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The Artifactual Nature of Fluoride Inhibition of Reverse Transcriptase and Associated Ribonuclease H

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ABSTRACT: Rauscher leukemia virus reverse transcriptase associated polymerization and ribonuclease H activities were totally resistant to sodium fluoride inhibition in contrast to AMV reverse transcriptase enzyme activities.We have found that the observed fluoride mediated inhibition is divalent-cation specific and is solely due to Mg2+ dependent precipitation of substrates and polynucleotides.Furthermore, the differential inhibition of AMV polymerase and RNase H could be explained on the basis of stoichiometry of fluoride to polynucleotide substrate or template primer used in the individual assay.The non-specific nature of fluoride mediated inhibition is further confirmed by the observation that the fluoride sensitivity of several DNA polymerases and RNase H activities is seen only when Mg2+ is an effective divalent cation.

INTRODUCTION: The association of DNA polymerase and RNase H activity on a single polypeptide of reverse transcriptase(RT)* and yet mechanistic independence of polymerization and nucleolytic action of RT has been clearly demonstrated(1-7).In most cases, the mechanistic distinction was demonstrated by the inhibition of polymerase reaction when catalysis of RNase H had remained unaffected. In an attempt to understand the structure-function relationship of various catalytic activities expressed by RT, we have begun an extensive investigation to characterize various inhibitors of this class of enzyme.Our studies thus far have indicated that RNase H expression by RT may involve the template binding site(4,8-10). Thus any reagent that will affect template binding function would be expected to inhibit both RNase H and polymerase reactions. This notion implied that no polymerase activity would be apparent if the RNase H site was the target of the inhibitor.Yet, an early report that suggested mechanistic independence of RNase H and polymerization had clearly shown that sodium fluoride is an inhibitory reagent for the nucleolytic but not for the polymerization reaction catalysed by AMV RT(1).We have therefore investigated in detail the mechanism of fluoride action on the RTs from avian and murine oncornaviral sources and have found that the inhibitory effects of NaF are: i) not exerted in a site-specific manner,ii)are Mg^{2+} dependent,iii) are due to

*Abbreviations Used: RT,reverse transcriptase;AMV,Avian myeloblastosis virus; RLV,Rauscher Murine leukemia virus;RNase H,Ribonuclease H;DTT,Dithiothreitol.

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the precipitation of template primer or RNA:DNA hybrid and iv) are quite non-specific in that a variety of DNA polymerases and calf thymus RNase H are quite sensitive for inhibition only in the presence of Mg^{2+}.

MATERIALS AND METHODS: All the radioactive deoxynucleoside triphosphates were obtained from New England Nuclear.Unlabeled triphosphates and template primers were the products of P.L.Biochemicals Inc. The molar ratio of template to primer was 1:1 in the case of Poly(rA).(dT)12-18 and 10:1 in the case of Poly(dC).(dG)12-18 or Poly(rC).(dG)12-18.Phage fd DNA was purchased from Miles laboratory,while E.coli RNA polymerase,holoenzyme was supplied by Enzo Biochemicals Inc. Sodium fluoride was purchased from Fisher Scientific Company. Rauscher leukemia virus and purified AMV polymerase was made available by the Division of Cancer Cause and Prevention ,National Cancer Institute.Purification of various DNA polymerases from viral or cellular sources and RNase H from calf thymus has been described earlier(3,11).Preparation of fd DNA: ^{3}(H)RNA as a substrate for RNase H was essentially as described before(10).

Enzyme Assays: DNA polymerase reactions were carried out in a final volume of 0.1 ml and contained the following components: 50 mM Tris-HCl(pH 7.8), 1 mM dithiothreitol,50 µg of bovine serum albumin,20 µM appropriate nucleoside triphosphate together with tritiated substrate(final specific activity adjusted to 1000 cpm/pmol), 0.5 µg of template primer and 10-20 ng of enzyme. Either Mg^{2+} or Mn^{2+} was used as an effective divalent cation at the optimal concentration determined for the individual template primer and enzyme(3,12). Five mM potassium phosphate was also included (pH 7.4) in Mg2+ containing reaction mixture to reduce the backgrounds observed in the presence of NaF (see results).Incubations were for 30 min at 37°C and reactions were terminated by the addition of 5% trichloroacetic acid containing 10 mM pyrophosphate. The acid insoluble material was collected on glass fiber discs and counted in toluene based scintillation fluid. Terminal deoxynucleotidyl transferase was assayed using oligo(dA)12-18 as a primer and tritiated dGTP as a substrate in the presence of Mg2+ or Mn2+(11). The reaction mixture for RNase H in a final volume of 0.1 ml contained:50 mM Tris-HCl(pH 7.8),1 mM DTT, 50 µg albumin,10 picomol of fd DNA:^{3}(H)RNA hybrid(as a total RNA nucleotides), 100 mM KCl and 0.5 mM $MnCl_2$ for RLV enzyme or 5 mM $MgCl_2$ for AMV enzyme. The reactions were terminated after incubation at 37°C for 30 min by the addition of trichloroacetic acid and acid insoluble counts were determined as above.

RESULTS AND DISCUSSION

Effect of fluoride on the polymerization and RNase H activities:The differential inhibitory effect of fluoride on the expression of polymerization and RNase H activities of AMV reverse transcriptase has been reported by Brewer and Wells(1) and further confirmed by Collett and Faras(13) using detergent disrupted virions as a source of enzyme.However, when purified RLV reverse transcriptase was used as a test enzyme,no inhibition of polymerization or RNase H activity could be obtained(see below).We, therefore,re-examined the sensitivity of purified AMV reverse transcriptase to fluoride inhibition. We found that the inhibition of AMV RNase H activity indeed occured prior to the inhibition of polymerase reaction,but that latter reactions exhibited high background in the presence of NaF.A dose response study revealed that addition of fluoride above 20 mM concentration results in the partial

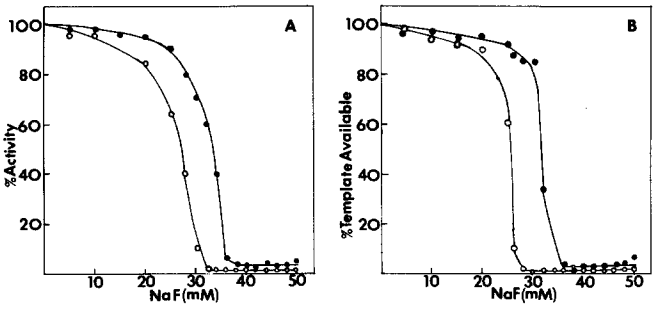

Figure 1:(A) Sodium fluoride mediated inhibition of AMV RNase H and RT : DNA polymerase and RNase H activities of AMV RT were measured using poly(dC).(dG)12-18(●——●) and fd DNA:^{3}(H)RNA hybrid (o——o) as described in materials and methods. Freshly prepared sodium fluoride was added to individual reaction mixture at desired concentration. KCl concentration in the reaction mixture was decreased with increasing concentration of fluoride such that final salt concentration remained at 100 mM.

Figure 1:(B) Precipitation of polynucleotide (template/hybrid) from AMV polymerase and RNase H reaction mixtures as a function of NaF concentration: 0.5 µg.of ^{3}(H)-poly(dC).(dG) representing about 30,000cpm/assay(●——●) and 25 ng of fd DNA:RNA hybrid(20,000cpm/assay) were incubated in a standard reaction mixture with increasing concentration of sodium fluoride in the absence of enzyme. The salt concentration in the individual reaction was maintained at 100 mM as described in 1A. After 15 minute incubation, reaction mixtures were centrifuged at 10,000 x g for 10 minutes and 25µL aliquots from the spernatent were acid precipitated to determine the % template-primer(●——●) or DNA:RNA hybrid(o——o) remanining in solution.

precipitation of substrate triphosphate. Since this precipitate was found to be acid insoluble, the conventional use of acid precipitation as a means of monitoring DNA synthesis was unsuitable. Several conditions for solubilization of precipitated triphosphate were attempted. While termination of reaction with 20% trichloroacetic acid or 1 N hydrochloric acid with or without 0.1 M sodium pyrophosphate did not reduce the radioactivity backgrounds, addition of 5-10 mM inorganic phosphate in the assay mixture prevented the formation of insoluble triphosphate complexes in the presence of NaF. Since inorganic phosphate itself has not been found to interfere in the polymerase or RNase H activity of AMV RT(3), we included 5 mM potassium phosphate(pH 7.4) as a standard component of assay systems used to measure either polymerase or RNase H activity in the fluoride inhibition studies. A typical dose response of fluoride addition on the RNase H and polymerase activity of AMV RT is presented in fig 1A. It may be seen that around 25-30 mM concentration of fluoride, expression of polymerization is minimally affected whereas RNase H activity exhibits significant inhibition. As the fluoride concentration is further increased, polymerase reaction is also inhibited. Thus, the results clearly demonstrate differential inhibition of

Table 1

Effect of Sodium fluoride addition on the Activity of Various DNA Polymerases and Calf Thymus RNase H

Enzyme	Divalent Cation	Activity(pmol/30 min)		% Inhibition
		Control	+30mM NaF	
AMV DNA Polymerase	Mg^{2+}	284	30	90
"	Mn^{2+}	43	44	-
E.coli DNA Pol.I	Mg^{2+}	403	29	93
"	Mn^{2+}	442	439	-
Calf Thymus DNA Polymerase β	Mg^{2+}	11	0.2	98
"	Mn^{2+}	337	341	-
Terminal Deoxynucleotidyl Transferase	Mg^{2+}	32	6	81
"	Mn^{2+}	578	549	5
Calf RNase H*	Mg^{2+}	5	0.2	96

All the polymerase assays were performed using Poly(rA).$(dT)_{12-18}$ as a template primer while Terminal deoxynucleotidyl transferase assay was carried out using oligo$(dA)_{12-18}$ as a primer and dGTP as a substrate in the presence of desired divalent cation.
*Activity for Claf thymus RNase H represents pmol acid solubilization of fd DNA:(3H)RNA.

two activities similar to that reported by Brewer and Wells(1).Since RLV enzyme was totally insensitive to fluoride inhibition, we decided to investigate if the fluoride effects were enzyme specific or if they were exerted via another reaction component unique to AMV RT assay mixture.

Properties of Fluoride Inhibition of AMV RT: Preincubation of enzyme with fluoride was neither required nor did it enhance the inhibitory effects of fluoride suggesting no interaction of the inhibitor with the active-site structure of the enzyme.Reactions carried out in different buffers(e.g. Tris-HCl,Hepes-KOH or Barbital)exhibited identical sensitivity of catalysis to fluoride addition(data not shown).Kinetic analysis indicated that fluoride inhibition was non-competitive with respect to both substrate triphosphate and template primer.Addition of NaF to ongoing reaction instantly curtailed further polymerization(data not shown).The most unusual finding was the requirement for a specific divalent cation,namely $Mg^{2+}$to obtain fluoride inhibition.Replacement of Mg^{2+} with Mn^{2+} resulted in the complete protection of AMV RT from fluoride inhibition(Table 1).Indeed, increasing Mg^{2+} concentration in the reaction mixture has been found to increase the sensitivity of AMV enzyme to fluoride(data not shown).

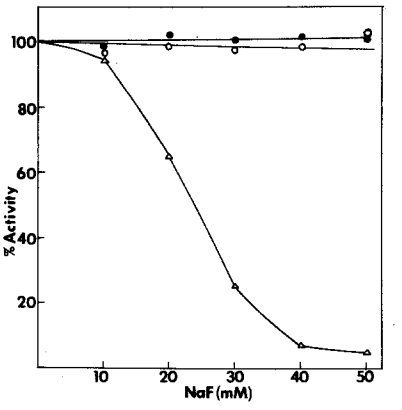

Figure 2:Sodium fluoride inhibition of RLV reverse transcriptase and associated RNase H in the presence of Mg^{2+} and Mn^{2+} :A standard DNA polymerase assay using poly(rA).(dT) as a template primer was carried out in the presence of either Mg^{2+}(△——△) or Mn^{2+}(●——●) and effect of increasing concentration of fluoride on the enzyme activity was determined. Similar analysis of RNase H activity was carried out except that catalysis in the presence of Mg^{2+}does not occur and hence not included.Dose response of RNase H activity with Mn^{2+} to the fluoride addition is represented by o——o. One hundred percent activity for polymerization with Mn^{2+} was 180 pmol while Mg^{2+}mediated reaction catalysed incorporation of 120 pmol. RNase reaction controls(100% activity) were equal to 5 pmol nucleotide solubilized.

Effect of fluoride on the RLV RT and other DNA polymerases with Mg^{2+} and Mn^{2+} as a divalent cation: Preference of RLV RT for Mn^{2+} as a choice of divalent cation for catalysis has been well established(3).In the presence of Mn^{2+}, catalysis of polymerization and RNase H is totally refractory to fluoride addition(figure 2). However, when Mn^{2+} is replaced by Mg^{2+}, an inhibition of RLV enzyme ,in a manner similar to AMV enzyme, may be obtained(fig.2).Thus, Mg^{2+} dependency of fluoride inhibition was clearly established. In order to further determine whether Mg^{2+} mediated fluoride inhibition is RT directed,we examined sensitivity of several DNA polymerases from eukaryotic and bacterial sources.The results presented in Table 1 clearly show lack of specificity of fluoride inhibition for all of the DNA polymerases including terminal deoxynucleotidyl transferase exhibited sensitivity to fluoride in the presence of Mg^{2+} and insensitivity in the presence of Mn^{2+}.Similarly, calf thymus RNase H from calf thymus which differs in its active site structure from that of RT associated RNase H (9,11) showed Mg-dependent fluoride inhibition(Table 1). Therefore, it appeared that polynucleotide component, similar to triphosphate moiety, may also be a target of Mg-mediated fluoride precipiation and thereby create a starvation of a polynucleotide substrate required for polymerzation as well as RNase reactions. Indeed, the well confirmed "Differential Inhibition" of RNase H and polymerase activities of AMV RT could be

explained on the basis of this polynucleotide precipitation phenomenon.

Mg^{2+} dependent precipitation of template primer: Usually assay system for RNase H employs relatively small quantity of polynucleotide substrate, namely, RNA:DNA hybrid since RNase H reaction is quite inefficient and small quantities of high specific activity substrate permit reliable measurement of nuclease activity.On the other hand, polymerase assay systems utilize saturating quantities of template primer polynucleotides. Thus,one may expect that the latter may require higher concentration of fluoride to form insoluble complex than that required for RNase H substrate(RNA:DNA hybrid).To test validity of this contention, we examined precipitibility of polynucleotides from the polymerase and RNase H assay mixtures as a function of fluoride concentration.Results are presented in fig 1B, for a ready comparison to enzyme activity pattern shown in Fig 1A , which clearly show that RNase H substrate precipitates out of solution at approximately 5 mM lower concentration of fluoride than that required for total precipiation of template primer.Therefore, within this concentration range, one may observe no catalysis of RNase H while polymerization reaction may still continue.The need for high concentration of fluoride,i.e. around 30 mM, to observe the inhibitory effects indicates that formation and stability of Mg-fluorophosphate may be optimal above this concentration of fluoride.The formation of insoluble Mg-fluorophosphate complexes has been well documented in early literature (14) and we attribute the inhibitory effect of fluoride, which is strictly Mg^{2+} dependent to this phenomenon.

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