ZINC IN REVERSE TRANSCRIPTASE

Bernard J. Poiesz, Narayana Battula, and Lawrence A. Loeb

The Institute for Cancer Research, The Fox Chase Center for Cancer and Medical Sciences, Philadelphia, Pennsylvania 19111

Received January 2,1974

SUMMARY

Purified DNA polymerase ("reverse transcriptase") from avian myeloblastosis virus was analyzed for zinc by atomic absorption spectroscopy. Tightly bound zinc was present in a stoichiometry approximating 1 gm atom/mole of enzyme. Inhibition by the chelating agent, ortho-phenanthroline, and lack of inhibition by its non-chelating analog, meta-phenanthroline, suggest that the bound zinc functions in catalysis.

Recent evidence indicates that DNA polymerases are zinc metalloenzymes. Zinc is present in homogeneous preparations of DNA polymerases of E. coli, sea urchin, and T_4 bacteriophages in amounts corresponding to 1.0, 4.2 and 1.0 gm atoms/mole of enzyme, respectively (1, 2). Inhibition of DNA polymerases by chelators implies that zinc functions in catalysis (1); studies measuring nuclear magnetic relaxation of bromide ions suggest that the enzyme-bound zinc coordinates with DNA. Most importantly, removal of zinc from E. coli DNA polymerase I is accompanied by a proportional loss of activity; addition of zinc to the apoenzyme results in full restoration of activity (2). If the DNA polymerases ("reverse transcriptases") from RNA tumor viruses also contain zinc, the mechanism of catalysis by these enzymes, which effectively copy RNA templates, may be identical with that of other DNA polymerases. We now report zinc to be present in a "reverse transcriptase".

Accurate analysis of zinc in DNA polymerase from avian myeloblastosis virus (AMV) was made difficult because the enzyme was obtainable in relatively small amounts and zinc, being a rather ubiquitous cation, is a contaminant of most reagents and laboratory apparatus. Accordingly, all solutions used in

the purification were rendered zinc free (i.e. less than 0.2 μM) by repeated treatment with the chelating resin Chelex-100 (Bio-Rad Laboratories, Richmond, California) (2). Isoelectric focusing was chosen as the final enzyme purification step since any contaminating unbound Zn^{2+} would be separated from the enzyme by migrating toward the cathode (3).

Avian myeloblastosis virus was initially isolated from the plasma of infected chickens (gift of Dr. Beard) by velocity and equilibrium centrifugation. Thereafter, the purified virions were disrupted with Triton-X and the DNA polymerase was purified by the procedure of Hurwitz (4). Two peaks of polymerase activity designated PC-I and PC-II were obtained after phosphocellulose chromatography. In accord with the results of Grandgenett et al. (5), we find that upon SDS-gel electrophoresis the minor activity, PC-I (M.W. 65,000), contains one contaminant which they report to be removable by glycerol gradient centrifugation. The major activity, PC-II, displayed on gel electrophoresis two distinct bands of molecular weight 65,000 and 110,000, designated as the α- and β-subunits, respectively. Specific inhibition by antisera (5) indicates that PC-I and the α-subunit of PC-II are enzymatically active, immunologically related, and presumably identical. The function of the β-subunit is unknown.

The distribution of zinc as determined by atomic absorption spectroscopy after isoelectric focusing of PC-I, compared with that from a blank column to which only buffer was added, is shown in Fig. 1. As indicated, free Zn^{2+} is concentrated at the cathode. The polymerase is found at an isoelectric point (pI) of about 7.0 and the amount of activity correlates closely with the concentration of zinc. It is unlikely that the PC-I fraction sequestered zinc during storage since it was present in a 50-fold excess of bovine serum albumin (pI, 5.5) and no zinc appears at this region of the column. Moreover, the zinc peak cannot be explained by coincidental binding of zinc to carrier ampholytes because no such zinc peak appears on the blank column. The amount of protein in the peak enzyme fraction was estimated from densitometry measure-

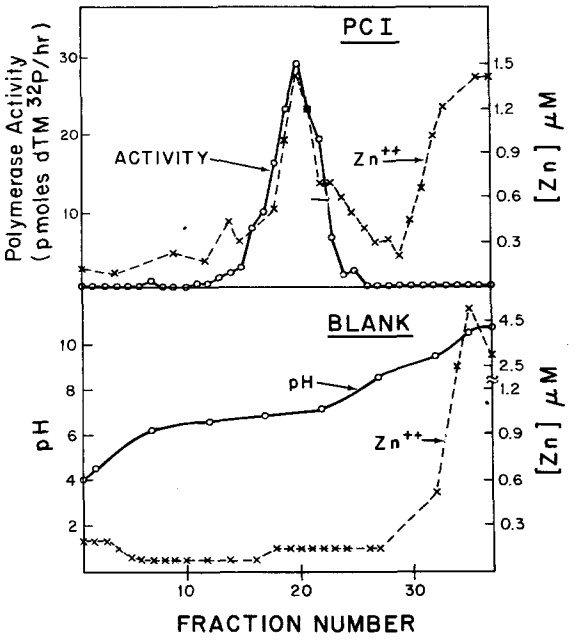

Fig. 1. Correlation of zinc content with polymerase activity upon electrofocusing AMV DNA polymerase (PC-I). Electrofocusing was carried out in plastic minicolumns with modification of the technique outlined by Cohen et al. (6). The columns consisted of two plastic pipettes (Falcon #7529) connected at the tips by Tygon tubing. The entire apparatus was extensively washed with 0.5 mM o-phenanthroline and distilled water to render it zinc free. The dense solution (0.2 M phosphoric acid in 80% w/v sucrose surrounded the anode and filled one pipette, tubing and the tip of the adjoining pipette. A 6 ml 10-40% glycenol gradient, containing 5 mM β-mercaptoethanol and 0.8% carrier ampolytes pH 3-10 (LKB) was layered upon the top of the dense solution in the tip of the second pipette. PC-I (50 μg protein) in 0.3 ml of 20% glycerol, 0.02 M dithiothreitol, 0.02 M tris-HCI (pH, 7.8) was introduced into the middle of the gradient from the top of the pipette by means of thin tygon tubing. Finally, 2 ml of 2% (V/v) ethylene diamine was placed at the cathode end of the gradient. Platinum electrodes were inserted in the top of each pipette and electrofocusing was carried out for only 2 hours at 600 V. Fractions (0.15 ml) were then collected into wells in Zn^{2+} free plastic plates from the anode end of the gradient. Greater resolution can be obtained by electrofocusing for longer times, 4 to 6 hours, however, zinc is progressively leached out of the apparatus and increases the background. Zinc content was determined on 1 μl samples of each fraction using a Techtron atomic absorption spectrometer equipped with a carbon rod attachment. The bottom graph is the zinc content of an identical gradient electrofocused without added enzyme. The pH distribution was the same in both gradients.

DNA polymerase activity was determined in a reaction mixture of 0.05 ml containing 2.5 γ moles Tris-HCI (pH, 8.4), 20 μmoles dithiothreitol, 2.5 μmoles α-^{32}P-dTTP, 10 μg bovine serum albumin, 1 μmole KCl, 0.5 μmoles $MgCl_2$, 2 μg poly rA·oligo dT (ratio 1:1.5) and 5 μl of each fraction. Incubation was for one hour at 37° and incorporation expressed in pmoles dTMP into an acid-insoluble and alkali resistant product.

ments on Coomassie blue stained SDS-gels of the enzyme after electrophoresis and removal of the ampholytes by extensive dialysis. The electrofocusing had not separated the contaminating protein. Estimating the enzyme to be 50% homogeneous and to have a M.W. of 65,000 from gel electrophoresis, the enzyme concentration would be 1.2 μM yielding a ratio of approximately 1 gm atom Zn/mole of enzyme. A similar stoichiometry was found in the electrofocusing analysis of two other separately purified samples of PC-I.

The electrofocusing profile of the PC-II fraction is shown in Fig. 2.

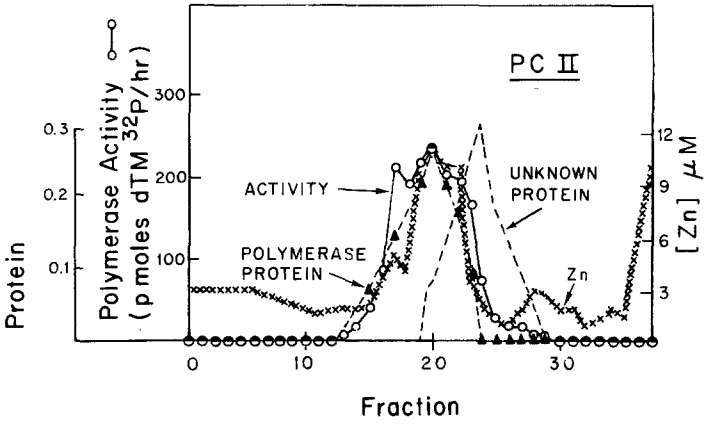

Fig. 2. Electrofocusing of AMV DNA polymerase (PC-II). Methods are detailed in legend to Fig. 1. In addition, aliquots of fractions 15 through 30 was subjected to SDS-polyacrylamide gel electrophoresis, stained with Coomassie blue, and the relative amounts of polymerase and an unknown protein were determined by densitometry.

This particular preparation had a large amount of a contaminating viral protein of M.W. 25,000, pI 7.8 (fractions 18-30). The specific nature of the association between zinc and AMV DNA polymerase is emphasized by the fact that the zinc correlated with polymerase activity and not with the contaminating protein. From the specific activity of a homogeneous sample of PC-II assayed at the same time and assuming its M.W. to be 175,000 we estimate the polymerase concentration in the peak fraction to be 6.4 μM. This gives a ratio of 1.3 gm atoms Zn/mole of PC-II. In addition a small quantity of homogeneous enzyme (PC-II)

was analyzed by electrofocusing; the location and amount of zinc coincided with polymerase activity.

The DNA polymerase of Rous sarcoma virus has already been shown to be inhibited by chelating agents (8, 9). We find that the DNA polymerase from avian myeloblastosis virus is also inhibited by ortho-phenanthroline (o-phen.), a chelator with a high affinity for Zn^{2+}, and not by its non-chelating analogue, meta-phenanthroline (m-phen.) (Fig. 3). For both PC-I and PC-II, 50% inhibition

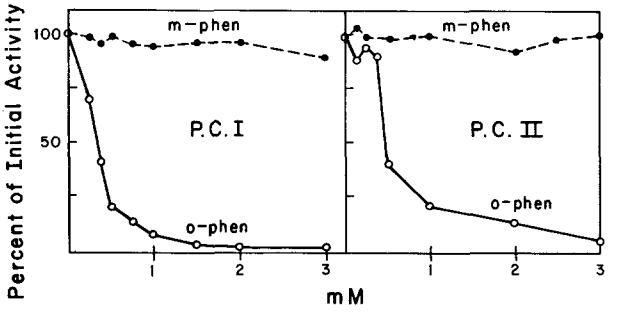

Fig. 3. Comparative effects of ortho- and meta-phenanthroline. PC-I or PC-II was incubated with the indicated concentration of o-phen or m-phen for 30 min at 10°C. Polymerase activity was then determined as described in Fig. 1 in the presence of the same concentration of either o-phen or m-phen. The initial activity with PC-I and PC-II was 20 and 140 pmoles $dTM^{32}P$/30 min at 37°C.

is attained between 0.3 - 0.4 mM and there is almost complete inhibition at 2-3 mM o-phen. This inhibition is not due to chelation of the added divalent cation since the Mg^{2+} was present in excess, 8 mM, and o-phen. has a 10^5 greater affinity for Zn^{2+} than for Mg^{2+} (7). Most importantly, no inhibition of either PC-I or PC-II is observed with m-phen., a non-chelating analogue having a similar aromatic structure, thereby suggesting that the inhibition by o-phen. is excerted on the active subunit is related to its chelating properties.

The presence of stoichiometric amounts of zinc in AMV DNA polymerase and the specific inhibition by o-phen. suggests but does not prove that the DNA polymerase from RNA tumor viruses, like other cellular DNA polymerases, are zinc

metalloenzyme. During the course of this work, Valenzuela et al. (8) reported that a variety of DNA and RNA polymerases are inhibited by chelators. Levinson et al. (9) reported that the "reverse transcriptase" from Rous sarcoma virus was inhibited by thiosemicarbazones and suggested that the inhibition might be related to the copper-binding properties of these antiviral agents. Further studies are necessary to determine if metals other than zinc are present on these enzymes and if any chelating agents interact differently with metals on cellular DNA polymerases and DNA polymerases from known animal tumor viruses.

Acknowledgements

We thank Drs. A. S. Mildvan and F. Zweidler for generous counsel. This investigation was supported by grants from The National Institutes of Health (CA-12818, CA-11835) and The National Science Foundation (GB-36812), by grants to this Institute from the National Institutes of Health (CA-06927, RR-05539), by an appropriation from the Commonwealth of Pennsylvania. B. Poiesz is a medical student, N. Battula is a predoctoral associate, graduate group on Molecular Biology, and L. A. Loeb is also a member of the Department of Pathology, University of Pennsylvania.

References

1. Slater, J. P., Mildvan, A. S., and Loeb, L. A. Biochem. Biophys. Res. Commun., 44, 37 (1971).
2. Springgate, C. F., Mildvan, A. S., Abramson, R., Engle, J. L., and Loeb, L. A. J. Biol. Chem. 248, 5987 (1973).
3. Vesterberg, O. Ann. N.Y. Acad. Sci. 209, 23 (1973).
4. Hurwitz, J. and Leis, J. P. J. Virol. 9, 116 (1972).
5. Grandgenett, D. P., Genand, G. F., and Green, M. Proc. Nat. Acad. Sci. U.S. 70, 230 (1973).
6. Cohen, L. H., Penner, P. E., and Loeb, L. A. Ann. N.Y. Acad. Sci. 209, 354 (1973).
7. Sillen, L. G. and Martell, A. E. Chem. Soc. Special Publ. 17 (1964).
8. Valenzuela, P., Morris, R. W., Faras, A., Levinson, W., and Rutter, W. L. Biochem. Biophys. Res. Commun. 53, 1036 (1973).
9. Levinson, W., Faras, A., Woodson, B., Jackson, J., and Bishop, M. J., Proc. Nat. Acad. Sci. U.S. 70, 164 (1973).