fraction H was incubated with varying amounts of α in the assay mixture for 20 min at 37°. With actinomycin D no incorporation of AMP was obtained. In case of core enzyme each assay mixture contained 2 μg of σ subunit. α or σ do not incorporate AMP in the assay. (B). *Kinetics of the AMP incorporation of fraction* H *and* H *with saturating amounts of* α *at* 37°. 2.0 ml of the incubation mixture contained 72 μg of fraction H and the same amount of fractions H and 45 μg of α. 0.25 ml of the reaction mixture were taken at different time.

fraction H and the core enzyme respectively, it is clear that β′ or β or both are inactivated by the complete removal of α either instantaneously or at least faster than the time of separation on the gradient. The activation of H by the addition of α could then be due either to the presence in this fraction of a complex $\beta'\beta\alpha$ which lacks only one α-subunit and is comparatively stable or to the content of not yet inactivated β′ and β-subunits. In the latter case the residual activity of H would be due to the presence of some $\beta'\beta\alpha_2$ besides $\beta'\beta$, in the first case to the formation of $\beta'\beta\alpha_2$ by the redistribution of α. Both possibilities can be described by the assumption of LiCl concentration dependent equilibria

$$\beta'\beta\alpha_2 \rightleftharpoons \beta'\beta\alpha + \alpha \rightleftharpoons \beta'\beta + 2\alpha$$

which would also explain that the subunits and especially α do not form distinct peaks on the gradient at intermediate LiCl concentrations (2.75–3 M).

3.2. *Binding of* ^{3}H-*labelled T4-DNA with RNA-polymerase and its subunits*

A simple test for the binding of RNA-polymerase to native DNA was developed by Jones and Berg [9] based on the finding that native DNA and the enzyme are not retained separately on nitrocellulose membrane filters, whereas a mixture of both is retained quantitatively. This technique has been applied to test the binding of the enzyme and its subunits to ^{3}H-labelled T4-DNA. The results are presented in fig. 4. The number of molecules of the core enzyme (PC), the $\beta'\beta$ fraction, the complete enzyme (E) and the α-subunit causing a 50% retention of 2 μg of T4-DNA are 1 X, 1 X, 5 X, and 90 X 10^{12} respectively; the σ subunit does not bind to DNA. The values for the $\beta'\beta$ fraction and the PC enzyme are very close to each other showing clearly that $\beta'\beta$ has the same affinity to the DNA as the core enzyme whereas the α-subunit binds 90 times less efficiently. The fact that σ, which reduces the affinity of the core enzyme-DNA complex to the filter [10] also

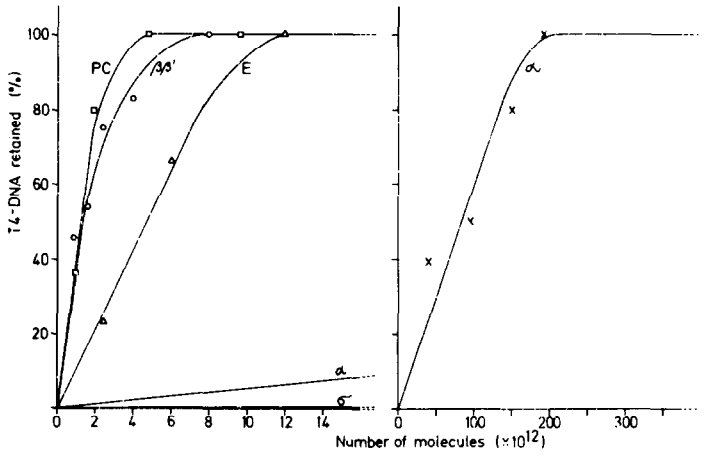

Fig. 4. Retention of ^{3}H-labelled T4-DNA by RNA-polymerase and its subunits on nitrocellulose filters. Reaction mixtures of 0.250 ml contained 2 μg of ^{3}H-labelled T4-DNA (approximately 3000 cpm), and different amounts of the enzyme and subunits in TMA buffer pH 7.3. They were incubated for 5 min at 37°, diluted with 2 ml of cold 0.01 M tris-HCl buffer pH 8.0 containing 0.05 NaCl and 200 μg/ml bovine serum albumin; filtered through MF 14 membrane filters, washed with 40 ml of the same buffer and counted in Packard liquid scintillation spectrometer. Assuming the stoichiometry and molecular weight of complete enzyme ($\beta'\beta\alpha_2\sigma \sim 500{,}000$), core enzyme ($\beta'\beta\alpha_2 \sim 400{,}000$), $\beta'\beta$ ($\sim 300{,}000$) and α ($\sim 40{,}000$) [1, 7], the number of molecules of the respective entities retained with 2 μg of T4-DNA on the filter were calculated. The $\beta'\beta$ was isolated from the core enzyme on a sucrose gradient containing 3–3.5 LiCl; this fraction had no activity of its own in the enzyme test and was not stimulated by α.

diminishes the affinity of the $\beta'\beta$-DNA complex to the same extent is a further support for the assumption that the retention of both complexes is due to the same specific protein DNA interaction. Therefore $\beta'\beta$ which cannot be stimulated for AMP incorporation is still active at least in this partial function. These results support the hypothesis that β' is the binding subunit of the enzyme to the DNA which has been proposed on the basis of other findings [1].

Acknowledgements

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