ZINC REVERSE TRANSCRIPTASES FROM MAMMALIAN RNA TYPE C VIRUSES

D. S. Auld,* H. Kawaguchi, D. M. Livingston+ and B. L. Vallee

*The Biophysics Research Laboratory, Department of Biological Chemistry, Harvard Medical School, and the Division of Medical Biology, Peter Bent Brigham Hospital, Boston, Massachusetts

Received November 15,1974

1,10-phenanthroline (OP) reversibly inhibits the RNA dependent DNA polymerases -- reverse transcriptases -- from murine, simian, feline and RD-114 RNA tumor viruses. The log of the OP inhibition constants, pK_I, vary from 4.7 to 4.9 and the number of moles of inhibitor bound to the metal, $\bar{n}$, vary from 1.3 to 2.2. These values are very similar to those previously reported for known zinc enzymes and imply that the mammalian reverse transcriptases are also zinc enzymes. Combined use of microwave-induced emission spectrometry and micro gel exclusion chromatography demonstrates the presence of stoichiometric amounts of zinc in the enzymes from murine leukemia and woolly monkey type C-viruses. These results parallel those previously reported for the enzyme from avian myeloblastosis virus.

Introduction

Previous postulates of a relationship between a zinc enzyme and the leukemic process (1,2) resulted in our identification of the RNA dependent DNA polymerase -- reverse transcriptase -- of avian myeloblastosis virus, AMV, as a zinc metalloenzyme (3,4). The demonstration that zinc is an intrinsic component of this enzyme and that copper, iron and manganese are absent was made feasible by the use of microwave emission spectrometry (5). This method of metal analysis is capable of measuring metals in the picogram range allowing metal determinations on μg quantities of protein. We have now used microwave emission spectroscopy in conjunction with studies of inhibition by metal binding agents to demonstrate that, similarly, reverse transcriptases from a number of mammalian type C RNA tumor viruses are also zinc metalloenzymes.

+Present address: Children's Cancer Research Foundation, Boston, Massachusetts.

*To whom correspondence should be addressed.

Materials and Methods

Viruses - Rauscher murine leukemia virus, MuLV, (from cell line JLS-V9); Rickard feline leukemia virus, FeLV, (from cell line F422); Woolly monkey Type C virus, WLV(WSV) (from cell line KW-23) and RD-114 virus were all banded on isopycnic sucrose gradients formed in the zonal ultracentrifuge.

Purification of Reverse Transcriptases The virus (3 ml) was disrupted by addition of 150 µl of 1M Tris·HCL, pH 7.8; 600 µl 3M KCl; 60 µl of 0.01M β-mercaptoethanol and 360 µl of 10% Triton X-100 followed by stirring for 1 hour at 4°. The resultant solution was dialyzed twice at 4° for 1 1/2 hr versus a buffer containing 0.05 M Tris·HCL, pH 7.8; 0.01% Triton X-100; 20% glycerol and 10^{-3} M DTT.

The dialyzed enzyme was applied to a P-11 phosphocellulose column (8 ml). The column was washed with 25 ml of the above dialyzing buffer and then eluted at 0.6 M NaCl in a buffer containing 0.05 M Tris, pH 7.8, 10^{-3} M DTT, 0.01% Triton X-100, 20% glycerol. 2 ml fractions were collected and analyzed for activity. The major fractions were pooled and concentrated in an amicon microfiltration apparatus using a pM 10 filter. The storage of the enzyme and assay procedures have been described previously (4).

$(rA)_n$ and $(dT)_{12-18}$ were obtained from Collaborative Research, Waltham, Massachusetts and (^{3}H) TTP, 56 Ci/mole, from New England Nuclear Corp. All chemicals were reagent grade. Micro-gel exclusion chromatography was performed using small diameter columns (4) and zinc was measured by means of microwave-induced emission spectroscopy on 5 µl samples according to methods described before (4,5).

Results and Discussion

Assays using the homopolymers $(rA)_n$ and $(dA)_n$ can differentiate the RNA dependent DNA polymerases of RNA tumor viruses from known cellular DNA dependent DNA polymerases (6-8). Thus, the AMV reverse transcriptase exhibits a 100-fold greater activity towards $(dT)_{10}$ $(rA)_n$ than towards $(dT)_{10}$ $(dA)_n$.

All of the purified reverse transcriptases used in this study were examined using $(dT)_{12-18}$ and $(dT)_{10}$ as initiators with $(rA)_n$ and $(dA)_n$ as templates. Using the $(rA)_n$ template in all cases the activity was from 500 to 2000 fold higher than that obtained when using $(dA)_n$ as template indicating the virtual absence of contamination from known cellular DNA dependent DNA polymerases. The assays were optimized for inhibition studies using $(rA)_n$ as template and $(dT)_{12-18}$ as initiators at pH 7.8 and 25°. Most of the constituents were kept at the same concentrations employed in the AMV assay (4). Since the KCL optimum for the mammalian polymerase catalyzed reaction is lower, about 0.03M-0.07M, a value of 0.05M was used for these studies.

Our previous results have indicated that 1,10-phenanthroline (OP) reversibly inhibits the AMV polymerase catalyzed reaction at 25° but irreversibly inhibits it at 37° (4). The inhibition of the mammalian reverse transcriptases by OP was therefore examined at 25°. OP reversibly inhibits the enzymes from the feline and RD-114 viruses with a pK_I value of 4.7 (Fig. 1). The results for all other reverse transcriptases examined are similar. OP has also been reported to be an inhibitor of the reverse transcriptase from Rous sarcoma virus (10). The pK_I values for OP inhibition of the reverse transcriptases are very similar to each

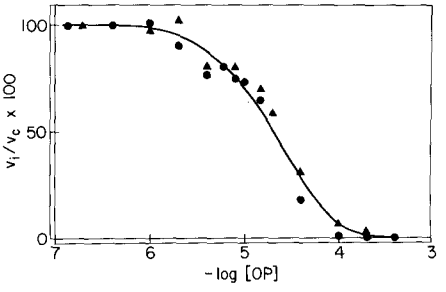

Figure 1. Instantaneous inhibition of the reverse transcriptases from FeLV (▲) and RD-114 (●) viruses by 1,10-phenanthroline (OP) at 25°. The agent is added only to the assay system. V_i is the velocity in the presence of the inhibitor, and V_c that in its absence. Standard concentrations in the 100 µl assay are: $MnCl_2$ 0.2 mM; $(rA)_n$, 0.1 µM; $(dT)_{12-18}$, 1 µM; [^{3}H]TTP, 2.4 µM; DTT, 2 mM; KCl, 0.05 M; Tris, pH 7.8, 0.11 M.

Table 1

1,10-Phenanthroline Inhibition of
Mammalian Reverse Transcriptases and Known Zinc Enzymes

Enzyme	pK_I [a]	$\bar{n}$
Bovine carboxypeptidase A [b]	4.2	2.3
B. Thermoproteolyticus Thermolysin [b]	4.4	1.8
E. coli Alkaline Phosphatase [b]	4.4	1.8
Horse Liver Alcohol Dehydrogenase [b]	4.7	1.2
AMV Reverse Transcriptase	4.2	1.5
Mammalian Reverse Transcriptases:		
MuLV	4.9	2.2
FeLV	4.6	1.2
RD-114	4.8	1.3
WLV(WSV)	4.9	1.5

a) pK_I is - log K_I where K_I is the OP concentration at 50% inhibition. The average number of moles of enzyme, $\bar{n}$, is calculated from $\phi = K_I I^{\bar{n}}$ where $\phi = (\frac{V_c}{V_I} - 1)$ (9).

b) Data taken from Coombs et al., Biochemistry 1, 899 (1962); Holmquist, B. and Vallee, B.L., J. Biol. Chem. 249, 4601 (1974); Plocke et al., Biochemistry 1, 373 (1962) for A.P.; and Vallee et al., J. Biol. Chem. 237, 262 (1959) for LADH.

other and to those obtained for the zinc enzymes, carboxypeptidase A, thermolysin, alkaline phosphatase and horse liver alcohol dehydrogenase (Table I). The values of $\bar{n}$, the number of moles of inhibitor bound to the metal, 1.3 to 2.2, are also very similar for all of these enzymes. In fact, the pK_I and $\bar{n}$ values for the mammalian reverse transcriptases are so closely similar to each other, to that of the avian reverse transcriptase and to those of other zinc enzymes as to suggest that these, too, are zinc enzymes. The fact that the values of $\bar{n}$ are

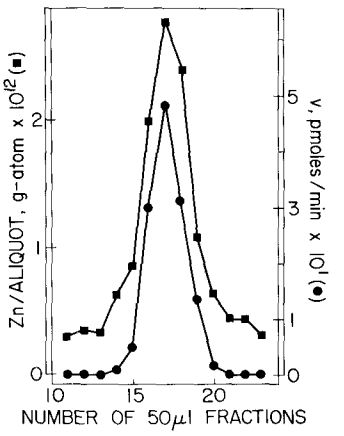

Figure 2. Distribution of murine polymerase activity (●), and zinc (■) in fractions from a G-100 sephadex column (0.4 cm x 18 cm). Metal contaminants of the column were removed by washing with 4 ml of 1,10-phanenthroline, 0.01 M in pH 7.8 Tris·HCl, 0.01 M, followed by 40 ml of Tris buffer. The column was equilibrated at 4° with pH 7.8 Tris·HCl, 0.01 M; KCl, 0.01 M; DTT, 1 mM, and Triton X-100; 0.001%. Approximately 10 μg of protein in 50 μl was placed on the column and eluted with the above buffer at a flow rate of 0.06 ml/min. Droplet fractions, 50 μl were collected for duplicate assays of enzyme activities, and triplicate zinc analyses. Zinc content was calculated by reference to the emission of solutions of zinc standards. Zinc content is expressed as g-atoms per 5 μl aliquot, and the velocity for 1 μl of enzyme added to the standard assay.

---

sometimes close to 1.0 may indicate that in these cases OP inhibits by binding to the zinc of the enzyme forming a mixed complex. This mechanism has been demonstrated for LADH (11,12). When the values of $\bar{n}$ are 2 or greater, OP likely inhibits by removing zinc from the enzyme (13).

The zinc content of the murine and woolly monkey enzymes was determined by the same procedure employed previously for the avian enzyme (3,4). Micro gel exclusion chromatography on G-100 served to remove metal quenching agents and low molecular-weight protein contaminants. Droplet fractions, 45 μl, were collected for activity measurements, zinc analysis and protein content by the method of Lowry (14). In both cases one major peak of enzyme activity and zinc is obtained

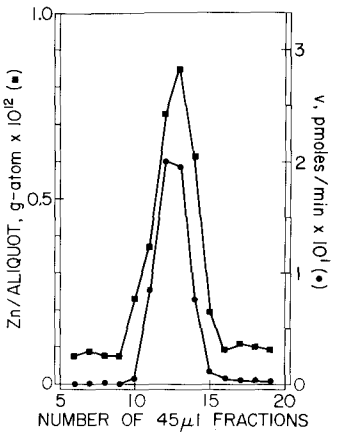

Figure 3. Distribution of woolly monkey polymerase activity (●) and zinc (■) in fractions from a G-100 column (0.3 x 25 cm, Altex microbore column). Approximately 5 μg of protein in 50 μl was applied to the column. Droplet fractions of 45 μl were collected for duplicate assays of enzyme activities and triplicate zinc analyses. All other conditions are the same as in Figure 2.

centered about fraction 17 (2.2 ml column) for the murine enzyme (Figure 2) and fractions 12 and 13 (1.4 ml column) for the woolly monkey enzyme (Figure 3). The elution patterns are very reproducible, but throughout most of the column, the protein content of the eluate is so low as not to be detectable even by the micro Lowry procedure. Only the protein content of the fractions containing the largest amount of zinc is sufficient to allow quantitative analysis. Assuming equivalent specific activities for the AMV and mammalian enzymes, these preparations of the murine and woolly monkey enzymes contain 1.4 and 1.0 g-atoms of zinc per mole of enzyme. Clearly, establishment of the exact stoichiometry will require extension of this work with larger amounts of purified enzymes. However, it is of particular interest that these studies demonstrate a catalytic role of zinc in these enzymes both by enzymatic and spectrometric means, even when the amounts of enzyme available are at the very limits of detection of the amount of protein to be determined. The present data suggest that the functional role of zinc may extend to many of these enzymes. This realization reinforces earlier suggestions of the

importance of zinc enzymes in the biochemistry underlying the leukemic process (1-4).

Acknowledgement

We are indebted to Dr. Jack Gruber for his aid in making these studies possible. The excellent technical assistance of Susan Shanahan and Thomas Kennedy is gratefully acknowledged.

This work was supported by Grant-in-Aid GM-15003 from the National Institutes of Health of the Department of Health, Education and Welfare.

References

1. Vallee, B.L., Fluharty, R.G. and Gibson, J.G., 2nd Acta Union Intern Contre Cancer 6, 869 (1949).
2. Gibson, J.G., 2nd., Vallee, B.L., Fluharty, R.G. and Nelson, J.E., Acta Union Intern Contre Cancer 6, 1102 (1950).
3. Auld, D.S., Kawaguchi, H., Livingston, D.M. and Vallee, B.L., Biochem. Biophys. Res. Commun. 57, 967 (1974).
4. Auld, D.S., Kawaguchi, H., Livingston, D.M. and Vallee, B.L., Proc. Nat. Acad. Sci. USA 71, 2091 (1974).
5. Kawaguchi, H. and Vallee, B.L., Anal. Biochem., in press.
6. Goodman, N.C. and Spiegelman, S., Proc. Nat. Acad. Sci. USA 68, 2203 (1971)
7. Baltimore, D. and Smoler, D., Proc. Nat. Acad. Sci. USA 68, 1507 (1971).
8. Robert, M.S., Smith, R.G., Gallo, R.C., Sarin, P.S. and Abrell, J.W., Science 176, 798 (1972).
9. Kistiakowsky, G.B. and Shaw, W.H.R., J. Am. Chem. Soc. 75, 2751 (1953).
10. Valenzuela, P., Morris, R.W., Faras, A., Levinson, W. and Rutter, W.J., Biochem. Biophys. Res. Commun. 53, 1036 (1973).
11. Drum, D.E. and Vallee, B.L., Biochemistry 9, 4078 (1970).
12. Bränden, C.-I., Eklund, H., Nordström, B., Boiwe, T., Söderlund, G., Zeppezauer, E., Ohlsson, I. and Åkeson, Å., Proc. Nat. Acad. Sci. USA 70, 2439 (1970).
13. Vallee, B.L. in The Enzymes, Eds., Boyer, P.D., Lardy, H. and Myrback, K. (Academic Press, Inc., New York), Vol. III, 2nd Ed., pp. 225-276.
14. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R. J., J. Biol. Chem. 193, 265 (1951).