Complexing Reverse Transcriptase with Polyspermine–Ribonuclease

Alok Kumar BANDYOPADHYAY,* Dalton WANG† and Carl C. LEVY*

*Laboratory of Molecular Biology, Baltimore Cancer Research Program, National Cancer Institute, Baltimore, MD 21201, U.S.A., and †The Rockefeller University, New York, NY 10021, U.S.A.

(Received 27 February 1980)

Polyspermine–ribonuclease ($M_r \sim 17000$) and the enzyme reverse transcriptase from Rauscher-leukaemia virus ($M_r \sim 70000$) form a complex (M_r approx. 160000) such that the molar ratio of polyspermine–ribonuclease to reverse transcriptase is 5:1. The most favourable condition for complex-formation is in a solution consisting of 0.01 M-Tris/HCl buffer, pH 7.5, 0.25 M-KCl and 1 mM- Mn^{2+} at 37°C. The association of the two enzymes retains full RNAase activity, but reverse-transcriptase activity is completely inhibited when ribonuclease-sensitive polymers such as $(dG)_{12} \cdot (rC)_n$ or viral 70S RNA are used as primer templates.

The observation (Bandyopadhyay *et al.*, 1977) that a plasma ribonuclease from patients with Hodgkin's disease has a tendency to form a complex with reverse transcriptase of Rauscher-leukaemia virus has led us to examine whether the polyspermine derivative of bovine pancreatic ribonuclease prepared by Wang & Moore (1977) might have an enhanced affinity for reverse transcriptase. Since tumour-forming viruses (type C) replicate via a DNA provirus copia from the RNA genome by the viral reverse transcriptase (Gallo, 1976), an RNAase in close association with reverse transcriptase might affect the replication process. There is considerable evidence that RNAases may influence tumour regression (Daoust & de Lamirande, 1975); the cross-linked dimer of bovine pancreatic RNAase, for example, has been found (Tarnowski *et al.*, 1976) to effect a measurable decrease in size of the enlarged lymph nodes and the spleen in AKR/J mice with advanced leukaemia. Polyspermine–RNAase (Wang & Moore, 1977) is a derivative that has the special property of hydrolysing double-stranded substrates much more effectively than does the native enzyme; susceptible substrates are the double-stranded RNA of reovirus 3 and the rU or rC strand of the hybrid substrate poly(rU)·poly(dA) or poly(rC)·poly(rI). The present paper is concerned with the physical and enzymic properties of admixtures of reverse transcriptase and polyspermine–RNAase.

Materials and Methods

Isopycally banded Rauscher-leukaemia virus (1000-times-concentrated, grown in JLS-V9 cells)

Abbreviation used: RNAase, ribonuclease.

was obtained from the Office of Program Resources and Logistic Branch, National Institutes of Health, Bethesda, MD, U.S.A. Unlabelled deoxyribonucleoside 5'-triphosphate, dithiothreitol, calf thymus DNA and yeast RNA were obtained from Calbiochem, Los Angeles, CA, U.S.A. The synthetic polynucleotides $(dT)_{12} \cdot (rA)_n$ and $(dG)_{12} \cdot (rG)_n$ were purchased from Collaborative Research, Waltham, MA, U.S.A. Poly(A)·poly(U) was purchased from P-L Biochemicals, Milwaukee, WI, U.S.A. Pancreatic RNAase was obtained from Worthington Biochemicals, Freehold, NJ, U.S.A. Phosphocellulose (P-11) was purchased from Reeve Angel and Co., Clifton, NJ, U.S.A. All radioactive compounds were obtained from New England Nuclear Corp., Boston, MA, U.S.A. Bovine serum albumin, ovalbumin, and chicken albumin were purchased from Sigma, St. Louis, MO, U.S.A. The chemically cross-linked dimer of pancreatic RNAase A and polyspermine–RNAase were prepared as described previously (Wang & Moore, 1977). Viral components, e.g. gp 69/71, p15 and p30 proteins, and reverse transcriptase, were purified from Rauscher-leukaemia virus as described previously (Deepak *et al.*, 1975; Strand & August, 1976; Bandyopadhyay, 1977). The quantity of each protein was measured by the radioimmunoassay described previously (Hunter, 1967; Strand & August, 1973). Viral 70S RNA was isolated from Rauscher-leukaemia virus by the technique of Biswal & Bennysh-Melnick (1969).

Enzyme assays

Polyspermine–RNAase activity was measured spectrophotometrically with poly(A)·poly(U) as the substrate (Wang *et al.*, 1976). The reverse-trans-

criptase activity was assayed in a 100 μ l reaction mixture that was 50 mM in Tris/HCl buffer, pH 8.1, 0.8 mM in MnCl_2 , 1 mM in dithiothreitol, 20 mM in KCl, 2 μ M in [^3H]TTP (or [^3H]dGTP), and contained 2 μ g of oligo(dT) $_{12}$ ·(rA) $_n$ [or oligo(dG) $_{12}$ ·(rC) $_n$] and 20 μ l of enzyme solution. After incubation of the reaction mixture for 60 min at 37°C, the reaction was stopped by the addition of 3 ml of cold 10% (w/v) trichloroacetic acid, and the entire mixture was chilled on ice for 10 min. The precipitate that formed was collected on a Millipore filter (HAWP25; 0.45 μ m pore size) and was washed with five 3 ml portions of 5% trichloroacetic acid in 0.02 M-sodium pyrophosphate, and finally with water. The radioactivity retained on the dried filter was counted with a Packard liquid-scintillation counter by using toluene-based fluor. Transcription of the viral 70S RNA was performed as described by Rothenberg & Baltimore (1976).

Determination of molar ratio of components in the complex

The method by which the molar ratio of reverse transcriptase to polyspermine-RNAase in the complex was determined was described previously (Bandyopadhyay & Levy, 1978). In brief, the ^{125}I -labelled reverse-transcriptase-[^{14}C]polyspermine-RNAase complex (3 μ g) was dissociated and the two proteins separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. After electrophoresis, the gel was sliced and the radioactivity for each protein was measured. From the known specific radioactivity of each component, the amount of protein could be determined and, by using mol.wts. of 70000 (Bandyopadhyay, 1977) and 17000 (Wang & Moore, 1977) for reverse transcriptase and polyspermine-RNAase respectively, the number of molecules of each protein in the complex could be calculated.

Results

Interaction between purified viral reverse transcriptase and polyspermine-RNAase

Preliminary studies with mixtures of polyspermine-RNAase and reverse transcriptase showed that RNAase activity (10 μ g of protein) was unchanged even in the presence of as much as 250 ng of reverse transcriptase. Reverse transcriptase, on the other hand, which is dependent on a primer template for its activity, may be severely affected by the presence of polyspermine-RNAase. If, for example, the primer template is an RNAase-sensitive one, such as (dG) $_{12}$ ·(rC) $_n$, then the polymerizing activity can be inhibited by as much as 90% as the RNAase concentration in the mixture is increased (Fig. 1). When the primer template used is insensitive to RNAase activity, as is (dT) $_{12}$ ·(rA) $_n$,

then reverse-transcriptase activity is unaffected (Fig. 1).

Since it is known that, under certain conditions, reverse transcriptase can form a complex or aggregate with an RNAase obtained from the plasma of patients with Hodgkin's disease (Bandyopadhyay *et al.*, 1977), we decided to determine whether the polymerizing enzyme could aggregate with polyspermine-RNAase as well. Accordingly, the two proteins were mixed under conditions that led to complex-formation with the plasma enzyme (Bandyopadhyay *et al.*, 1977) and the mixture was subjected to glycerol-gradient centrifugation. As shown in Fig. 2, some of the reverse-transcriptase activity moved from its normal position near the top of the gradient (fractions 24–26) to one sedimenting with a considerably higher *s* value (fractions 13–20). Some 70% of the RNAase activity migrated near the top of the gradient (fractions 26–30), whereas the remainder co-sedimented with reverse transcriptase suggesting that a complex between the two enzymes had formed. The complex possesses RNAase activity and could in consequence neither transcribe viral 70S RNA nor use (dG) $_{12}$ ·(rC) $_n$ as a primer template. Only the RNAase-insensitive polynucleotide (dT) $_{12}$ ·(rA) $_n$ could be used to measure reverse-transcriptase activity.

Fig. 1. Reverse-transcriptase activity in the presence of polyspermine-RNAase

The reaction mixture contained 50 ng of reverse transcriptase. ●, Incorporation of [^3H]TMP with the RNAase-resistant primer template (dT) $_{12}$ ·(rA) $_n$; ○, inhibition of the incorporation of [^3H]dGMP when the primer template contains poly (C) and (dG) $_{12}$ ·(rC) $_n$.

Factors affecting complex-formation

After some preliminary studies, maximum complex-formation was found to take place in 0.01 M-Tris/HCl buffer, pH 7.4, at 37°C, 1 mM in Mn^{2+} and 0.25 M in KCl (Table 1). The salt concentration is an important variable in complex-formation, for in its

Fig. 2. Interaction of reverse transcriptase (RT) and polyspermine-RNAase (PR)

Reverse transcriptase (100 ng) was mixed with polyspermine-RNAase (25 μ g) in a solution consisting of 0.01 M-Tris/HCl buffer, pH 7.5, 1 mM in $MnCl_2$ and 0.25 M in KCl. The mixture was incubated for 30 min at 37°C and then layered over a 4.4 ml glycerol gradient [5–20%]. Centrifugation was performed at 0°C and 60000 rev./min (= 125000 g) in a Beckman L2 65 ultracentrifuge with an SW65 rotor for 2.5 h. Fractions (200 μ l each) were collected by puncturing the bottom of the tube. A sample (20 μ l) from each fraction was taken for assaying the activity of reverse transcriptase or polyspermine-RNAase: O, polyspermine-RNAase activity; ● and △, reverse transcriptase activity with (dT)₁₂·(rA)_n or (dG)₁₂·(rC)_n respectively.

absence no detectable amount of complex could be found. When the concentration of KCl is adjusted to 0.25 M, complex-formation reaches a maximum value and thereafter remains at that value even if the KCl concentration is raised to 2.0 M. Substitution of NaCl for KCl caused no significant change in the results just cited, so long as the concentrations of salt remained the same.

As shown in previous work, Mn^{2+} at 1 mM enhances complex-formation between reverse transcriptase and a serum RNAase isolated from patients with Hodgkin's disease (Bandyopadhyay *et al.*, 1977). In the present study, however, the absence of Mn^{2+} had only a slight effect on the binding between the proteins, if the concentration of Mn^{2+} is increased to 10 mM, then complex-formation is greatly inhibited (Table 1). Other cations, such as the polyamine spermidine [also shown to cause dissociation between reverse transcriptase and human serum RNAase (Bandyopadhyay *et al.*, 1977)], have no apparent effect at any concentration tested, on either the formation or dissociation of the reverse-transcriptase-polyspermine-RNAase complex.

Under the conditions defined for Table 1, the influence of pH on complex-formation was examined with several 0.01 M buffers: Tris/HCl, phosphate, Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] and Bes [2-[bis-(2-hydroxyethyl)amino]ethanesulphonic acid]. At a pH value below 6.5, irrespective of the buffer used, complex-formation could not be detected. Above that pH, the amount of complex formed increased until, in Tris/HCl buffer at pH 7.5, complex-formation reached a maximum value. At a higher pH value there was a steady decline in the amount of complex formed until, at pH 9.0, complex-formation was negligible.

Table 1. Factors affecting the formation of polyspermine-RNAase (PS-RN)-reverse-transcriptase (RT) complex. About 100 ng of reverse transcriptase were mixed with 15 μ g of polysperminase-RNAase in each case in 200 μ l of 0.01 M-Tris/HCl, pH 7.4. The reaction mixture was incubated for 30 min at 37°C and subjected to glycerol-gradient centrifugation as described in Fig. 2. The polyspermine-RNAase-reverse-transcriptase complex migrated to the 160000-dalton region, whereas the individual components (reverse transcriptase and polyspermine-RNAase) migrated to the 70000- and 17000-dalton regions respectively. The percentage complex-formation refers to the percentage of the reverse transcriptase present in the complexed form.

Reaction conditions	Complex-formation (%)
RT + PS-RN, 1 mM in Mn^{2+} and 0.2 M in KCl	100
RT + PS-RN, 10 mM in Mn^{2+} and 0.2 M in KCl	50
RT + PS-RN, 0.2 mM in Mn^{2+}	85
RT + PS-RN	0
RT + PS-RN, 1 or mM in Mn^{2+}	0
RT + PS-RN, 20 μ M in spermidine	0
RT + PS-RN, 1 mM in Mn^{2+} , 0.2 M in KCl, and 20 μ M in spermidine	95
RT + PS-RN, 1 mM in Mn^{2+} , 0.2 M in KCl, and 1 mM in spermidine	95

Fig. 3. Dissociation of reverse transcriptase-polyspermine-RNAase complex

Approx. $3\mu\text{g}$ of polyspermine-RNAase-reverse-transcriptase complex was exposed to 0.25 M -Tris/HCl buffer, pH 7.2. The mixture was processed as described in Fig. 2. ●, Reverse-transcriptase activity; ○, RNAase activity. The arrows at the top of the panel indicate the peak of enzyme activity for either reverse transcriptase (RT) or polyspermine-RNAase (PR) when each one was run separately in the gradient.

Dissociation of the complex

The reverse-transcriptase-polyspermine-RNAase complex is not dissociated by such reagents as spermidine or high concentrations (above 0.8 M) of KCl known to dissociate other complexes of reverse transcriptase (Bandyopadhyay, 1977; Bandyopadhyay *et al.*, 1977). Variations in the concentration of Tris/HCl buffer, however, did cause dissociation of the complex into its two component enzymes. Fig. 3 shows the pattern obtained after subjecting the complex to glycerol-gradient centrifugation in 0.25 M -Tris/HCl buffer, pH 7.2; the two enzyme components were recovered near the top of the gradient. An increase in buffer concentration to 0.4 M has the same effect, but if the concentration of buffer is raised to 0.5 M , then the amount of complex dissociation is substantially decreased. In phosphate buffer much the same effects were seen; at 0.01 M , little if any dissociation of the complex is apparent, whereas at concentrations ranging between 0.1 and 0.3 M the complex is dissociated completely. Any increase in molarity of buffer beyond this (i.e. to 0.4 M) had no effect on dissociation.

Molecular weight of the complex

Although the molecular weights of Rauscher-leukaemia reverse transcriptase and polyspermine-RNAase are known to be $70\,000$ and $17\,000$ respectively (Bandyopadhyay, 1977; Wang & Moore, 1977), the complex between the two proteins sediments in a region of the glycerol gradient in which its molecular weight can be estimated to be about $160\,000$. By the use of ^{125}I -labelled reverse

transcriptase and $[^{14}\text{C}]$ polyspermine-RNAase it was found that $1.4\mu\text{g}$ of reverse transcriptase were bound to $1.6\mu\text{g}$ of polyspermine-RNAase, thereby giving the molar ratio $1:5$ for reverse transcriptase/polyspermine-RNAase [see Bandyopadhyay & Levy (1978) and the Materials and Methods section].

Displacement by polyspermine-RNAase of p30 protein within a reverse-transcriptase-protein-p30 complex

In previous work (Bandyopadhyay, 1977; Bandyopadhyay & Levy, 1978) it was shown that p30, the major internal protein of Rauscher leukaemia virus, can bind specifically with reverse transcriptase to form a protein-protein complex with an estimated mol.wt. of $400\,000$. An important characteristic of the complex is the observed increase in the rate of DNA synthesis over that seen with reverse transcriptase alone (Bandyopadhyay, 1977; Bandyopadhyay & Levy, 1978). Since the two proteins are present in the virion core, it was postulated that the more efficient synthesis of virally induced DNA by the bound form of reverse transcriptase might indicate some precursor role for the protein-p30-reverse-transcriptase complex in viral assembly (Bandyopadhyay, 1977; Bandyopadhyay & Levy, 1978). Because of the possible importance of this complex, we decided to examine the effect of exposing it to polyspermine-RNAase. Under conditions similar to those for line 1 in Table 1, $0.5\mu\text{g}$ of the complex was not dissociated by the presence (15 , 25 or $50\mu\text{g}$) of polyspermine-RNAase.

Discussion

The original observation that the complex formed between Rauscher-leukaemia-virus reverse transcriptase and the major internal viral protein, p30, may be important in early phases of viral assembly (Bandyopadhyay, 1977; Bandyopadhyay & Levy, 1978) led us to examine the uniqueness of the complexing ability of reverse transcriptase. Of a variety of other viral and non-viral proteins examined (Bandyopadhyay *et al.*, 1977; Bandyopadhyay, 1977), only serum RNAase from Hodgkin's disease patients could complex with the enzyme (Bandyopadhyay *et al.*, 1977). Neither bovine pancreatic RNAase nor the human RNAase when isolated from normal patients could do so. Yet, as shown in the present study, the bovine pancreatic enzyme, if modified by linkage to spermine (Wang & Moore, 1977), can form a rather tight complex with the polymerizing enzyme, and the complex formed had a molar ratio reverse-transcriptase/polyspermine-RNAase of $1:5$.

The reverse-transcriptase-polyspermine-RNAase

complex has several characteristics that differ considerably from those of the complexes described previously (Bandyopadhyay *et al.*, 1977). It is unaffected by high salt concentrations (above 0.5M KCl) or by such cations as spermidine. Secondly, since the polyspermine-RNAase retains activity within the complex, heteropolymeric transcription with 70S viral RNA as template is obviously not possible.

The reverse-transcriptase-polyspermine-RNAase complex may be useful as a probe in the study of viral assembly and viral replication.

The preparation of polyspermine-RNAase by D. W. was supported in part by U.S. Public Health Service grant no. GM 07256. The experiments were conducted with the technical assistance of Ms. Grace Dean (Rockefeller University) and Ms. Trisha Payer (National Cancer Institute). Thanks are due to Ms. Linda Mueller (National Cancer Institute) for typing the manuscript. We are highly indebted to Dr. Stanford Moore (Rockefeller University) for counsel in the preparation of the manuscript.

References

- Bandyopadhyay, A. K. (1977) *J. Biol. Chem.* **252**, 5883-5887
- Bandyopadhyay, A. K. & Levy, C. C. (1978) *J. Biol. Chem.* **253**, 8285-8290
- Bandyopadhyay, A. K., Levy, C. C. & Mardiney, M. (1977) *J. Biol. Chem.* **252**, 7783-7787
- Biswal, N. & Bennysh-Melnick, M. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **64**, 1372-1379
- Daoust, R. & de Lamirande, G. (1975) *Sub-Cell. Biochem.* **4**, 185-211
- Deepak, J., Comer, J., Bowling, M., Dobbs, J., Aldenderfer, P., Fish, D. C. & Bandyopadhyay, A. K. (1975) *Biochem. Biophys. Res. Commun.* **63**, 400-408
- Gallo, R. C. (1976) in *Modern Trends in Human Leukemia II* (Neth, R., Gallo, R. C., Mannweiler, K. & Moloney, W. C., eds.), pp. 431-455, J. F. Lehmann Verlag, München
- Hunter, W. M. (1967) in *Handbook of Experimental Immunology* (Weir, D. M., ed.), pp. 608-642, R. A. Davis, Philadelphia
- Rothenberg, E. & Baltimore, D. (1976) *J. Virol.* **17**, 168-174
- Strand, M. & August, J. T. (1973) *J. Biol. Chem.* **248**, 5627-5633
- Strand, M. & August, J. T. (1976) *J. Biol. Chem.* **251**, 559-564
- Tarnowski, G. S., Kassel, R. L., Mountain, I. M., Blackburn, P., Wilson, G. & Wang, D. (1976) *Cancer Res.* **36**, 4074-4078
- Wang, D. & Moore, S. (1977) *Biochemistry* **16**, 2937-2942
- Wang, D., Wilson, G. & Moore, S. (1976) *Biochemistry* **15**, 660-665