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RNA-Dependent DNA Polymerase (Reverse Transcriptase) from Avian Myeloblastosis Virus: A Zinc Metalloenzyme

(microwave excitation spectrometry/zinc metabolism/leukemia)

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ABSTRACT **RNA tumor viruses contain a characteristic RNA-dependent DNA polymerase (reverse transcriptase) which has been thought to be related to the induction of leukemia by this virus. A disturbance in a zinc-dependent enzyme system was first postulated to account for the demonstrated differences in zinc metabolism of normal and leukemic leukocytes [Vallee *et al.* in (1949) *Acta Unio. Int. Contra Cancrum* 6, 869 and (1950) *Acta Unio. Int. Contra Cancrum* 6, 1102]. In order to investigate the relationship between zinc and the initiation of leukemia in chickens by avian myeloblastosis virus, we have examined the metalloenzyme nature of its reverse transcriptase. The present data show that this protein is a zinc metalloenzyme demonstrating the postulated relationship between zinc and a leukemic process. Paucity of purified enzyme generated the design of a novel system of analysis incorporating microwave-induced emission spectrometry combined with gel exclusion chromatography. It provides precision, reproducibility, and remarkable limits of detection on μl samples containing 10^{-12} to 10^{-14} g-atoms of metal, and is thus orders of magnitude more sensitive than other methods. The chromatographic fraction with highest enzymatic activity contains 1.8×10^{-11} g-atoms of zinc per 1.6 μg of protein, corresponding to either 1.8 or 2.0 g-atoms of zinc per mole of enzyme for a molecular weight previously determined either as 1.6 or 1.8×10^5. Copper, iron and manganese are absent, i.e., at or below the limits of detection, 10^{-13} to 10^{-14} g-atoms. Agents known to chelate zinc inhibit the enzyme, while their nonchelating isomers do not. The data underline the participation of zinc in nucleic acid metabolism and bear importantly upon the lesions that accompany leukemia and zinc deficiency.**

Zinc is essential for the growth of species in all phyla. Growth arrest resulting from its deficiency presumably reflects important roles for this metal in critical metabolic steps (1). The biological essentiality of zinc can be discerned at various steps of cell growth and development, and it has been known for some time that the element plays a significant role in the metabolism of leukocytes (2). Differences in zinc metabolism of normal and leukemic leukocytes first led to the postulate that the disturbance of a zinc-dependent enzyme system is critical in the pathophysiology of myelogenous and lymphatic leukemia (3, 4). Zinc is now known to be an integral component of numerous macromolecules through which it exerts its major, known physiological roles. Among many other enzymes (5), the DNA and RNA polymerases of *Escherichia coli* (6, 7) have also been shown to contain zinc essential for their catalytic activities. Moreover, zinc and other divalent metal atoms stabilize the structures of both DNA and RNA (8, 9).

Type C oncogenic RNA viruses, such as avian myeloblastosis virus (AMV), are associated with lymphomas and leukemia. The existence of an RNA-dependent DNA polymerase—reverse transcriptase—in these RNA tumor viruses (10, 11) has greatly stimulated study of the initiation, biochemical basis, and maintenance of malignant transformations and of the manner by which viral RNA is transcribed into a DNA copy. Although a role for zinc in this process is unknown, the above indications of its importance in normal and leukemic leukocyte metabolism have prompted us to study the RNA-dependent DNA polymerase from avian myeloblastosis virus from this point of view. This minimally requires the analytical demonstration of the presence of a functional metal atom. The paucity of the material seemed to present formidable problems in regard to metal analysis, but the marked inhibition of the enzyme's activity by metal-binding agents here reported led us to devise novel means capable of quantitative metal determinations at the 10^{-14} g-atom level. This allowed the identification of stoichiometric amounts of zinc in this enzyme, which was available to us in quantities of the order of 10^{-9} moles. The limits of detection of the procedures are sufficiently low, ultimately to permit quantitative studies of metals and their metabolism at the cellular level. Preliminary communications of this work have been made (25, 26).

MATERIALS AND METHODS

AMV polymerase was prepared from the purified virus by a modification of the procedure of Ross *et al.* (12). The Triton X-100 disrupted, isopycnically banded virus (10 ml) was applied to a 2.2 × 30-cm P-1 phosphocellulose column and eluted with a linear NaCl gradient from 0.2 to 0.6 M. The polyethylene glycol extraction step was eliminated, and ethylenediaminetetraacetic acid (EDTA) was not used in any of the purification steps. The enzyme was stored under liquid N_2 in pH 6.8 buffer containing 0.05 M imidazole, 0.30 M KCl, 1 mM dithiothreitol (DTT); 20% glycerol and 0.01% Triton X-100. Immediately before assay, it was diluted into a buffer containing 0.05 M Tris·HCl, pH 7.8 and all the above constituents, with the exception of imidazole and glycerol.

$(rA)_n$ and $(dT)_{12-18}$ were obtained from Collaborative Research, Waltham, Mass., and [^{3}H]TTP, 70 Ci/mole, from New England Nuclear Corp. Protein was measured on 15-μl aliquots using the method of Lowry *et al.* (13). All chemicals

Abbreviations: AMV, avian myeloblastosis virus; DTT, dithiothreitol; OP, 1,10-phenanthroline (*ortho*).

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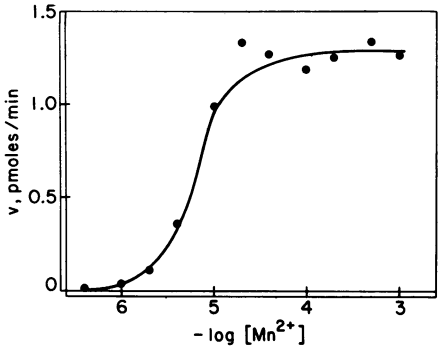

FIG. 1. Effect of Mn^{2+} on incorporation of TMP at 25° and pH 7.8 catalyzed by RNA-dependent DNA polymerase of AMV. In the following figures, unless otherwise noted, standard concentrations in the 100-μl assay are: $MnCl_2$, 0.2 mM; $(rA)_n$, 0.1 μM; $(dT)_{12-18}$, 1 μM; [^{3}H]TTP, 2.4 μM; DTT, 2 mM; KCl, 0.10 M; Tris·HCl, pH 7.8, 0.11 M. Approximately 0.2 μg of protein were added to initiate the reaction, which was terminated at 60 min with 10% trichloroacetic acid. In Figs. 1–5 the rates given are for a protein concentration of 1 μg/100-μl assay mixture.

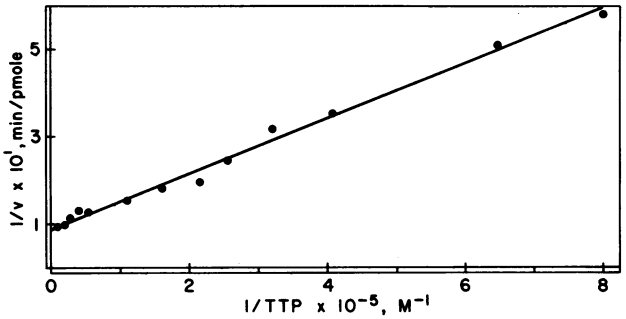

FIG. 2. The Lineweaver–Burk plot for the polymerase-catalyzed incorporation of TMP, 0.8 to 50 μM TTP, using a $(rA)_n$ template and $(dT)_{12-18}$ initiator. In this paper, the convention is that numbers on the axes or in columns are the products of the experimental values and the factors indicated.

were reagent grade. Potential contamination by adventitious metal ions was controlled by prior extraction of solutions with dithizone (14). Zinc was measured by means of microwave-induced emission spectroscopy on 5-μl aliquots according to principles discussed previously (15) but using experimental facilities designed by Dr. George Wooten, Monsanto Research Corp., Dayton Laboratories, Dayton, Ohio.

RESULTS AND DISCUSSION

With $(rA)_n$ as the template and $(dT)_{12-18}$ the initiator, the rate of the AMV-polymerase-catalyzed incorporation of TMP at 25°, pH 7.8, is linear over a 20-fold range of enzyme concentration. The effect of the various constituents of the assay mixture on the rate of polymerization was examined at this pH and temperature in order to optimize conditions for metal-binding studies. Variation of Triton X-100 from 0.001 to 0.1% and DTT from 0.2 to 6 mM had a small ($\leq$20%) effect on the rate. For KCl, a broad optimum in activity is found between 0.05 M and 0.2 M. At higher KCl concentrations, activity decreases rapidly. The rate of nucleotide polymerization is maximal at Mn^{2+}, about 0.1 mM, for a TTP concentration of 2.4 μM (Fig. 1). Activity is constant between 0.1 and 1 mM Mn^{2+}.

A 20-fold increase in the TTP concentration shifts the midpoint of the Mn^{2+} versus activity curve only to a 2-fold higher metal ion concentration, maximal activity being obtained then at 0.2 mM Mn^{2+}. The effect of changes in TTP concentration on the rate of the reaction was therefore examined at this Mn^{2+} concentration. All other constituents were held at their "standard" assay values. A Lineweaver–Burk plot is linear over a 100-fold concentration of TTP (Fig. 2). The K_m for TTP is about 10 μM and the V_{max} is 12 pmoles of TTP incorporated per min/μg of protein, 25°.

The effects of variation of template and initiator concentrations on the rate of the reaction are nearly identical, resulting in curves with midpoints at approximately 4 nM, i.e., of the same order of magnitude as the enzyme concentration in the reaction (Fig. 3). In the absence of either $(rA)_n$ or $(dT)_{12-18}$ no rate is detectable. These results are consistent with a mechanism involving a tight ternary complex of $(dT)_{12-18}$, $(rA)_n$, and enzyme. Measurement of activity as a function of template and/or $(dT)_{12-18}$ concentrations may, in fact, allow a determination of the active enzyme concentration by kinetic means.

Complexing agents, long employed to explore the functional role of metals in enzymes by kinetic methods (5), served to investigate a possible role of a metal ion other than Mn^{2+}. Such agents inhibit the AMV-polymerase-catalyzed reaction both instantaneously and reversibly (Figs. 4 and 5) and also in a time-dependent, irreversible manner (Fig. 4). The stability of the enzyme in the absence and presence of 1,10-phenanthroline (OP), 1 mM, was examined at a protein concentration of 20 μg/ml in the standard diluting buffer at both 25° and 37°. Aliquots were withdrawn from the incubation mixtures at various times over a 60-min interval and diluted 10-fold into a standard assay mixture not containing OP. At either temperature, in the absence of OP, the enzyme is completely stable for 60 min (Fig. 4). In the presence of OP at 25° and at zero time of incubation, inhibition is instantaneous and of the same magnitude as that obtained if OP, 0.1 mM, is added to the assay system without preincubation. Activity remains essentially constant for the entire 60-min preincubation period (Fig. 4). Preincubation of enzyme and OP at 37°, however, results in both an instantaneous and also a progressive, irreversible decrease in activity (Fig. 4).

The instantaneous, reversible inhibition of 1,10-phenanthroline at 25° can be examined by adding it only to the assay mixture¶. The midpoint of the OP inhibition curve is 70 μM (Fig. 5).

The inhibition cannot be accounted for, however, simply by removal of the activating Mn^{2+} ion through complexation with the metal-binding agent. An OP concentration of 0.1 mM causes 60% inhibition of the polymerization reaction when activated by Mn^{2+}, 0.2 mM (Fig. 5). Based on the known stoichiometry of $[(OP)_nMn]^{2+}$ complexes this concentration of OP cannot remove a sufficient amount of free Mn^{2+} to lower the activity of the enzyme by means of eliminating this activating metal ion (Fig. 1). It also does not seem possible that $[(OP)_nMn]^{2+}$ complexes could account for the inhibition, since an increase in the concentration of free Mn^{2+} ions from 0.2 to 1 mM decreases inhibition by OP, as would be expected if free OP were responsible for the inhibition. The metal-

¶ OP also inhibits the polymerizations of TTP on an $(rA)_n \cdot (dT)_{12-18}$ template and of dGTP on an $(rC)_n \cdot (dG)_{12-18}$ template catalyzed by the Mg^{2+}-activated polymerase of AMV.

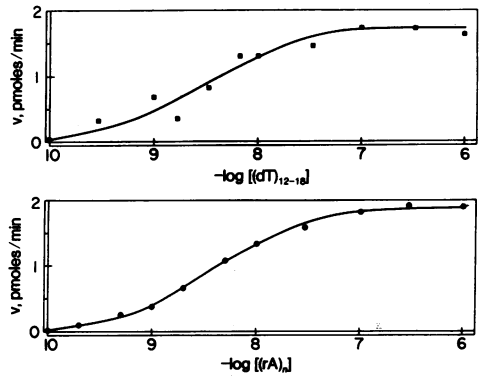

FIG. 3. The dependence of polymerase activity on the concentration of $(dT)_{12-18}$ and $(rA)_n$. The concentration of $(rA)_n$ was held constant at 0.1 μM for the $(dT)_{12-18}$ study. The $(dT)_{12-18}$ concentration was held constant at 1 μM when the $(rA)_n$ concentration was varied.

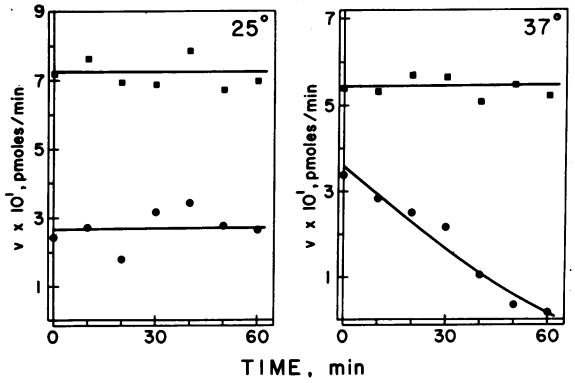

FIG. 4. The activity of the polymerase in the presence (●) and absence (■) of 1,10-phenanthroline, 1 mM. Protein samples (approximately 20 μg/ml) were incubated in the normal diluting buffer ± 1 mM 1,10-phenanthroline. The enzyme was diluted 10-fold into a standard assay at the times given.

complexing properties of OP account for the inhibition: its isomers 1,7- and 4,7-phenanthroline bind metals poorly and do not inhibit the nucleotide polymerization reactions under conditions where 1,10-phenanthroline inhibits completely (Table 1). In summary, it is the capacity of OP to bind to a metal other than manganese that must account for the inhibition. The interpretation is supported by the fact that a number of other, structurally different, metal-binding agents also markedly and instantaneously inhibit the polymerase activity (Table 1). Ethylenediaminetetraacetic acid (EDTA), 8-hydroxyquinoline-5-sulfonate, 8-hydroxyquinoline, and ethyleneglycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA), at concentrations from 0.1 to 0.3 mM inhibit polymerase activity 60% or more.

All of the inhibition data presented here strongly support the hypothesis that the enzyme contains a hitherto unidentified functional and/or structurally indispensible metal of the first transition series or II-B group. However, while necessary, such data are not sufficient, of course, to prove the metal's presence and essentiality. Analytical demonstration of the presence of metal is a prerequisite to such conclusions (5). The paucity of (AMV) RNA-dependent DNA polymerase available to us seemed to preclude definitive metal analyses by existent procedures.

Based on the analysis of standard solutions, microwave-induced emission spectrometry seemed capable of extending the detection limits to 10^{-14} g-atoms of metal (15). The potential of lowering the current limits of sensitivity by a factor of 10^6 offered the possibility of precise, quantitative metal analyses on the μg amounts of enzyme available to us for this purpose. This availability of only small quantities of protein is a prototype of a problem encountered frequently by enzymologists. The presence of zinc, copper, or iron could account for observed inhibition of AMV polymerase (Table 1, Figs. 4 and 5), and these elements and manganese were determined by microwave-induced emission spectrometry. Both metal quenching agents and low-molecular-weight protein contaminants were removed by means of gel exclusion chromatography, resulting in 45-μl fractions, which were analyzed for metals, activity and protein, as under *Methods* (Fig. 6). Enzyme activity emerged in a sharp, narrow band with maximal activity, v, of 2.4 pmoles/min per μl of enzyme. Remarkably, zinc was measured quantitatively with high precision at absolute amounts of from 10^{-11} to 10^{-12} g-atoms. For the most active fractions (Fig. 6, fractions 18, 19, and 20) the Zn/activity and Zn/protein ratios and, hence, specific activity (16) remain nearly constant, indicating purification concomitant with gel exclusion chromatography. Completely analogous results have been obtained repeatedly on samples of this enzyme (25). Zinc is a stoichiometric component of the purified enzyme, while the transition metals, Cu, Fe, and Mn, are present only at or close to the limits of their detection, 10^{-13} to 10^{-14} g-atoms (Table 2). The specific activity, 7.5 pmole/min per μg of protein of the most active fraction (Fig. 6, fraction 19), is three times

TABLE 1. *Effect of metal-binding agents on the (AMV) RNA-dependent DNA polymerase activity**

Metal-binding agent	Concentration (M × 10^4)	v_i/v_c, %
1,10-Phenanthroline	2	4
1,7-Phenanthroline	2	101
4,7-Phenanthroline	3	98
8-Hydroxyquinoline-5-sulfonate	1	33
8-Hydroxyquinoline	3	40
Ethylenediamine-*N,N'*-tetraacetic acid (EDTA)	3	30
Ethyleneglycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA)	3	3

* All rates measured at 25°. See caption of Fig. 1. for other conditions.

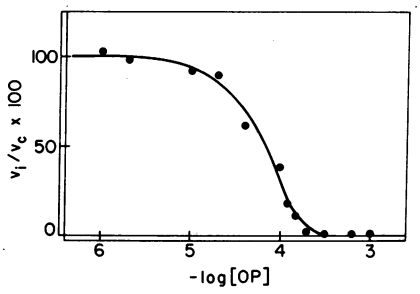

FIG. 5. Instantaneous inhibition of the polymerase activity by 1,10-phenanthroline (OP) at 25°. The agent is added only to the assay system. v_i is velocity in presence of inhibitor, v_c is velocity of control.

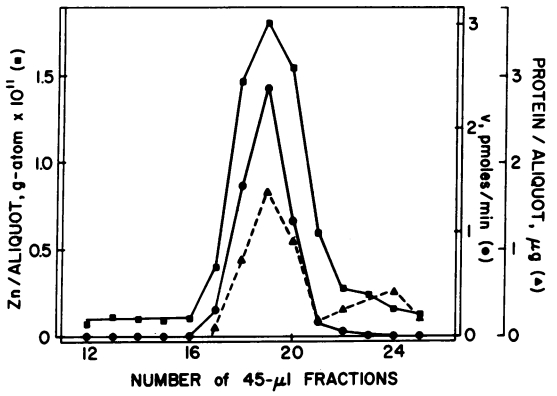

FIG. 6. Distribution of polymerase activity, zinc, and protein in fractions from a G-100 Sephadex column (0.4 cm × 18 cm). Metal contaminants were removed from the column by washing with 4 ml of 1,10-phenanthroline, 0.01 M in pH 7.8 Tris·HCl, 0.01 M, followed by 40 ml of Tris buffer. The column was equilibrated 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 75 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, 45-μl, were collected for duplicate assays of enzyme activities (●), and triplicate zinc analyses (■) and protein content (▲) by the method of Lowry *et al.* (13). The zinc content was calculated by reference to the emission of standard zinc solutions. Zinc and protein content are expressed as g-atoms and μg/5-μl aliquot, respectively, and the velocity for 1 μl of enzyme added to the standard assay.

higher than that of the enzyme placed on the column, and is closely similar to that of highly purified preparations of the enzyme employed for determinations of molecular weight, which ranged from 1.6 to 1.8 × 10^5 (17–19). The detection of 1.8 × 10^{-11} g-atoms of zinc in 1.6 μg of protein corresponds to from 1.8 to 2.0 g-atoms of zinc per molecular weights of enzyme ranging from 1.6 to 1.8 × 10^5 (17–19). The validity and accuracy of the method was established by determination of zinc in a series of zinc metalloenzymes of known metal content and stoichiometry. Table 3 compares the zinc content of carboxypeptidase, carbonic anhydrase, alkaline phosphatase, and alcohol dehydrogenase determined on μg quantities of protein by microwave-induced emission spectrometry and on mg quantities of protein by atomic absorption. The agreement is excellent. Further investigations should permit verification of the stoichiometry and the analysis of the mechanism underlying the reversible and irreversible OP inhibition, which might be due to formation of a mixed complex, E·Zn·OP, or removal of the metal of the enzyme for formation of an $[(OP_{2 \text{ or } 3})Zn]^{2+}$ complex, respectively.

TABLE 2. *Analysis of fraction no. 19, which exhibits maximal activity*

Metal	(g-atom/aliquot) × 10^{13}	[Metal]/[protein], g-atom/mole*
Zn	180	1.8
Mn	5	0.05
Cu	<0.1	<0.001
Fe	<1	<0.01

* Calculations are based on 1.6 μg of protein per aliquot used for metal analysis and on a molecular weight of this polymerase of 1.6 × 10^5 (18). The specific activity of enzyme in fraction 19 of Fig. 6 is 7.5 pmoles/min per μg of protein—similar to that reported for comparable substrates (19)—as compared to 2.5 pmoles/min per μg of protein for the enzyme applied to the column. Assay conditions are those given in the caption of Fig. 1.

TABLE 3. *Microwave-induced emission and atomic absorption spectrometric determination of zinc stoichiometry in zinc metalloenzymes**

	Microwave emission	Atomic absorption
	g-atom Zn/mole of protein	
Bovine carboxypeptidase A	1.0	1.0
Human carbonic anhydrase	1.1	1.1
Horse-liver alcohol dehydrogenase	4.2	3.9
E. coli alkaline phosphatase	3.7	3.6

* Zinc content determined in 0.01 M KCl, 0.01 M Tris on μg quantities of protein for microwave-induced emission spectrometry and mg quantities of protein for atomic absorption.

The role of Mn^{2+} and Mg^{2+} also deserves comment. These ions generally form metal–enzyme complexes (16). They have long been recognized to be important in phosphoryl transfer reactions which they activate, but in such instances they have generally not been found to be intrinsic constituents of the enzymes (16), as in the present instance. However, the requirement for them, in addition to that for the intrinsic zinc in the AMV-polymerase-catalyzed reaction, is not surprising. Mn^{2+} and Mg^{2+} are known to activate a number of different zinc enzymes (6–7, 20–23). In addition, it has become evident that intrinsic metals of metalloenzymes may be essential to their function in two distinct ways. They may either participate directly in catalysis as components of active enzymatic sites or control structure and affect function indirectly. Indeed, atoms of the same metal ion may serve both roles, or atoms of different metal ions may each serve one of these roles in a given enzyme (24). For the nucleotidyl polymerases, the prevalence of these alternatives remains to be determined.

The importance of the role of zinc in the formation of RNA and DNA from DNA templates would seem established. The present studies on the reverse transcriptase from AMV show the importance of zinc in the formation of DNA from RNA templates and demonstrate a relationship between zinc and a leukemic process postulated long ago (1–4). The data underline the participation of zinc in nucleic acid metabolism and bear importantly on the lesions which accompany leukemia and zinc deficiency.

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