RECONSTITUTION OF BACTERIAL DNA-DEPENDENT RNA-POLYMERASE FROM ISOLATED SUBUNITS AS A TOOL FOR THE ELUCIDATION OF THE ROLE OF THE SUBUNITS IN TRANSCRIPTION*

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1. Introduction

The role of subunits β', β and α of the core enzyme of bacterial DNA-dependent RNA-polymerase in the transcription process has been investigated by testing if isolated subunits suffice for the performance of partial functions [1–4]. This procedure cannot be applied to functions which depend on previous events in which other subunits participate. In such cases it appeared possible to study the properties of mixed reconstituates of a subunit with a defined functional alteration with the other subunits from normal enzyme.

This approach requires the availability of dissociation and separation techniques yielding native subunits or of a reconstitution procedure allowing renaturation of denatured subunits. As shown by Sethi the dissociation by LiCl in moderate concentration leads to subunits, which can be recombined directly to form active enzyme [2, 3]. Since the LiCl-method yields only two fractions α and $\beta + \beta'$ and the reconstitution efficiency is low, we investigated the possibility to obtain active enzyme from subunits obtained by dissociation with urea and separated by electrophoresis on cellulose acetate sheets [4]. This attempt was stimulated by the finding of Lill and Hartmann [5], that enzyme inactivated by urea in dithiothreitol can be reactivated.

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2. Experimental

2.1. *Dissociation and separation technique*

Minimal or full enzyme was dissociated by dialysis against a buffer containing 0.01 M tris acetate, 0.01 M magnesium acetate, 0.022 M NH_4Cl, 0.02 M β-mercaptoethanol, 6 M urea, 20% (v/v) glycerol adjusted to pH 8. The dissociation products (20 to 50 μg of protein/ml) were separated by electrophoresis on cellulose acetate sheets or blocks (Cellogel strips or blocks, Chemetron, Milano) in the following way (fig. 1): The carrier material was thoroughly equilibrated with a buffer containing 0.5 M tris acetate, 0.01 M magnesium acetate, 0.02 M β-mercaptoethanol, 0.001 M EDTA, 20% (v/v) glycerol and 6 M urea adjusted to pH 8. The strips (or blocks) were positioned, with the shiny (impermeable) side down, onto the surface of a cooled brass electrophoresis chamber covered with a teflon sheet (H. Hölzel, Dorfen). Excess buffer was removed by careful blotting with filter paper. The sample (up to 20 μl over a width of 4 cm on the analytical strips and up to 1.0 ml over a width of 6 cm on the blocks) was applied with a soft tipped polyethylene pipette. The strips (or blocks) were then connected to the electrode vessels by filter paper (or cardbord) bridges, the (moist) chamber was closed with a tight fitting cover just not touching the strips and electrophoretic separation was performed on the strips at 1000 to 1200 V (~ 60 V per cm), 10–20 mA per strip for 1.5 to 2 hr or on the blocks at maximally 400 V, allowing not more than 100 mA per block, for 10 hr. Bands were visualized by printing the edges of the sheets with dry cellulose acetate strips (Selecta,

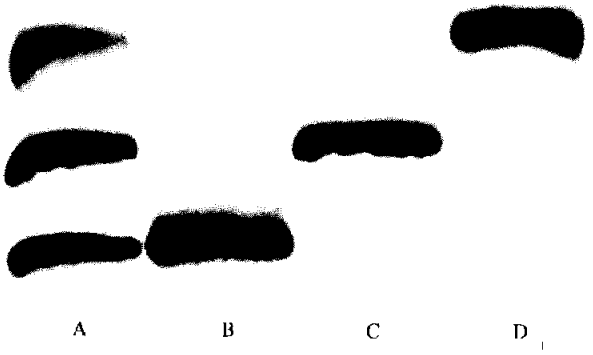

Fig. 1. Comparison of minimal enzyme with separated subunits β', β and α by cellogel electrophoresis on analytical strips. A = minimal enzyme; B = β'; C = β; D = α. Separation conditions and procedure for separation of subunits as described in the text.

Schleicher u. Schüll) staining the prints with 0.24% amidoschwarz in 45% methanol, 45% water, 10% glacial acetic acid for 5 min and destaining in the same solvent without the dye. Segments containing the subunits were excised and positioned into polyethylene funnels with narrow tips adapted to centrifugation tubes (Amicon) and then submitted to centrifugation in a Servall centrifuge at 20,000 rpm for 5 min. More than 95% of the buffer containing more than 50% of the subunits was liberated in this way. The protein content of these solutions was estimated by a micro procedure specifically developed for this purpose: 1 to 5 μl of the solutions (containing 0.5 to 5 μg of protein) were pipetted onto dry cellulose acetate strips (Selecta, Schleicher u. Schüll). The sheets were dried, stained for 10 min with amidoschwarz, then destained, as described above and dried again. The blue spots were excised and dissolved in 0.5 ml of 80% glacial acetic acid, 10% formic acid, 10% water, 1% TCA. The extinction was measured at 630 nm and compared to that of albumin standards treated in the same way. This method allows a rather accurate estimation of down to 0.25 μg of protein also from solutions yielding a high background in the Lowry reaction. The possible inaccuracy introduced by differences of the binding capacities for amidoschwarz can be eliminated by proper standardization and is negligible for our purpose.

Table 1
Reconstitution of DNA-dependent RNA-polymerase from separated subunits.

Minimal enzyme or equivalent amount of subunit	mU./mg
Experiment 1:	
Original enzyme	124
Subunit present during dialysis	
$\alpha_S + \sigma$	1.6
$\beta_S + \sigma$	3.2
$\beta'_S + \sigma$	1.7
$\alpha_S + \beta_S + \beta'_S + \sigma$	127*
$\alpha_S + \beta_S + \beta'_S - \sigma$	7.0
Combined after dialysis	
$\alpha_S + \beta_S + \beta'_S + \sigma$	25.6
Experiment 2:	
Original enzyme	242
Subunit present during dialysis	
$\alpha + \sigma$	1.9
$\beta + \sigma$	1.4
$\beta' + \sigma$	2.9
$\alpha + \beta + \beta' + \sigma$	74**

The techniques for preparation of subunits and for reconstitution were as described in the text. *Experiment* 1: 1.15 μg α, 2.3 μg β, 2.3. μg β' and 1.15 μg σ; *Experiment* 2: 1.85 μg α, 3.7 μg β, 3.7 μg β' and 1.85 μg σ were present in 50 μl of reconstituate solution tested in a 0.25 ml incubation volume as described [6].

* 100% reconstitution of original activity.
** 31% reconstitution of original activity.

2.2. *Reconstitution technique*

For reconstitution the subunit solutions were mixed in stoichiometric ratio, an equivalent amount of σ was added and the mixture was dialysed over night at 0° against a buffer containing 0.05 M tris acetate, 0.01 M magnesium acetate, 0.001 M EDTA, 0.15 M NH_4Cl, 20% (v/v) glycerol, 0.02 M β-mercaptoethanol, adjusted to pH 8.0 and containing a small amount of silicon antifoam (Wacker) to avoid surface denaturation. After 30 to 60 min at room temperature [5] it was tested for enzyme activity [6] either directly, or after concentration by precipitation with ammonium sulfate at 55% saturation.

The separation of subunits is more efficient at

pH 9 in the absence of magnesium ions. Subunits prepared by separation in a buffer as that described above but free of magnesium acetate and adjusted to pH 9 still yielded active enzyme. In this case there was, however, no clear requirement for the presence of σ during codialysis.

3. Results and discussion

3.1. *Requirements for reconstitution*

As shown in table 1, the yield of reconstituted enzyme, expressed in mU./mg ranges from 20 to 100%. Codialysis of the separated subunits is essential. When the subunits are dialysed singly or in groups of 2 and combined after dialysis, only a small fraction of the activity is recovered. The activity obtained by codialysis is 20 to 40 fold higher than the sum of the background activities of singly dialysed subunits which probably is due to incomplete separation. For a high yield reconstitution, the presence of σ during dialysis is required. Since σ still stimulated minimal enzyme even when obtained from full enzyme by urea dissociation, cellogel electrophoresis and acetone precipitation this can be explained by assuming that its structure is very rigid and thus provides a nucleus for the reestablishment of correct conformation.

The reconstituted enzyme is indistinguishable from the original polymerase in sucrose gradient centrifugation as well as in cellogel electrophoresis in urea neutral buffer.

3.2. *Mixed reconstitution and the role of subunit β in transcription*

It had been shown by D. Rabussay, that out of five rifampicin resistant RNA-polymerases from *E. coli* mutants one contained a β-subunit which in cellogel electrophoresis exhibited a mobility different from that of wild type β [7]. The possibility of a chance coincidence has been excluded by the finding, that radioactive rifampicin in a sucrose gradient exclusively cosediments with partially renatured β [4, 8]. Since thus the subunit responsible for rifampicin action is known, this case appeared particularly suited for testing the validity of our mixed reconstitution technique.

In table 2, the specific activities of reconstituates prepared from subunits obtained from wild type and rifampicin resistant enzyme (*E. coli* B, a gift of Wehrli and Knüsel) tested both in the absence and in the presence of rifampicin are listed. The reconstituate from the subunits of sensitive enzyme is fully sensitive, that from the subunits of resistant enzyme is fully resistant. Of the three mixed reconstituates formed from either one of the subunits of the resistant enzyme with the others from normal enzyme, those containing β from sensitive enzyme are fully sensitive, that containing β from resistant enzyme is resistant. This is the expected proof for the conclusion that β is the subunit responsible for the interaction with rifampicin and thus is involved in the initiation step specifically inhibited by the drug.

Table 3 lists the specific activities of all possible reconstituates obtained from subunits of normal enzyme and of a partially streptolydigin resistant polymerase prepared from an *E. coli* mutant (RFS 528), kindly donated by Schleif [9], again in the absence and in the presence of the drug. It is clearly seen that whenever β is from normal enzyme and no matter from where the other subunits are taken the reconstituate is fully sensitive to the drug. When on the other hand β comes from the partially resistant enzyme, the residual activity characteristic for this enzyme at the chosen streptolydigin concentration is observed without any influence of the origin of the other subunits. The same result was obtained with another streptolydigin resistant mutant, RFS 529. Thus streptolydigin like rifampicin exerts its action on polymerase over the β subunit. Therefore strains producing enzymes resistant to either of both drugs are β mutants. This explains the closeness of the genetic mapping positions for both features [9, 10]. Since streptolydigin specifically blocks translocation, β is involved in this partial function as it is in initiation. It had been shown by other methods, that β also contains the binding sites for α and for σ [1, 4]. Thus β exhibits multiple functions.

This approach should allow a simple and fast correlation of genetic mapping positions of mutants to the different peptide chains and also of the subunits to their functions if the functional alterations of the mutants are known.

Table 2
Mixed reconstitution of RNA-polymerase from separated subunits of rifampicin sensitive and rifampicin resistant enzyme.

	Reconstituted minimal enzyme (mU./mg)		
	Rifampicin	+ Rifampicin (100 μg/ml)	% Resistant activity
Original sensitive enzyme	242	1.5	0.6
Original resistant enzyme	124	120	97
Subunits present during dialysis			
$\alpha + \beta + \beta' + \sigma$	52	1.4	2.0
$\alpha_r + \beta_r + \beta'_r + \sigma$	27	25.6	95
$\alpha_r + \beta + \beta' + \sigma$	40	0.6	1.5
$\alpha + \beta_r + \beta' + \sigma$	88	69	78
$\alpha + \beta + \beta'_r + \sigma$	17.5	1.4	8.0

Preparation of subunits, reconstitution technique and test (with 0.45 μg α, α_r; 0.9 μg β, β_r; 0.9 μg β', β'_r in 25 μl/0.25 ml test volume) as described in table 1. Subunits with the index *r* are derived from resistant enzyme.

Table 3
Mixed reconstitution of RNA-polymerase from separated subunits of streptolydigin sensitive and resistant enzyme.

Subunits present during dialysis	Reconst. (mU./mg) Streptolydigin (4 mM)		% Resist. activity	Reconst. (mU./mg) Streptolydigin (1mM)		% Resist. activity
	−	+		−	+	
$\alpha + \beta + \beta' + \sigma$	65	0.78	1.2	84	1.8	2.1
$\alpha_s + \beta_s + \beta'_s + \sigma$	112	13.6	12.1	123	35.2	29.0
$\alpha_s + \beta + \beta' + \sigma$	29	0.52	1.8	36	1.1	3.0
$\alpha + \beta_s + \beta' + \sigma$	84	10.9	13.0	86	26	30.2
$\alpha + \beta + \beta'_s + \sigma$	103	1.25	1.2	113	4.2	3.6
$\alpha + \beta_s + \beta'_s + \sigma$	135	19.2	14.2	132	38	29.0
$\alpha_s + \beta + \beta'_s + \sigma$	58	1.1	1.9	75	1.2	1.6
$\alpha_s + \beta_s + \beta' \sigma$	33	3.5	10.6	31	11.5	36.6

Conditions as described in table 1: 1.9 μg α, α_s; 3.8 μg β, β_s and β', β'_s and 1.9 μg σ in 5 μl (conc. by $(NH_4)_2SO_4$ precipitation)/0.25 ml test volume. Subunits with the index *s* are derived from resistant enzyme.

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