ESSENTIAL ARGINYL RESIDUES IN REVERSE TRANSCRIPTASE

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Summary

The RNA dependent DNA polymerases -- reverse transcriptases -- from avian, feline and simian type C RNA viruses are rapidly inactivated by butanedione in borate buffer, a reagent which is highly selective for the modification of arginyl residues. The pronounced enhancement by borate on the inactivation of reverse transcriptase implicates essential arginyl residues, and substrate protection experiments with the polymerase from avian myeloblastosis virus indicate that these residues function in binding the RNA template to the active site.

Introduction

Previous studies have demonstrated a catalytic role for zinc in the RNA dependent DNA polymerases -- reverse transcriptases -- from avian myeloblastosis virus (AMV) (1,2) and mammalian type C RNA viruses (3,4). The effect of butanedione on the activity of these polymerases has been examined in order to determine the identity of amino acid residues which participate in the catalytic process. This reagent reacts specifically with arginyl residues and has been found to inactivate numerous other enzymes which depend on anionic cofactors or act on anionic substrates (5-9). In each of these cases, inactivation was shown to be due to modification of essential arginyl residues. We have now obtained similar results with three different reverse transcriptases suggesting that arginyl residues are critical for the action of these enzymes as well.

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Materials and Methods

AMV reverse transcriptase was prepared and kindly provided by Dr. J.W. Beard. The enzyme was stored at -20°C in 50% glycerol, 0.2 M potassium phosphate, pH 7.2, 2 mM dithiothreitol and 0.2% Triton X-100. Assays were carried out at pH 7.8 and 25°, and contained the following concentrations of reagents in a total volume of 100 µl: Tris-Cl, 100 mM; KCl, 80 mM; $MnCl_2$, 0.2 mM; dithiothreitol, 2 mM; poly rA, 1 µM; oligo dT_{12-18}, 1 µM; [^{3}H]TTP, 2.4 µM. Assays were terminated with 10% trichloroacetic acid. The polymerases from woolly monkey Type C virus, WLV(WSV), (from cell line KW-23) and from Rickard feline leukemia virus, FeLV, (from cell line F422) were prepared and stored as previously described (3). The assay for these enzymes was identical to that for the AMV polymerase, except that the concentration of KCl was 60 mM. Poly rA, poly dA and oligo dT_{12-18} were obtained from Collaborative Research, Waltham, Mass., and [^{3}H]TTP, 55 Ci per mole, from Schwarz Mann. 2,3-Butanedione was a product of Aldrich Chemical Corp. All chemicals were reagent grade.

The polymerases were modified with butanedione at 25°. All modification mixtures contained the following concentrations of reagents in a final volume of 60 µl: HEPES, 50 mM, pH 8.3; KCl, 10 mM; dithiothreitol, 1 mM; Triton X-100, 0.001%; butanedione, 15 mM. Other reagents also included in selected modification mixtures were: borate, 50 mM, pH 8.3; poly rA, 1.3 µM; oligo dT_{12-18}, 2.5 µM; TTP and MnTTP, 0.2 mM. At various times 10 µl aliquots were withdrawn and diluted into the standard assay mixture. Activity is expressed as a ratio of the activity of the modified enzyme, v, to that of the unmodified enzyme (subjected to identical conditions but in the absence of butanedione), v_c, multiplied by 100.

Results and Discussion

Butanedione selectively modifies arginyl residues of enzymes, and this modification is enhanced in borate buffer (5,9). The time course for the inactivation of AMV reverse transcriptase by 15 mM butanedione at pH 8.3 is shown in Figure 1. When the modification is carried out in 50 mM HEPES buffer (which itself does not react with butanedione), inactivation of the enzyme is very slow. However, in the presence of 50 mM borate, but under otherwise identical conditions, the polymerase is inactivated rapidly with a $t_{1/2}$ of 4 minutes. After 10 minutes of modification in the absence of borate, approximately 90% of the control activity is retained, but only 15% activity remains in its presence. Borate alone, in the absence of the diketone, does not affect activity. The known specificity of butanedione, together with the capacity of borate to enhance its reactivity, strongly suggests that inactivation of AMV reverse transcriptase is due to the modification of one or more essential arginyl residues. Direct evidence of the modification of

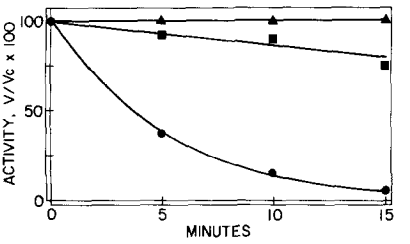

Figure 1. Time course of the inactivation of AMV reverse transcriptase by 15 mM butanedione in 50 mM HEPES, 10 mM KCl, 1 mM dithiothreitol, 0.001% Triton-X-100, pH 8.3. Modification was carried out in the presence (●) or absence (■) of 50 mM borate. The controls (▲) were subjected to identical conditions, but in the absence of butanedione.

arginyl residues can generally be obtained by amino acid analysis, but this was precluded in the present study by insufficient quantities of enzyme.

Butanedione in borate buffer similarly inhibits the reverse transcriptases from mammalian type C RNA viruses. Modification of the enzyme from feline leukemia virus with 15 mM butanedione in 50 mM borate, pH 8.3, under conditions identical to those employed for the AMV polymerase, results in a loss of activity with a $t_{1/2}$ of 8 minutes, while the polymerase from woolly monkey virus is inactivated with a $t_{1/2}$ of 16 minutes. In both cases borate is required for maximal inactivation, suggesting again that these reverse transcriptases, like that from AMV, also contain essential arginyl residues.

It has been proposed that arginyl residues play a general role in binding anionic substrates and cofactors to enzyme active sites (7). We have tested this hypothesis by carrying out the modification of AMV reverse transcriptase with 15 mM butanedione, 50 mM borate, pH 8.3, in the presence of various combinations of substrates (Table I). Neither 0.2 mM TTP, 0.2 mM MnTTP, nor the oligo dT_{12-18} initiator, at 2.5 μM, provide significant protection against inactivation. However, in the presence of 1.3 μM poly rA, 69% of the control activity is retained after modification for 10 minutes compared with only 15% activity in its absence. The presence of 0.2 mM MnTTP plus 1.3 μM poly rA provides no more protection against inactivation than poly

TABLE I: Effect of Substrates on the Inhibition of AMV Reverse Transcriptase Activity by Butanedione-Borate*

Substrate	Concentration x 10^6, M	Activity, V/V_C x 100
NONE	-	15
TTP	200	15
MnTTP	200	15
oligo dT_{12-18}	2.5	25
poly rA	1.3	69
poly rA +MnTTP	1.3 200	62
poly rA +oligo dT_{12-18}	1.3 2.5	90
poly rA +MnTTP +oligo dT_{12-18}	1.3 200 2.5	88

*Modification was performed for 10 minutes in 15 mM butanedione, 50 mM borate, 50 mM HEPES, 10 mM KCl, 1 mM dithiothreitol, 0.001% Triton-X-100, pH 8.3.

rA alone. However, when 2.5 μM oligo dT_{12-18} is present together with 1.3 μM poly rA, 90% of the control activity remains after modification for 10 minutes. If template, initiator and nucleotide all are present, the same degree of protection is found as with poly rA plus oligo dT_{12-18}.

Thus, of the three individual components required for DNA synthesis -- template, initiator, and nucleotide -- poly rA provides the most significant protection against inactivation by butanedione-borate. The poly rA concentration (1.3 μM) is insufficient to produce this effect by interacting with butanedione or borate; rather it likely binds to the RNA template site

of reverse transcriptase, thereby shielding the essential arginyl residue(s) from modification. This hypothesis is supported by the observation that poly dA (1.3 μM), a very poor template for AMV reverse transcriptase (3,11), presumably due to poor binding to the template recognition site, offers no protection against butanedione-borate inactivation. Oligo dT_{12-18} alone provides very little protection against inactivation, possibly due to low affinity for the polymerase in the absence of template. However, it does enhance the protective effect of poly rA. This additional protection could be due to the shielding of additional arginyl residues on the binding of oligonucleotide in the presence of template or, alternatively, to a conformationa change induced by the oligonucleotide in the presence of template.

The failure of TTP to protect against inactivation, or to enhance the protection provided by template or template plus initiator, even at a concentration which is twenty times above its K_M (1), would seem to imply that arginyl residues do not play a role in binding this nucleotide to the active site. This would seem to contrast with results obtained with several other nucleotide-dependent enzymes (7-10). It is interesting to note that chemical modification of E. coli DNA polymerase I with N-carboxymethylisatoic anhydride, a modification thought to involve lysyl residues, almost completely abolishes polymerase activity concomitant with a marked reduction in the affinity of the enzyme for deoxyribonucleoside triphosphates. The same modified enzyme retains exonuclease activity, which requires DNA template but not triphosphates (12).

The possibility remains that arginyl residues could be involved in deoxyribonucleotide triphosphate binding in reverse transcriptase, but are modified much more slowly than those involved in template binding. Protection by TTP would thus be insignificant compared to protection by poly rA template. The rates of enzyme inactivation by modification of essential arginyl residues are known to vary by as much as three orders of magnitude (6,9).

The results of these protection experiments implicate essential arginyl residues in the functional binding of the RNA template. Based on experiments

with other systems, these residues likely interact with the negatively charged phosphate moiety of the phosphodiester backbone of the template. Recent x-ray crystallographic studies of model arginine-phosphodiester systems show that arginyl residues are well suited for this type of interaction (13). Further, inactivation of reverse transcriptases from mammalian type C RNA viruses by butanedione in borate buffer indicates that arginyl residues may play a general role in the formation of DNA from RNA templates.

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References

1. Auld, D.S., Kawaguchi, H., Livingston, D.M. and Vallee, B.L. (1974) Proc. Nat. Acad. Sci. USA 71, 2091-2095.
2. Poiesz, B.J., Seal, G. and Loeb, L.A. (1974) Proc. Nat. Acad. Sci. USA 71, 4892-4896.
3. Auld, D.S., Kawaguchi, H., Livingston, D.M. and Vallee, B.L. (1975) Biochem. Biophys. Res. Commun. 62, 296-302.
4. Kawaguchi, H. and Auld, D.S. (1975) Clin. Chem. 21, 591-594.
5. Riordan, J.F. (1973) Biochemistry 12, 3915-3923.
6. Daemen, F.J.M. and Riordan, J.F. (1974) Biochemistry 13, 2865-2871.
7. Lange, L.G., III, Riordan, J.F. and Vallee, B.L. (1974) Biochemistry 13, 4361-4370.
8. Borders, C.L., Jr. and Riordan, J.F. (1975), Fed. Proc. 34, 647.
9. Borders, C.L., Jr. and Riordan, J.F., Biochemistry, submitted.
10. Powers, S.G. and Riordan, J.F. Proc. Nat. Acad. Sci. USA 72, in press.
11. Goodman, N.C. and Spiegelman, S. (1971), Proc. Nat. Acad. Sci. USA 68, 2203-2206.
12. Jovin, T.M., Englund, P.T. and Kornberg, A. (1969), J. Biol. Chem. 244, 3009-3018.
13. Furberg, S. and Solbakk, J. (1974) Acta Chem. Scand. 28B, 481-483.