Purification and Properties of the RNA Polymerase-Template Complex of an Influenza Virus

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The enzyme-template complex of influenza RNA polymerase (fowl plague virus) was purified 200-fold. The sole virus component found in this preparation was RNP-antigen. All attempts to remove the internal template led to an irreversible loss of enzyme activity. The complex was essentially free of nucleases. It synthesized exclusively viral minus strand RNA and was unable to initiate more than one cycle of RNA synthesis. The lag phase at the beginning of RNA synthesis *in vitro*, present in crude enzyme preparations, was abolished with the purified complex. The enzyme was sensitive to sulfhydryl reagents and it was able to accept α-S-ATP in place of ATP.

Introduction

After infection of chick embryo cells with influenza A viruses, an RNA-dependent RNA-polymerase is induced[1–5]. The enzyme is found mainly in the microsomal fraction of the cell. Although attempts have been reported to purify the enzyme from cells infected with fowl plague virus[3], data are not available concerning the specific activity, yield of enzyme activity, or properties of the enzyme.

In this paper we will present a method developed for the purification of the enzyme-template complex and some information on its properties.

Material and Methods

Preparation of a microsomal fraction from cells infected by fowl plague virus

The Rostock strain of fowl plague virus, an influenza A virus, was used throughout. Chick embryo cells were prepared from 11 day old embryos by trypsinization and were used after removal of the enzyme at a cell density of $2 \cdot 10^6$ cells/ml at 34 °C in suspension according to Zwartow and Algar[6]. They were infected with an input multiplicity of about 5 plaque forming units/cell. Seven hours after infection, when cell associated material had an hemagglutination titer on the average of 2^{-10}, the cells were harvested and the microsomal fraction was prepared as described[2]. In later experiments cell-monolayers prepared in large roller flasks (7 cm in diameter and 35 cm in length, 20 ml medium/flask) were used and harvested 6 hours after infection. In the latter instance enzyme purification was more efficient.

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Polymerase test

20 µl of the sample were added to 20 µl of a mixture containing 5 mM creatine phosphate, 0.05 µg creatine phosphokinase, 1 mM of each of the three non-labelled nucleoside triphosphates, 8 mM $MgCl_2$, 100 mM KCl, 10 mM mercaptoethanol, 66 mM tris-HCl buffer, pH 8.0, and 10 µCi of [^{3}H] GTP. The reaction mixture was incubated at 32 °C or 28 °C, as indicated by the legends in the Figs and Tables. The reaction was stopped by cooling the reaction tubes to 0 °C and the addition of carrier protein and 6% trichloroacetic acid (TCA). After several washings with TCA, containing 0,1 M pyrophosphate, the samples were digested with 0.2 M NaOH and counted in a Packard Tricarb scintillation counter as described[7].

During purification several fractions were also tested using homopolymeres as external templates. In those instances the labelled complementary nucleoside triphosphate, without the other non-labelled nucleoside triphosphates was added to the assays.

Hybridization of viral RNA

The hybridization procedure was followed as described[7]. After incubating 0.5 ml of the purified polymerase-template complex with [^{3}H] GTP and cofactors for 30 min at 32 °C, the labelled RNA was extracted twice with phenol plus SDS, was precipitated with two volumes of ethanol, and was dissolved in 2 ml of 0.3 M NaCl containing 0.03 M sodium citrate (2 · SSC). Aliquots of 0.2 ml were used for the hybridization, as described in the legend of Table III. After addition of non-labelled plus-strand RNA the tubes were sealed and heated over night at 65 °C. If not otherwise stated, the samples were digested with 0.1 mg/ml of pancreatic RNase at 20 °C for

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20 min. After addition of carrier protein the TCA-insoluble material was worked up and counted as described above.

Other analyses

The hemagglutination test was performed according to DAVENPORT *et al.*[8]; RNP-antigen was determined according to BECHT[9]. Protein was measured according to LOWRY *et al.*[10]. Neuraminidase activity was determined according to DRZENIEK *et al.*[11].

Reagents

The following isotopically labelled compounds were used: [^{3}H] GTP (1.38 Ci/mM) from Schwarz BioResearch, Orangeburg, USA; [^{3}H] CTP (4.39 Ci/mM and [^{3}H] UTP 13 Ci/mM) from the Radiochemical Centre; Amersham, England.

The nucleoside triphosphates, the homopolymeres, and the ATP-regenerating system were obtained from Boehringer, Mannheim, Germany. Digitonin was obtained from Merck, Darmstadt, Germany. Polyethylene glycol (PEG) (6000) and dextran (500) were obtained from Mann, Orangeburg, USA, and Pharmacia, Uppsala, Sweden, respectively.

The α-S-ATP (adenosine-5'-triphosphorothioate)[12] was kindly supplied by Dr. F. ECKSTEIN, Max-Planck-Institut für Medizinische Forschung, Göttingen, Germany.

Results

A. Attempts to separate the enzyme from the internal template

For the purification of the influenza polymerase, column chromatography or procedures yielding large volumes could not be used, since the enzyme was immediately and irreversibly inactivated, if the protein concentration was below 0.5 mg/ml.

Since the enzyme template complex consists of two components of completely different properties, the first step in the purification should be the separation of the nucleic acid from the protein. The separation from the internal template is obtained, if the protein component polymerizes again nucleoside triphosphates only after the addition of exogenous template. The most promising technique, which was successful for the corresponding Qβ-system, is the two phase system used by EOYANG and AUGUST[13]. When this procedure was employed at low salt concentrations, the enzyme activity was found in the dextran phase. The internal template was not removed (Table I). After addition of 4 M NaCl to the system, as used with the Qβ-enzyme, the influenza polymerase activity could not be recovered at all, neither in the upper nor in the lower phase. Dialysis against standard buffer containing 50 mM tris-HCl-buffer, pH 8, 5 mM $MgCl_2$, 1 mM EDTA, and 5 mM 2-mercaptoethanol, and addition of influenza RNA or homopolymeres like poly-C, poly-A, or poly-I did not restore the enzyme activity. Probably the high concentration of salt was responsible for this effect, since 0.5 M KCl inhibits 80% of the enzyme activity. This loss of enzyme activity could be restored only partially by dialysis.

Table I. Distribution of polymerase activity between the two phases polyethylene glycol/dextran without NaCl. Microsomes were rehomogenized in standard buffer to give a concentration of 150 mg/ml protein. After treatment with digitonin (s. Table II. and text) and removal of the precipitate, 0.5 ml of the supernatant was mixed with 0.15 ml of a 30% PEG-solution in water and 0.12 ml of 20% dextran in water. After 10 min of stirring the two phases were separated by centrifugation and the enzyme activity was determined in both phases by incubation at 32° C for 10 min with [^{3}H] reaction mixture.

	Volume [ml]	dpm
supernatant	0.5	48000
upper phase (PEG)	0.4	490
lower phase (dextran)	0.3	52000

Removal of the internal template by protamin sulfate, the method used by HARUNA *et al.*[14] for the Qβ-enzyme, led to a quantitative precipitation of the influenza enzyme-template complex without inactivation and separation of the internal template. Streptomycin sulfate, which was used by CHAMBERLIN *et al.*[15] for the DNA-dependent RNA-polymerase of the T 7 phage, had the same effect in our system as protamin sulfate. This step can be used to remove internal RNase activity (see below).

B. Purification of the enzyme-template complex

Since methods for the separation of the internal template from the enzyme used in other viral RNA-polymerase systems were not applicable for the influenza enzyme, purification of the complex was tried as shown in Table II.

Compared with a crude cell extract the specific enzyme activity found in the microsomal fraction was increased by a factor of 7 to 10. About 75% of the total enzyme activity is present in this fraction[2].

The microsomes prepared from cells in monolayer harvested from 150 flasks were rehomogenized in 1.5 ml of standard buffer to give a protein concentration of 150 mg/ml. 0.5 ml of a digitonin solution (16 mg/ml) were added and the precipitate, containing mainly lipids, was removed by centrifugation

Tab. II. Purification of the enzyme-template complex. For experimental details see text. After precipitation with digitonin the supernatant was treated with streptomycin sulfate. The solubilized complex was layered on a sucrose gradient.

Sample	Vol. [ml]	Protein [mg/ml]	Dpm [20 μl]	HA-titer	RNP-antigen titer	Neuraminidase activity
microsomes plus digitonin	2	93.0	600,000			
supernatant after digitonin treatment	1.5	40.0	300,000			
fraction No. of the sucrose gradient						
1	1	1.08	6,430	2	128	
2	1	1.50	19,624	2	256	
3	1	1.20	33,000	<2	256	
4	1	0.80	69,000	<2	1,024	
5	1	1.20	91,200	<2	1,024	
6	1	0.75	116,000	<2	1,024	<0.5 μg/ml*
7	1	0.76	75,000	<2	512	
8	1	0.48	27,500	<2	512	
9	1	0.40	8,000	<2	256	
10	1	0.38	5,600	<2	128	
11	1	0.90	2,300	<2	128	
12	1	0.90	1,460	<2	128	
sediment after rehomogenization	1	2.00	10,000			
		sum	465,114			

*At equal protein concentration in cell homogenates the HA-titer was 2^{-10} and the neuraminidase activity was 725 μg/ml sialic acid liberated from sialolactose under standard conditions[11].

at 10 000 rpm for 10 min. In some experiments the supernatant was mixed with 0.02 M sodium acetate at pH 5. The enzyme-template complex was precipitated quantitatively and could be dissolved again in standard buffer without any loss in enzyme activity. This technique was found particularly useful for concentrating the enzyme-template complex.

After addition of streptomycin sulfate (final concentration of 1%) the complex was precipitated quantitatively. Enzyme activity could not be recovered from the supernatant after centrifugation at 10 000 rpm for 10 min, neither with virus RNA nor with homopolymeres as external templates. Sometimes difficulties were encountered to dissolve the precipitated complex. In those cases the addition of 0.2 to 0.4 M KCl was necessary, although some enzyme activity was lost by this procedure. The enzyme complex (20-30 mg protein/ml) was layered onto a 5 to 20% linear sucrose gradient and the tubes were centrifuged for 120 min at 40 000 rpm in an SW 41 swinging bucked rotor. The distribution of the enzyme activity in the fractions of the gradient collected are shown in Table II. The maximal purification of the fraction with the highest enzyme activity was 200-fold.

As shown in Table II, the fractions of the gradient were tested also for other virus-specific proteins. It can be seen that primarily the RNP-antigen could be detected in significant levels in the enzyme complex.

C. Kinetics of RNA-synthesis

In Fig. 1 incorporation of [^{3}H] GTP using the purified enzyme complex is shown. At 32 °C an incorporation of the labelled precursor into viral RNA proceeded for about 120 min. No loss of label was observed during incubation for additional 3 hours. Pulses given at different times after the onset of polymerization (Fig. 1) revealed that synthesis of viral RNA ceased almost completely after 2 hours of the start of incubation. This suggests that [^{3}H] GTP incorporation is not a net result of synthesis and breakdown of RNA. Addition of new enzyme complex 2 hours after the start of incubation led to

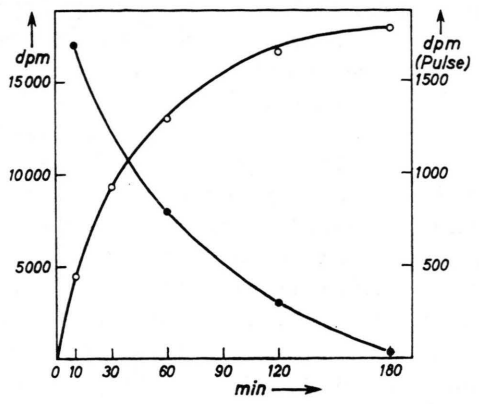

Fig. 1. Incorporation of [^{3}H] GTP into virus-specific RNA *in vitro*. Fractions 5 to 7 of a sucrose gradient (s. Table II) were pooled. The standard polymerase test was performed using incubation times at 32 °C as indicated on the abscissa (total incorporation, ○—○). The purified polymerase was incubated with reaction mixture containing instead of [^{3}H] GTP the same concentration of non-labelled GTP. At the times indicated on the abscissa 10 μCi [^{3}H] GTP was added to aliquots and the incubation at 32 °C was continued for 5 min. Thereafter the samples were processed (= pulse, ●—●).

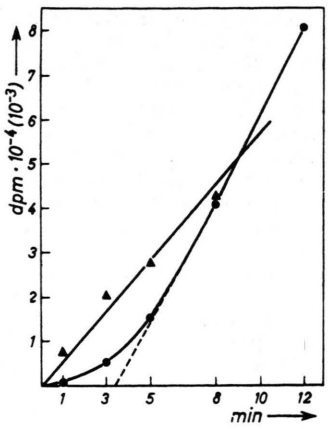

Fig. 2. Disappearance of the lag phase after purification of the enzyme-template complex. Either a microsomal fraction or the pooled fractions 5, 6 and 7 of a sucrose gradient (s. Table II) were prewarmed to 28 °C and incubated at this temperature with [^{3}H] reaction mixture. At the times indicated on the abscissa aliquots were removed and processed. ▲—▲ = purified enzyme-template complex (dpm · 10^{-3}). ●—● = crude microsomal fraction (dpm · 10^{-4}).

further incorporation of [^{3}H] GTP into RNA with normal kinetics (not shown here). This indicates that cessation of incorporation 2 hours after the onset of incubation is not due to lack of substrate.

In crude enzyme preparations at relatively low temperatures, a consistent but pronounced lag phase of incorporation *in vitro* was found[16]. On the other hand with the purified enzyme complex [^{3}H] GTP incorporation was linear from the beginning (Fig. 2).

D. The product of the enzyme reaction in vitro

With a crude enzyme preparation, it was reported that the *in vitro* synthesized RNA had a base sequence complementary to the RNA isolated from virus particles[7]. Subsequently conflicting reports appeared concerning the nature of the *in vitro* synthesized RNA[4,5]. We, therefore, reexamined the product formed by the purified enzyme complex by the hybridization technique. As shown in Table III, the *in vitro* product has a base sequence exclusively complementary to viral RNA.

By sucrose gradient centrifugation the newly synthesized RNA was found to have a sedimentation coefficient of 10 to 11S, which is in agreement with the finding of others[3].

Table III. Hybridization of the *in vitro* synthesized virus-specific RNA. RNA was synthesized with a purified enzyme-template complex as described in *Materials and Methods*. Aliquots of 0.2 ml were hybridized with increasing doses of virus particle RNA (0.7 mg/ml) at 65 °C over night and digested by RNase[7].

Virus particle RNA added [μl]	dpm in RNA	Remarks
25	14 570	no RNase (= total RNA)
25	14 600	
10	15 000	
1	14 800	
0	720	self-annealed
0	760	not heated to 65 °C

E. Involvement of sulfhydryl groups in the enzyme reaction

As shown in Table IV, the enzyme activity can be inhibited by *p*-chloromercuribenzoic acid (PCMB). This effect can be reversed, at least partially, by the addition of mercaptoethanol.

F. Replacement of ATP by α-S-ATP

When ATP was substituted by α-S-ATP the enzyme activity was reduced but not abolished (Fig. 3). Thus the enzyme is able to accept the sulfur derivative of ATP as substrate.

Table IV. Effect of *p*-chloromercuribenzoic acid on the polymerase. A microsomal fraction after treatment with digitonin (s. Table II) was used for the test. To 20 μl of the enzyme preparation 1 mM PCMB as indicated in the table was added. After 10 min at room temperature the sample was either mixed with [^{3}H] reaction mixture or with 5% mercaptoethanol as indicated. In the latter case the [^{3}H] reaction mixture (20 μl) was added 5 min later. After 10 min at 32° C the samples were processed.

μl added	dpm
none	87 600
1 μl mercaptoethanol	85 300
10 μl mercaptoethanol	86 300
1 μl PCMB	3 880
1 μl PCMB, after 5 min 1 μl mercaptoethanol	36 000
1 μl PCMB, after 5 min 10 μl mercaptoethanol	40 000

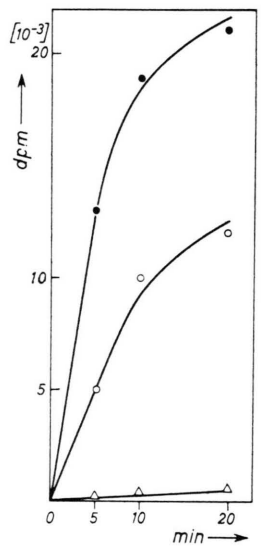

Fig. 3. Influence of α-S-ATP on the incorporation of [^{3}H] GTP into virus-specific RNA. Fractions 5, 6 and 7 of the sucrose gradient (s. Table II) were pooled. Aliquots were incubated with [^{3}H] raction mixture lacking the ATP-regenerating system (●—●); or with a [^{3}H] mixture in which ATP was replaced by α-S-ATP (○—○); or with a [^{3}H] mixture lacking ATP (△—△). The two latter mixtures also did not contain an ATP-regenerating system. Aliquots were removed and processed at the times as indicated on the abscissa.

Discussion

The only RNA-dependent RNA polymerase which can be obtained free of internal template and which is able to synthesize infectious RNA *in vitro* is that of the phage Qβ[17]. This enzyme is highly specific for its template, which cannot be replaced by any other naturally occurring RNA. This specificity has been postulated to be necessary for the biosynthesis of the viral RNA in the infected cell in the presence of so many different species of other RNA molecules[14].

Many unsuccessful attempts have been made to purify corresponding enzymes from cells infected by animal RNA-containing viruses [3,18-20]. In a series of experiments with the RNA polymerase of fowl plague virus, we were unable to remove the internal template without an irreversible loss of enzyme activity. Thus the enzyme-template complex of animal viruses seems to be relatively stable, and the question was raised whether a similar stability also exists *in vivo*. The failure of rescue to occur *in vivo* among different influenza strains, probably at the level of exchange between polymerase and template, supports the latter hypothesis[21].

A 200-fold purification of the enzyme-template complex has been achieved. The purified complex *in vitro* synthesized exclusively an RNA complementary to the RNA isolated from virus particles. The incorporation curve of [^{3}H] GTP indicates (Fig. 1), that the complex is not able to reinitiate RNA synthesis. The product is stable during prolonged incubation. Thus the preparation is essentially free of RNases.

The lag phase of RNA synthesis consistently observed with crude enzyme preparations at relatively low temperatures[16] was absent if purified enzyme complex was used. Thus protein(s) which might have regulatory functions have been removed during one of the purification steps. It should be mentioned that the purified enzyme complex loses its activity within a few hours, while in the crude complex the template seems to be rather protected. Crude preparations can be stored in the refrigerator for a week without significant loss of enzyme activity.

The enzyme activity can be inhibited reversibly by PCMB. Thus free sulfhydryl groups are necessary for enzyme activity.

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Note added in proof: The immediate *in vitro* product synthesized by the purified polymerase-template complex is up to 70% double stranded RNA as determined by RNase treatment prior to deproteinization or extraction by diethyl pyrocarbonate according to Paffenholz and

SCHOLTISSEK[16]. The immediate *in vitro* product of the crude enzyme preparation, however, is exclusively single stranded RNA[16]. There seems to exist a correlation of the lag phase and the synthesis of single stranded RNA.

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