Volume 3 no.9 September 1976 Nucleic Acids Research

Influence of phosphate on activity and stability of reverse transcriptase from avian myeloblastosis virus

Chris M. Tsiapalis, G. Edwin Houts and Joseph W. Beard

Life Sciences Research Laboratories, 1509½-49th Street South, St. Petersburg, FL 33707, USA

Received 14 May 1976

ABSTRACT

Activity of RNA-dependent DNA polymerase (RDDP) from avian myeloblastosis virus (AMV), either in purified form or in virus lysates, was increased by phosphorylation. Stability of RDDP in lysates buffered with phosphate was much greater (no loss of activity in 48 hours at 4°) than that in lysates buffered with Tris-Cl (76% loss). Activity lost in the Tris-buffered extracts was completely restored by phosphorylation. The findings suggested that AMV RDDP activity is influenced by the degree of phosphorylation of the enzyme or enzyme-associated proteins and that this chemical modification is mediated by protein phosphokinase and phosphoprotein phosphatase present in crude extracts of purified AMV. Application of these results provided the basis of procedures whereby RDDP can be recovered in significantly higher yield and purity than formerly.

INTRODUCTION

Enzyme modification is important in the regulation of many metabolic processes. One of the most widely studied modifications is protein phosphorylation-dephosphorylation (1). The enzymatic machinery that can carry out this protein modification in AMV and other oncornaviruses has been identified (2-4) and recent observations *in vitro* (5) indicate that RDDP from Rous sarcoma virus (RSV) may be one of these modified proteins. The possibility exists that phosphorylation and dephosphorylation occurs with RDDP of other and perhaps all oncornaviruses and thus may constitute a regulatory mechanism of considerable biological significance. Effects of phosphorylation and dephosphorylation on RDDP in oncornaviruses was not alluded to in the many published reports on purification and characterization of this enzyme (6-13). This reversible chemical modification may be partly responsible for variations observed on the stability, specific activity, and purity of the enzyme (6-13).

The possibility that RDDP may also be subject to irreversible chemical modification will be discussed elsewhere.

The present communication provides evidence that AMV RDDP activity in both crude and purified form is influenced by phosphorylation and dephosphorylation in vitro. On this basis, appropriate changes in the methods used for isolation of RDDP, have resulted regularly in a 6-10 fold increase in yield and purity, and in the identification of a small, non-dialyzable acidic phosphoprotein. The properties of this protein and its implications on the RDDP-catalysed DNA synthesis will be reported elsewhere (C.M.T., submitted to Nature). Results on the purification of a protein kinase, detection of a phosphoprotein phosphatase and phosphoproteins in purified AMV will appear elsewhere (C.M.T., G.E.H. and J.W.B. submitted to J. Virol).

MATERIALS AND METHODS

Purification of AMV and RDDP. Plasma from blood drawn into heparin from the heart of chicks with myeloblastosis was spun twice at 3,000 X g for 10 minutes, and virus was sedimented at 59,000 X g for 40 minutes. Pellets resuspended by stirring with a teflon pestle and homogenization by 8-10 strokes in a loose-fitting tissue grinder were centrifuged at 3,000 X g for 10 minutes. The resulting pellet was resuspended and spun at 3,000 X g for 10 minutes, the suspension was pooled with the first low speed supernate, and the virus was sedimented again at 59,000 X g for 40 minutes. This cycle of low and high speed spinning was repeated twice more. The final pellet was suspended in either 10 mM Tris-Cl (pH 8.3) (TNE) or 10 mM potassium phosphate (pH 7.2) (PNE) in 0.15 M NaCl, 1 mM EDTA, and virus concentration was adjusted to 0.02-0.05 gm per ml wet weight determined by ATPase activity (14) . Virus was lysed at 4° with Triton X-100, in KCl, and DOC and chromatographed on DEAE (DE-52, 4.2 X 8 cm) and phosphocellulose (P-11, 2.6 X 10 cm) as described (7). Active fractions from an additional chromatographic step on phosphocellulose (1.6 X 15 cm) were concentrated by dialysis vs. 50% glycerol containing 0.2 M

potassium phosphate (pH 7.2), 0.2% Triton X-100, and 2 mM dithiothreitol (DTT) and then passed through a 2.6 X 90-cm column of Sephadex G-200 equilibrated with the same buffer solution but with 10% glycerol. The product was re-concentrated as described above. One unit of RDDP activity is expressed as that incorporating 1 nanomole of dTMP into an acid-insoluble product in 10 minutes at 37°. The reaction medium was 50 mM Tris-Cl (pH 8.3), 0.5 mM [^{3}H]-TTP, 6 mM $MgCl_2$, 0.2 mM poly(A)$_n$.dT$_{12-18}$ (10:1), 40 mM KCl and 100 µg BSA per ml.

Phosphorylation. The addition of phosphate to protein(s) in crude lysates (66.6 µg) or in purified RDDP (2.3 µg) was detected as acid precipitable [^{32}P]- formed during incubation at 37° under the following reaction conditions: 25 mM Tris-Cl (pH 8.3); 20 mM $MgCl_2$; 10 mM DTE; 0.02% Triton X-100; 38 µM [^{32}P]-ATP (7,675) c/m/pmole); purified AMV as phosphoprotein kinase (75 µg virus protein). Aliquots of the 100 µl reaction mixture were transferred to GF/C discs and processed for scintillation counting as described (15). When correlating phosphorylation with RDDP activity, duplicate samples were phosphorylated with non-radioactive ATP and then assayed for polymerization of [^{3}H]-TMP (15).

Dephosphorylation. The effect of E. coli alkaline phosphatase (BAP) on the activity of purified RDDP was tested in the presence of 20 mM orthophosphate (pH 7.2) or after its removal. To remove orthophosphate prior to treatment with BAP, RDDP was dialysed vs. 10 mM Tris-Cl (pH 8.3) containing 2 mM DTT, 0.1 mM EDTA, 0.2% Triton X-100, and 50% glycerol. Aliquots of RDDP (4.6 µg protein) then were incubated for 10 minutes at 35° in the presence of 50 mM Tris-Cl (pH 8.3), 6 mM MgCl , and 20 µg of BAP (active or heat denatured). The samples were chilled in ice and assayed for RDDP activity as described above except 50 mM potassium phosphate (pH 8.0) and 0.5 mM $MnCl_2$ were used in place of Tris-Cl and $MgCl_2$.

Materials. Synthetic polyribonucleotides, polydeoxyribonucleotides, and oligodeoxyribonucleotides were obtained from P-L Biochemicals and/or Collaborative Research, Inc.; [^{3}H]-TTP (47 Ci/mM) and [^{3}H]-poly(A) (8.5 mCi/mmole of polynucleotide phosphorous) from Schwarz/Mann; and poly(A) was purchased from Miles Laboratories. Bacterial alkaline phosphatase (free of ribonuclease activity) was purchased from Worthington Biochemical Co. Whatman DEAE-cellulose (DE-52), phosphocellulose (P-11), and GF/C paper for filter discs came from Reeve Angel; and Sephadex G-200 and blue dextran were obtained from Sigma Chemical Co. Gamma [^{32}P] labeled ATP was obtained from New England Nuclear.

RESULTS

Purification and stability. Yields of RDDP from AMV vary from about 240 to about 5,000 units per gm of virus (11-13). Efforts to increase the yield were undertaken by systematic alterations of the isolation procedures. Factors first noted strongly to affect the outcome were (a) reduction of the period of lysis in TNE ordinarily used prior to addition of phosphate and (b) adjustment of AMV concentration in the lysis mixture to 0.02 to 0.05 gm per ml. Table 1

TABLE I

Isolation and Activity of Reverse Transcriptase from Avian Myeloblastosis Virus*

Fraction	Protein (mg)	Units of Activity	Specific Activity
1 Crude Lysate (TNE)	1,112	541,557	487
2 DEAE	58.8	495,232	8,274
3 First Phosphocellulose	18.1	400,970	22,200
4 Second "	12.8	376,476	29,529
5 G-200	4.8	295,562	61,193

*All values were derived from 9.3 gm of purified AMV in suspension adjusted to 0.044 gm per ml prior to lysis.

summarizes the results with a specimen of 9.3 gm of virus lysed in TNE for 1 hour and then diluted with 10 mM potassium phosphate (pH 7.2), 2 mM DTT, 0.2% Triton X-100 and 10% (v/v) glycerol. Enzyme recovery, 295,562 units, from the crude lysate, 541,557 units, was about 55% or about 31,781 units per gm of virus. The specific activity, 61, 193 nmoles per 10 minutes per mg, reveals

an enzyme purification of about 125 fold from the detergent lysate with a 232-fold decrease in protein. Such preparations have already being successfully used in gene synthesis by others (16-18).

In contrast to rapid inactivation of RDDP in TNE, the enzyme was highly stable in lysates prepared in phosphate buffer (Fig. 1A). After 1-hour at 4^{o}, the activity of RDDP in PNE and TNE were 55,312 and 46,640 units per gm, respectively, of AMV. After 4 days at 4^{o}, RDDP activity in PNE remained at 46,419 units per gm of AMV (84% of the initial value) but had declined to 4,751 units per gm (10% of the original activity) in TNE.

Phosphorylation and dephosphorylation. Although phosphate consistently exerted a stabilizing influence on RDDP, the mechanism was obscure. A possible explanation was suggested by the observation of Lee et al (5) showing that RDDP from RSV was inactivated by alkaline phosphatase from E. coli and, moreover, that the enzyme could be reactivated by a protein phosphokinase from RSV-transformed chick embryo fibroblasts. To test this possibility, the PNE and TNE lysates (Fig. 1A) were phosphorylated with gamma $[^{32}P]$-ATP (Fig. 1B). Significant incorporation of trichloroacetic acid-insoluble $[^{32}P]$ radioactivity was observed only with the TNE lysate (Fig. 1B). The results of this experiment indirectly suggested dephosphorylation of AMV phosphoproteins in the TNE lysate and inhibition of this reaction in the PNE lysate.

On the basis of the above observations and the knowledge that AMV virions contain enzymes that catalyze the turnover of phosphoproteins (2-4), the following experiments were done to determine if AMV RDDP activity is affected by phosphorylation-dephosphorylation. Fig. 1C compares the rates of protein phosphorylation of two aliquots of TNE lysate (4,751 units RDDP activity per g AMV see Fig. 1A) in the presence and absence of endogenous AMV protein kinase. At the completion of phosphorylation with nonradioactive ATP, the rates of $[^{3}H]$-TMP incorporation were examined (Fig. 1D). On the basis of these polymerization rates, essentially complete restoration of RDDP activity (about

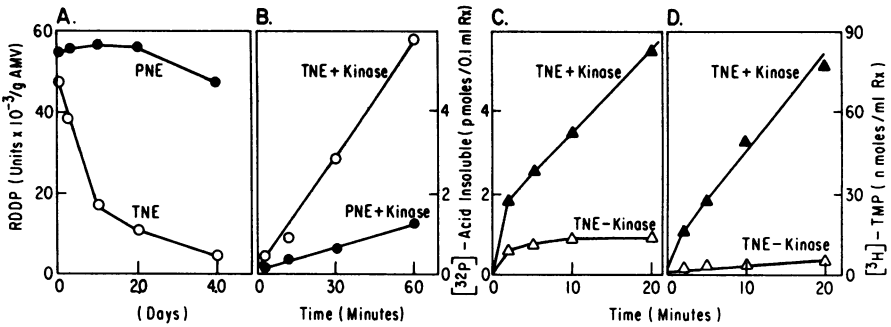

Fig. 1. A. Stability of RDDP activity at 4° in detergent lysates of AMV (0.044 g/ml) prepared in PNE and TNE. After 1:5 dilution in the respective buffer of lysis, 5-μl aliquots (6.66 μg of protein) were assayed for RDDP activity as described in Methods. B. Comparison of protein phosphorylation between PNE and TNE lysates after 4 days incubation at 4° (See Fig. 1A). C. Kinetics of protein phosphorylation in aliquots from the TNE lysate (Fig. 1A) in the presence and absence of endogenous AMV protein kinase. D. Effect of protein phosphorylation on the activity of crude RDDP prepared in TNE (Fig. 1A). At the completion of the phosphorylation reaction in the presence of nonradioactive ATP, RDDP was assayed by measuring the rate of [^{3}H]-TMP polymerization as described in Methods.

47,000 units per g AMV) was obtained after phosphorylation. The same experiment was repeated with purified RDDP enzyme (Fraction 5, Table 1). Fig. 2D compares the rates of protein phosphorylation of two identical aliquots of enzyme in the presence and absence of endogenous AMV protein kinase. At the completion of an identical experiment with nonradioactive ATP, the rates of [^{3}H]-TMP incorporation were examined (Fig. 2C). A significant increase in the rate of polymerization (about 2-fold) is shown with the enzyme subjected to phosphorylation. Furthermore, when purified RDDP enzyme stored in Tris buffer was subjected to treatment with bacterial alkaline phosphatase (BAP) under conditions allowing hydrolysis of esterified phosphate groups from protein, a decrease in RDDP activity was observed (Fig. 2B). In a similar experiment (Fig. 2A), no detectable change in RDDP activity was observed when exposed to BAP in the presence of 20 mM phosphate buffer.

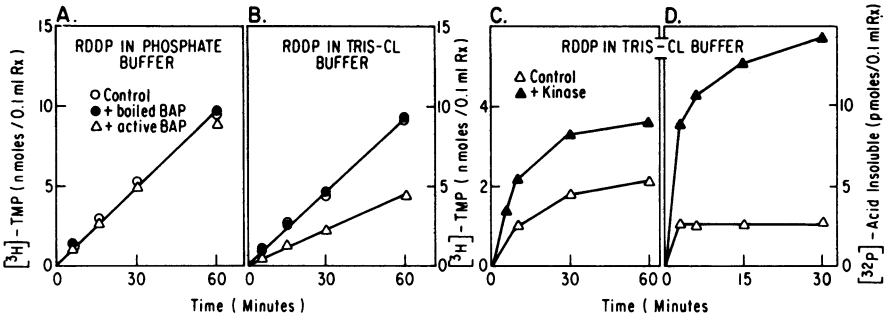

Fig. 2. A,B. The effect of bacterial alkaline phosphatase (BAP) on the activity of purified RDDP (Fraction 5, Table 1) in the presence of orthophosphate or Tris-Cl. C. Effect of protein phosphorylation on the activity of purified RDDP (Fraction 5, Table 1). D. Kinetics of protein phosphorylation of an enzyme fraction identical to that used in Fig. 2C.

DISCUSSION

The studies reported here clearly demonstrated the stabilizing influence of phosphate on RDDP of AMV and showed that enzyme activity lost in the absence of phosphate could be restored by phosphorylation. The influence of phosphate in the maintenance of RDDP activity is well illustrated by the high levels of activity, about 32,000 units per gm of AMV, recovered from preparations of virus lysed in Tris-Cl and quickly exposed to phosphate (Table 1). Effects of phosphate in the protection of RDDP activity was especially evident by comparison of the yields of activity from lysates prepared in phosphate buffer with those obtained in Tris-Cl (Fig. 1A). Although the initial activities were approximately the same, i.e., 50,000 to 56,000 units per gm of virus, activity of the lysate in Tris-Cl declined rapidly, while at least 80% of that in the lysate in phosphate buffer persisted for the period of at least 4 days at 4°. The basis for the influence of AMV concentration in the lysate mixture on yield of RDDP is not clear, but may be accounted by the deleterious effect of proteinase activity as will be discussed elsewhere. In addition, results in this communication indicate that AMV RDDP activity is influenced by the degree of phosphorylation of the enzyme or enzyme associated proteins, as is the case for

RSV RDDP activity (5). These results show that the influence of phosphate observed in the maintenance of RDDP activity (Fig. 1A) may very well be due to its function as a potent inhibitor of endogenous phosphoprotein phosphatase (2-4 and our results to be published elsewhere). On the other hand, the rapid decline in RDDP activity observed with AMV lysates prepared in Tris-Cl (Fig. 1A), is well correlated with protein dephosphorylation (2-4), since essentially complete reactivation resulted after phosphorylation of the lysate (Fig. 1C). The same results were obtained with purified RDDP enzyme (Fig. 2C, D). Furthermore, we obtained evidence that alkaline phosphatase decreased the activity of purified RDDP (Fig. 2B). Thus, the phosphorylated RDDP form of the enzyme appears to be more active (Fig. 2C) than the dephosphorylated form RDDP (Fig. 2B). Chemical proof for the existence of these two enzyme forms and the mechanism of RDDP activation by phosphorylation will be presented elsewhere (C.M.T. submitted to Nature). Reversible chemical modification of several enzyme systems *via* phosphorylation-dephosphorylation represents a regulatory mechanism of biological significance (1).

Exploitation of the above findings has afforded refinement of procedures of practical value in the mass production of RDDP from AMV. Needs for the enzyme, scarcely possible to meet with yields of 2,000 to 5,000 units from 1 gm of virus, are readily satisfied by recoveries of 30,000 or more units from the same amount of the agent. This becomes the more striking in view of the difficulty and expense attending collection of AMV from small chicks with myeloblastic leukemia. Recognition of the influence of phosphate has encouraged further efforts already undertaken to identify and characterize the nature of the reversible chemical modifications involved and their effects on the structure and function of RDDP both *in vitro* and *in vivo*.

ACKNOWLEDGEMENTS

The authors thank Mrs. Julie Norman, Mr. Gary Hardy and Mrs. Dorothy Beard for assistance in obtaining virus and preparing RDDP. This study was con-

ducted under Contract N01 CP 33291 within the Virus Cancer Program of the National Cancer Institute, N.I.H., P.H.S.

REFERENCES

1. Segal, H. L. (1973) Science 180, 25-32.
2. Strand, M. and August, J. T. (1971) Nature (New Biology) 233, 137-140.
3. Silberstein, H. and August, J. T. (1973) J. Virol. 12, 511-522.
4. Silberstein, H. and August, J. T. (1973) Abstr. Annu. Meet. Amer. Soc. Microbiol. p. 256.
5. Lee, S. G., Miceli, M. V., Jungmann, R. A. and Hung, P. P. (1975) Proc. Nat. Acad. Sci. U.S.A. 72, 2945-2949.
6. Temin, H. M. and Baltimore, D. (1972) Advances in Virus Research vol. 17, 129-186.
7. Temin, H. M. and Mizutani, S. (1974) in The Enzymes vol. 10, pp. 211-235, Academic Press, New York.
8. Baltimore, D. (1974) Cold Spring Harbor Symposia on Quantitative Biology vol. 39B, pp. 1187-1200.
9. Green M. and Gerard, G. F. (1974) in Progress in Nucleic acid Research and Molecular Biology, vol. 14, pp. 187-334, Academic Press, New York.
10. Grandgenett, D. P., Gerard, G. F. and Green, M. (1973) Proc. Nat. Acad. Sci. U.S.A. 70, 230-234.
11. Hurwitz, J. and Leis, J. P. (1972) J. Virol. 9, 116-129.
12. Kacian, D. L. and Spiegelman, S. (1974) in Methods in Enzymology Vol. 29, pp, 150-173.
13. Kacian, D. L., Watson, K. F., Burny, A. and Spiegelman, S. (1971) Biochem. Biophys. Acta 246, 365-383.
14. Beaudreau, G. S. and Becker, C. (1958) J. Natl. Cancer Institute 20, 339-349.
15. Bollum, F. J. (1959) J. Biol. Chem. 234, 2733-2734.
16. Krueger, L. J., Weiss, G. B., Merrick, W. C., Lloyd, M. A., and Anderson, W. F. (1976) Nature 260, 363-365.
17. Monahan, J. J., Harris, S. E. Woo, S. L. C., and O'Malley, B. W. (1976) Biochemistry in press.
18. Efstratiadis, A., Kafatos, F. C., Maxam, A. M., and Maniatis, T. (1976) Cell 7, 279-288.