Journal of Solid-Phase Biochemistry, Vol. 5, No. 4, 1980

STUDIES ON COMPLEXATION OF RNA WITH POLYCATIONIC COMPOUNDS AND USE OF COMPLEXED RNA AS A SUBSTRATE FOR RIBONUCLEASE

CHANAN SINGH, K. ANJANEYULU,[1] V. RAMESH, and R. D. DUA

Biochemistry Division,
Central Drug Research Institue,
Lucknow, India 226001

Biochemistry Laboratory,
Indian Institute of Technology,
New Delhi, India 11006

Accepted August 11, 1980

The effect of a wide array of positively charged compounds, comprising antibiotics, basic proteins, metal coordinates and drugs, on the activity of pancreatic ribonuclease has been studied. All the tested compounds inhibit the enzyme activity in a range of 30–70%, when used in optimal concentrations at pH 4.8. The fall in the enzyme activity in homogeneous mileu could be due to the inhibition of the enzyme by these polycations or to an altered state of the substrate on reacting with polycations. For this purpose, optimal conditions for complexation of RNA with these polycations were determined, and the rates of hydrolysis of the insolubilized RNA by RNase have been monitored in a heterogeneous system. The highest rate of hydrolysis is obtained in the case of protamine-RNA (11%) and the lowest in the case of propamidine-RNA (3%). Rates of hydrolysis of insolubilized RNA show an exponential fall when the reaction is prolonged up to 2 h. Moreover, the level of inhibition obtained in all the cases was nearly the same, despite considerable variance in size and conformation of the compounds used. It is proposed that these compounds, on complexing with RNA, make the hydrolyzable bonds unavailable by forming a protective layer upon the RNA surface. Biological implications of these investigations are also discussed.

INTRODUCTION

In an earlier study, we had reported that DNA on complexation with a variety of oppositely charged compounds exhibited a greatly altered response to the action of hydrogen peroxide, DNase, sonification, on protonation (1). In most of the cases, complexed DNA exhibited a marked resistance to the action of DNase. The complexed DNA has a compact structure similar to in vivo native DNA (2), hence such resistance to the above degradative agents was thought to be of biological significance. In

[1]Present address: Laboratory of Experimental Medicine, University of Brussels, 115 Boulevard de Waterloo, B-1000 Brussels, Belgium.

0146-0641/80/1200-0245$03.00/0

view of the above, it was considered appropriate to extend this concept to the RNA–RNase system, because RNA, also an anionic biopolymer like DNA, could be insolubilized by complexation with oppositely charged compounds such as antibiotics, basic proteins, etc. (3, 4).

Furthermore, a number of drugs are known to complex with macromolecules, including the nucleic acids (3–6). It was thought worth investigating, therefore, whether the complexed RNA responds to the action of RNase differentialy from the soluble one or not. The premise, that it may, is amply corroborated by the results of the present study, which show that, on macroionic complexation, RNA becomes variedly resistant to the action of RNase. Such changes in behavior of the substrate to the action of the enzyme as a result of its complexation have biological implications, and suggest to us that concentration and other structural features such as compactness must be taken into account when studying enzyme–substrate interaction. Further, results must be interpreted with caution, as these parameters with in vivo and in vitro experiments are not similar.

MATERIAL AND METHODS

Pancreatic RNase and RNA were purchased from E. Merck, Germany, and Sigma, U.S.A., respectively. Metal coordinates were prepared according to the methods of Sutherland and Meller (1, 3, 4). All antibiotics and polypeptides were gifts as indicated below under acknowledgments. The various basic compounds were used as aqueous solutions after adjusting the pH of each solution to the required value, which in most cases was pH 4.8, the pH selected for the assay of RNase.

Ribonuclease Assay

As pH values higher than 4.8 lead to nonenzymic hydrolysis of RNA, pH 4.8 was selected for the assay of ribonuclease (7, 8). The reaction mixture consisted of (2–10 μg) enzyme protein and 2 mg of ribonucleic acid or complexed RNA in 0.05 M acetate buffer, pH 4.8, in a total volume of 2 ml. Reaction mixtures were incubated at 37°C for 10 min, and pentose equivalents liberated from RNA were determined using Orcinol reagent (9).

Determination of the Effect of Polycations on Ribonuclease in a Homogeneous System

For studying the behavior of insolubilized RNA as a substrate on lines similar to those of insolubilized DNA, polycationic compounds found earlier to be precipitants of RNA were chosen (3–5, 10). However, before

investigating complexed/insolubilized RNA as a substrate, the effect of these polycationic compounds on RNase activity was determined in solution as follows.

One ml aliquots of RNA (2 mg/ml) in 0.05 M acetate buffer, pH 4.8, were made up in a number of test tubes. Increasing amounts of a given polycationic compound (dissolved in deionized distilled water) was added serially with shaking to each tube until turbidity appeared. The last test tube was ignored and, to start the enzymic reaction, 2 μg of RNase was added to each of the remaining test tubes in a total volume of 2 ml. The reaction mixture was incubated at 37°C for 10 min, when the reaction was stopped by adding 1 ml of 1 N HCl, and pentose equivalent liberated was determined as before. Percentage inhibition in each case was determined by taking activity of the control (in the absence of polycationic compounds) value as 100. Percentage inhibition and the concentration of each cationic compound that gave maximum inhibition in solution are shown in Table 1.

TABLE 1. **Effect of Polycationic Compounds on the Activity of Ribonuclease in the Homogeneous Phase**[a]

S. no.	Polycation added	Maximum concentration (μg/reaction mixture)	RNA/polycation	Inhibition (%)
1.	—	—	—	0.0
2.	Protamine	75	26.6	60
3.	Viomycin	100	20.0	45
4.	Neomycin	100	20.0	50
5.	Kanamycin	125	16.0	50
6.	Streptomycin	100	20.0	65
7.	Lysozyme	50	40.0	62
8.	Histone	50	40.0	70
9.	Hemoglobin	75	26.6	65
10.	Polymyxin	100	20.0	48
11.	Bovine serum albumin	75	26.6	55
12.	Tofranil (Imipramine)	125	16.0	40
13.	Samarium chloride	150	13.3	35
14.	Propamidine	125	16.0	45
15.	$[Co_4(en)_6(OH)_6]^{6+}$	100	20.0	40
16.	$[Co(NH_3)_6]^{3+}$	125	16.0	32
17.	$[Co(NH_3)_5(NO_2)]^{2+}$	125	16.0	30
18.	Phenylbutazone	100	20.0	40
19.	Neodymium	100	20.0	45
20.	Zirconium	125	16.0	35

[a]Two milligram samples of RNA in 0.05 M acetate buffer, pH 4.8, were treated with increasing amounts of cationic compounds dissolved in deionized distilled water until turbidity appeared. Ignoring the last tube, rates of hydrolysis of RNA in the presence of different concentrations of cationic compounds were determined, assaying the ribose released by incubating the tube at 37°C for 10 min after addition of 2 μg of RNase to each test tube. The control had no complexing agent. Its activity was taken as 100.

Complexation/Insolubilization of RNA with Polycationic Compounds

For determining the optimal time for complexation, 1 ml aliquots of RNA (2 mg/ml) were incubated at 30°C with 1 mg of the polycationic compounds in 0.05 M acetate buffer, pH 4.8, in a total volume of 2 ml for different time intervals and centrifuged at $10,000 \times g$ for 10 min. Concentration of RNA remaining uncomplexed in the supernatant was determined by assaying the pentose equivalent released on incubating it with 10 μg of RNase for 10 min. Ten minutes was found optimal for completing the complexation between RNA and positively charged compounds.

Increasing volume of a solution of a polycationic compound was added to 1 ml aliquots of RNA (2 mg/ml in 0.05 M acetate, pH 4.8) till turbidity appeared. The mixture was incubated for 10 min and centrifuged at $10,000 \times g$ for 10 min. To the supernatant, the solution of a polycationic compound was again added till turbidity appeared, and the solution was then centrifuged again. The process repeated till the supernatant showed no turbidity on further addition of the same polycation. The supernatant was then rejected, and the residue was resuspended in 2 ml of 0.05 M acetate buffer, pH 4.8, by gentle shaking and recentrifuged. Washing was repeated once again.

Evaluation of Complexed/Immobilized RNA as a Substrate

Insolubilized RNA in each case was suspended in 1 ml of the 0.05 M acetate buffer, pH 4.8, by gentle shaking for 5 min. The tube was transferred to a thermostatic bath maintained at 37°C. The tube was shaken at 50 strokes per min and allowed to attain the bath temperature. The reaction was then started by adding 2 μg of RNase in a total volume of 2 ml. After 10 min, the tube was chilled in an ice bath, centrifuged, and the supernatant assayed for released pentose. The residue was resuspended in 1 ml of 0.05 M acetate buffer, pH 4.8, as before and the enzymic reaction restarted by addition of a fresh aliquot of RNase solution. At the end of 2 h, the tube was cooled, centrifuged, and released pentose assayed in the supernatant. The process was repeated for determining hydrolysis of the complexed RNA up to 24 h.

RESULTS AND DISCUSSION

Positively charged compounds interact with oppositely charged (anionic) macromolecules like RNA and DNA to form insoluble complexes (3, 4), which need to be investigated as substrates for RNase for reasons

discussed below. However, before investigating the hydrolysis of insolubilized RNA, the effect of complexing cations on RNase activity was investigated, so that the results of hydrolysis of insolubilized RNA can be interpreted properly. The hydrolysis of RNA in the presence of various cationic compounds at which maximum inhibition was achieved was determined. It is shown in Table 1 that all the polycations studied inhibit RNA hydrolysis by RNase, this inhibition being only 30% (lowest) in the case of a cobalt coordinate and 70% (highest) in the case of histone. Inhibition by other cationic compounds falls within these two limits. This decrease in rates of hydrolysis of RNA can be due to inhibition (3, 10–12) of the enzyme RNase by polycations or due to the altered state of the substrate on complexation with these compounds, or both. Positively charged compounds can interact with polyanionic RNA, altering its conformation (without forming a precipitate) in such a way that the points hydrolyzable by RNase get imbedded. Attack by the enzyme becomes more difficult, or alternatively and more likely, these compounds can bind upon the RNA surface, forming a protective film. Macromolecular interactions have been known to bring about alterations in the characteristics of the interactants (1, 5, 6, 13, 14).

To eliminate the possiblity of enzyme inhibition by these polycations during hydrolysis of insolubilized RNA, only well-washed RNA polycation precipitates were used. Moreover, the reaction was initially run for only 10 min, so that concentration of the released polycation, if any (even the fragments obtained from RNA after hydrolysis by RNase will be polyanionic and capable of retaining polycations) will be too low to influence the rate of hydrolysis significantly. By washing the substrate after each specified period of incubation with the enzyme, the concentration of a polycation was kept low. The pH for the hydrolysis of insolubilized RNA in each case was also kept at 4.8, and shaking during incubation was fairly vigorous. It is evident from Table 2 that the RNA insolubilized by complexation with polycations is hydrolyzed at far slower rates than the uncomplexed RNA.

Highest rates of RNA hydrolysis were obtained in the case of protamine-RNA and lowest in case of propamidine-RNA complexes. Even these were only 11.0% and 3.0% of the control, respectively. The hydrolysis of the insolubilized RNA, when studied for prolonged periods (Fig. 1 is representative of similar studies that were made on other insolubilized RNA complexes), indicated that the rates of hydrolysis of RNA fall off exponentially with time, even when only a small fraction of the RNA has been hydrolyzed, thus supporting our view that the cationic compounds, on complexing with RNA, make the hydrolyzable bonds unavailable, either by imbedding them through conformational changes or by forming a protective layer. The latter view is further confirmed by the fact that the level of

TABLE 2. **Hydrolysis of Complexed/Immobilized RNA by RNase**[a]

S. no.	Polycations added	% Hydrolysis after 10 min (*a*)	2 h (*b*)	24 h (*c*)	Total hydrolysis ($d = a + b + c$)	% Inhibition ($100 - d$)
1.	Viomycin	7.7	4.6		12.3	87.7
2.	Neomycin	9.6	7.3		16.8	83.2
3.	Streptomycin	6.8	11.9		18.7	81.3
4.	Kanamycin	6.8	10.0		16.8	83.2
5.	Bovine serum albumin	5.5	8.5		14.0	86.0
6.	Lysozyme	6.8	11.9		18.7	81.3
7.	Polymyxin	7.7	8.3		16.0	84.0
8.	Histone	6.8	14.5		21.3	78.7
9.	Hemoglobin	6.5	11.0		17.5	82.5
10.	Tofranil (Imipramine)	4.0	7.5		11.5	88.5
11	Samarium chloride	8.5	7.5		16.0	84.0
12.	Propamidine	3.0	4.0		7.0	93.0
13.	$[Co_4(en)_6(OH)_6]^{6+}$	5.0	7.5		12.5	87.5
14.	$[Co(NH_3)_6]^{3+}$	8.5	15.0		23.5	76.5
15.	$[Co(NH_3)_5(No_2)]^{2+}$	6.5	13.5		20.0	80.0
16.	Phenylbutazone	8.5	15.0		23.5	76.5
17.	Protamine	11.0	2.0		13.0	87.0

[a] One milliliter aliquots of RNA (2 mg/ml in 0.05 M acetate buffer, pH 4.8) were incubated with an optimal quantity of each cationic compound in a total volume of 2 ml for 10 min at 30°C, and centrifuged at 4000 × *g* for 10 min, and the supernatant was then rejected. The residue was resuspended in 2 ml of 0.05 M acetate buffer, pH 4.8, by shaking and recentrifuged. The process was repeated, and finally insolubilized RNA was resuspended in 0.05 M acetate buffer, pH 4.8. Two μg of RNase was added in a total volume of 2 ml. Hydrolysis was followed by determining the released nucleotides in the supernatant with Orcinol. The reaction was started by addition of a fresh aliquot of RNase solution (2 μg) each time. The control had uncomplexed RNA.

inhibition effected by compounds varying considerably in shape and molecular size is nearly the same; for example, inhibition in case of kanamycin and histone or lysozyme is 93.2%. Such altered susceptibility to enzyme (DNase) action has been reported in the case of DNA complexed with drugs (15–20). Only limited investigations have been done on the RNA–RNase system (21).

These findings have certain biological implications. In the case of injury, for instance, the decompartmentalization of cellular material may allow the oppositely charged (or mutually incompatible) molecules to interact, forming complexes/insolubilized products; for example, proteins may interact with nucleic acids (17). Such insolubilized materials in many cases might be resistant to enzymic action (1, 5, 6, 18–20) and may stay in the system long enough to give side reactions or trigger immunological

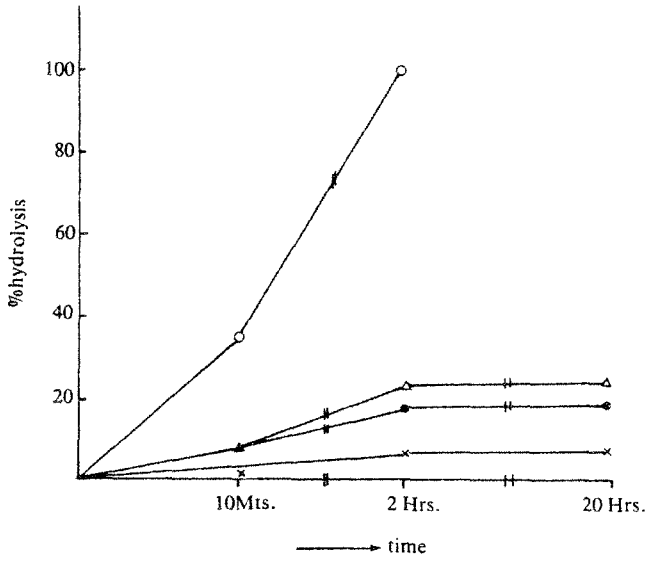

FIG. 1. Rate of hydrolysis of complexed/immobilized RNA by RNase. Two milligrams of RNA were incubated with an optimal concentration of complexing agent in 0.05 M acetate buffer, pH 4.8, in a total volume of 2 ml for 10 min at 30°C and centrifuged at 4000 × *g* for 10 min. The supernatant was rejected, and the precipitate was resuspended in 2 ml of 0.05 M acetate buffer, pH 4.8, by shaking and recentrifuged. Washing of the precipitate was repeated once again. Finally, the precipitate was resuspended in 2 ml of 0.05 M acetate and the reaction was started by the addition of 2 μg of RNase. Rate of hydrolysis of complexed RNA was followed by cooling the reaction mixture in ice after different time intervals, centrifuging it at 4000 × *g* for 10 min, and determining the released ribose in the supernatant. Each time, the reaction was restarted by addition of fresh aliquots of RNase (2 μg) in a total volume of 2 ml. Control had uncomplexed RNA. RNA complexed with (●—●) streptomycin, (×—×) propamidine, and (△—△) $(Co(NH_3)_6)^{3+}$. (○—○) represents the rate of hydrolysis of uncomplexed RNA.

response. Similarly, in the case of prolonged drug administration or overdosage (5, 6), some of the macromolecules may get complexed, with consequent altered susceptibility to enzyme action, giving rise to pathological symptoms. This may make diagnosis difficult, if not impossible. Conversely, the living system may regulate the metabolic processes by modulating the enzymic reaction through complexation of the substrate (1, 5, 6).

These investigations also suggest that in vitro studies on enzyme kinetics or mechanism should be applied to a living system with caution and scepticism, as substrate or enzyme may occur in the living system as

bound/complex form(s), or may have different conformations. For example, RNA occurs as a nucleoprotein in ribosomes, or some proteins occur as lipoproteins in the cell or mitochondrial membrane.

The present study also indicates the potentiality of these polycationic compounds for purification of RNase by affinity chromatography (22). Insolubilization of RNA with polycationic compounds may make enzyme substrate interaction possible, but without hydrolysis of the latter. Kanamycin and neomycin should especially be effective for this purpose. Insolubilized/complexed RNA may also be used for the rapid assay of RNase, where large samples are required to be analyzed, and accuracy requirements are not stringent.

ACKNOWLEDGMENTS

The authors are thankful to the following organizations for making available various chemicals and biochemicals as gifts: viomycin sulphate, Alembic Co., India; Hemoglobin, Centron Chemicals, India; kanamycin sulphate, Alembic Co., India; neomycin sulphate, Upjohns Co., U.S.A.; streptomycin sulphate, Alembic Co., India; propamidine, May and Baker, U.K.; protamide sulphate, Prof. E. W. Jones, U.K.; histone sulphate and polymyxin, Pfizer Co., U.S.A.

Two of the authors (K.A. and V.R.) are thankful to the Council of Scientific and Industrial Research, New Delhi, for providing the financial assistance.

REFERENCES

1. SINGH, C. (1970) Ind. J. Biochem. 7 : 255.
2. EISINGER, J., Bell. Teleph. Co. (U.S.A.), personal communication.
3. SINGH, C. (1971) Ind. J. Biochem. Biophys. 8 : 45and 8 : 71.
4. SINGH, C., and MISHRA, J. (1978) Arzneim-Forsch 28 : 1320.
5. EHRENPREIS, S. (1970) *In* Progress in Drug Research, Vol. 14, ARIENS, E. J. et al. eds.), Birkhauser Verlag, Basel, p. 59.
6. HAHN, F. E., PUCK, T. T., SPRINGER, G. F., SZYBALSKI, W., and WALLENFELS, K. (1971) *In* Progress in Molecular and Subcellular Biology, Vol. 2, HAHN, F. E. (ed.), Springer-Verlag, Berlin.
7. ALTMAN, P. L., and DITTMER, D. S. (eds.) (1974) Biology Data Book, Vol. III, Fed. Am. Soc. Exp. Biol., Bethesda, Maryland.
8. ANFINSEN, C. B., and WHITE, F. H. (1961) *In* The Enzymes, BOYERS, P. D., and LARDY, H. (eds.), Academic, New York, p. 95.
9. JOSEFSSON, L., and LAGERSTED, S. (1962), *In* Methods of Biochemical Analysis, Vol. IX, GLICK, D. (ed.), Interscience Publishers, New York, p. 39.
10. ALTESCU, E. J., (1964) Analy. Biochem. 8 : 373; (1966) Enzymol. Biol. Clin. 6 : 305.

11. MASSART, L., PEETERS, G., and LAGRAIN, A. (1948) Arch. Int. Pharmacodyn. Ther. 76 : 72.
12. SOBER, H. A., SCHLOSSMAN, S. F., YARON, A., and RUZHIZKY, G. W. (1965) Science (N.Y.) 50 : 384.
13. ARIENS, E. J. (1970) *In* Progress in Drug Research, Vol. 14, AREINS, E. J. et al. (eds.), Birkhauser Verlag, Basel, p. 11.
14. LEES, C. W., and VON HIPPEL, P. H. (1968) Biochemistry 7 : 2480.
15. KURNICK, N. B. (1962) *In* Methods of Biochemical Analysis, Vol. IX, GLICK, D. (ed.), Interscience Publishers, New York.
16. SELA, M., and KATCHALSKI, E. (1959) Adv. Prot. Chem. 14 : 391.
17. HAUROWITZ, F. (1963) *In* Chemistry and Functions of Proteins, Academic, New York, p. 287.
18. MANDL, I., and NEUBERG, C. (1956) *In* Advances in Enzymology, Vol. 17, NORD, F. F. (ed.), Interscience Publishers, New York.
19. WEBB, J. L. (1966) *In* Enzyme and Metabolic Inhibitors, Vol. II, Academic, New York, p. 461.
20. WIKLANDER, L., *In* Ion Exchangers in Organic and Biochemistry, CALMON, C., and KRESSMAN, T. R. E. (eds.), Interscience Publishers, New York.
21. SURYANARAYANA, T., and BURMA, D. P. (1975) Biochem. Biophys. Res. Commun. 65 : 708.
22. TAKASHIS, H., LAESON, T. J., and DOWNAN, W. (1976) Biochemistry 15 : 5205.