Study of the interactions between avian myeloblastosis virus reverse transcriptase and primer tRNA. Affinity labeling and inactivation of the enzyme by periodate-treated $tRNA^{Trp}$

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SUMMARY.

Reverse transcriptase from avian myeloblastosis virus can react with periodate-treated primer $tRNA^{Trp}$(beef) to form a Schiff's base between an ε-NH_2 lysine group whithin the active center of the enzyme and the dialdehyde derivative of the 3' terminal ribose of tRNA. In the presence of cyanoborohydride the reversible imminium moiety of the Schiff's base is reduced to a more stable adduct. Non-primer tRNAs were not able to reduce the extent of primer fixation to the enzyme.

Complete inactivation of the enzyme was attained when the ratio enzyme:tRNA in the complex was 1:1. When the 1:1 adduct was analyzed by polyacrylamide gel electrophoresis , radioactivity from the terminal adenosine of tRNA was found exclusively associated with the α subunit. At longer times of labeling the β subunit was also found linked to the oxidized primer tRNA.

INTRODUCTION.

Retroviruses contain a DNA polymerase (reverse transcriptase) able to copy the viral genome RNA into complementary DNA (1). Like all other DNA polymerases, reverse transcriptase needs a primer with a 3' OH end to initiate DNA synthesis. In the case of reverse transcriptase this primer is a specific tRNA which is found strongly bound to the viral genome. When 35 S viral RNA is used to synthetise DNA *in vitro*, primer tRNA is found covalently linked to the nascent DNA chain (2-5).

Reverse transcriptase from avian myeloblastosis virus (AMV), used in our work, is a dimer of the $\alpha\beta$ type. The α subunit can be isolated by phosphocellulose or poly U-cellulose chromatography. This subunit is able to catalyze all the reactions of the holoenzyme: DNA polymerase RNA dependent; DNA polymerase DNA dependent and ribonuclease H activity. The β subunit seems to play an important role in increasing the affinity of the enzyme for the natural primer-template complex (6).

AMV reverse transcriptase copies the 35 S viral genome

by initiating DNA synthesis on a $tRNA^{Trp}$ primer (7). The affinity of the enzyme for free $tRNA^{Trp}$ is high as shown by the studies of complex formation in sucrose gradients, gel chromatography and nitrocellulose binding (8-11).

We have previously studied the topography of the interactions between reverse transcriptase and primer $tRNA^{Trp}$ by determining the regions of the primer covalently linked to the enzyme after U.V. irradiation (12,13). From these experiments we concluded that the α and β subunits were probably bridged by primer tRNA. Moreover two regions of $tRNA^{Trp}$ were found to interact very strongly with the enzyme: the anticodon region and the CCA end. Evidence has also been presented that reverse transcriptase unwinds the acceptor stem of tRNA thus facilitating the positioning of primer tRNA on the viral genome (14).

We report in this article the affinity labeling and inactivation of reverse transcriptase by periodate-treated $tRNA^{Trp}$(beef). Other authors have successfully used this approach for the study of the interactions between tRNA and aminoacyl-tRNA synthetases (15-18). Enzyme inactivation is attained through the formation of a reversible Schiff's base between an ε-NH_2 group of a lysine residue in or near the active site of the enzyme, and the 2'-3' aldehyde group formed by oxidation of the 3' terminal ribose. A more stable covalent adduct can be obtained by reducing the Schiff's base with $NaBH_4$. In the present work we have used $NaCNBH_3$ as the reducing agent. This reagent has proven to be very suitable for this type of study since, contrary to $NaBH_4$, it reacts specifically with the imminium group involved in the Schiff's base without modifying the free 2'-3' aldehyde groups, thus shifting the reaction towards a higher yield of nucleic acid-protein adduct (19).

MATERIALS.

AMV pellets and purified reverse transcriptase were obtained from Life Science Inc.Florida.U.S.A. E.coli tRNA nucleotidyl transferase was a kind gift of Dr.D.S.Carré and was purified as described before(20). Radiochemicals were purchased from New England Nuclear Co. The purification of beef tRNAs has been published (21), and $tRNA^{Trp}$(yeast) was a kind gift of Dr.G.Keith. AMV 35 S RNA was purified as described before(14); poly C and oligo dG_{12} were purchased from Boehringer or P.L.Biochemicals and annealed as described previously(22). Sodium cyanohydridoborate was bought from Merck.

METHODS.

Inactivation of AMV reverse transcriptase. Reverse transcriptase (0.6 μM)was incubated in 60 mM potassium phosphate pH 7.5 buffer containing 5 mM $MgCl_2$, 0.6 mM DTT, 0.05% Triton X-100 and 15% glycerol. The $tRNA^{Trp}$(beef) dialdehyde derivative ($tRNA^{Trp}_{ox}$) was 9 μM and the sodium cyanohydridoborate,5mM. The incubation was carried out at 37°C, and at different times, two microliters of the incubation mixture were tested for enzyme activity. Controls wre done in the presence of 9 μM untreated $tRNA^{Trp}$(beef) or with $tRNA^{Trp}_{ox}$ in the absence of $NaBH_3CN$.

Periodate oxidation of tRNA. Unlabeled or radioactive tRNA (10 $O.D._{260\ nm}$) were treated with 10 mM sodium periodate in 500 μL of 100 mM sodium acetate pH 5. The reaction was performed at room temperature for 60 min in the dark; glucose was added to a final concentration of 10 mM to destroy excess periodate and tRNA was recovered by ethanol precipitation.

3' labeling of tRNA. 34 nmoles of $tRNA^{Trp}$(beef) were incubated with 37 mUnits of venom phosphodiesterase (Worthington) in a 50 mM Tris-HCl pH 8.0 buffer containing 10 mM $MgCl_2$. The incubation was for 10 min at room temperature.The reaction was stopped with 1 volume of saturated phenol and the tRNA solution precipitated with 3 volumes of cold ethanol and left overnight at -20°C.

The venom phosphodiesterase treated tRNA was labeled with (^{3}H)ATP by the E.coli tRNA nucleotidyl transferase. The reaction mixture in 1 ml contained 50 mM Tris-HCl pH 9.0, 10 mM $MgCl_2$, 2 mM DTT, 10 μM CTP and 200 μCi of tritiated ATP (34 Ci/mmol). The reaction was started by the addition of 0.4 units of the enzyme; incubation was carried out at 37°C for 90 min; then 100 nmoles of unlabeled ATP were added and the reaction continued for 30 min more followed by phenolic extraction. Free nucleotides were separated from (^{3}H)tRNA on a 2 ml B.D.cellulose column. Labeled tRNA was recovered by precipitation with 3 volumesof ethanol and stored at -20°C. The specific activity of (^{3}H)tRNA was about 1700 cpm/pmole.

Labeling of reverse transcriptase by (^{3}H)$tRNA^{Trp}$(beef) dialdehyde derivative. The labeling of reverse transcriptase by tritiated $tRNA^{Trp}_{ox}$ was performed as follows: a 150 μL reaction mixture contained different concentrations of labeled $tRNA^{Trp}_{ox}$, 5 mM $NaBH_3CN$ and 0.6 μM of the enzyme. Incubation was at 37°C for different lenghts of time. The reaction was followed by filtration on nitrocellulose membranes (Scleicher and Schuell, 0.45 μ). Ten μL aliquots were diluted with 1 ml of cold 20 mM potassium phosphate pH 6.5 buffer containing 5 mM magnesium acetate and 1 M NaCl and immediately filtered on nitrocellulose membranes under gentle suction, then washed twice with 10 ml

of the same buffer. Under these conditions only the covalent complex were retained on the filters (13). The membranes were dried and counted in a toluene PPO, POPOP scintillation mixture.

Polyacrylamide gel electrophoresis of labeled AMV reverse transcriptase. Reverse transcriptase was labeled with (^{3}H) $tRNA_{ox}$ as described above. Eighty µg of pancreatic RNase were added and the tRNA digested for 10 min at room temperature. The reaction mixture (200 µL) was dialized for one hour against 60 mM Tris-HCl pH 6.8, 10% glycerol and 5% 2-mercaptoethanol; the dialysis was continued overnight against the same buffer containing 2% SDS. The sample was made 0.001% in bromophenol blue, heated at 100°C for 30 seconds and loaded on a 10% polyacrylamide slab gel (0.1x20x12 cm) as described by Laemli (23). Electrophoresis was carried out at 20 mA and continued for one hour after the bromophenol blue marker had left the bottom of the gel. Bovine serum albumin, sarcoplasmic reticulum ATPase and ovoalbumin were used as standards (68,000; 110,000; and 42,000 daltons respectively).The labeled subunits of reverse transcriptase were analyzed by fluorography using a R.P.Royal X-Omat Kodak film (24).

Reverse transcriptase assay. The activity of reverse transcriptase during periodate-treated tRNA modification was followed using the synthetic template-primer and 35 S natural template-primer complexes, as previously described (14).

Sephadex G-100 chromatography of tRNA-reverse transcriptase complex. The column (0.8x51 cm) was equilibrated in a 100 mM potassium phosphate buffer pH 7.5 containing 5 mM magnesium acetate, 10 mM 2-mercaptoethanol, 0.1 mM EDTA, 0.1% Triton X-100 and 10% glycerol. V_o was determined with Dextran Blue. The specific activities of the different tRNAs labeled in the terminal adenosine as described above were between 750 and 850 cpm/pmole. About 35 pmoles of tRNA and 45 pmoles of enzyme were used in each experiment. Fractions were counted in 5 ml of Bray's scintillation mixture (25).

RESULTS AND DISCUSSION.

The study of the interactions between tRNA and their cognate aminoacyl tRNA synthetases have shown some common structural features. From these studies it appears likely that the major part of the binding site for most or all synthetases is along and around the diagonal side of the tRNA structure which contains the acceptor stem, dihydrouridine loop, and anticodon (26). Since the 3' terminus of the acceptor stem is the site of amino acid attachment, contact by the enzyme must be made at this site. A similar

situation is found for the interactions between reverse transcriptase and its primer tRNA since terminal adenosine is the point of initiation of DNA synthesis. We have reported previously that a covalent complex between AMV reverse transcriptase and $tRNA^{Trp}$(beef) is obtained by irradiation of the nucleic acid-protein complex at 283 nm. The regions of tRNA most strongly bound to the enzyme are the acceptor stem and the anticodon region. Moreover tRNA is photochemically crosslinked to both subunits of the viral polymerase (12,13). Bearing all these facts in mind, we have attempted to localize the enzyme site involved in the recognition of the CCA end of primer tRNA. For that purpose we have labeled the enzyme by using periodate-treated $tRNA^{Trp}$ as an affinity probe; $tRNA^{Trp}_{ox}$ was incubated with reverse transcriptase; the reversible Schiff's base formed is reduced with $NaCNBH_3$ to give a stable complex. This radioactive complex is detected by nitrocellulose membrane binding . As seen in Figure 1 the rate and extent of primer tRNA fixation to the enzyme is dependent on the tRNA concentration. At a concentration of 9 μM tRNA a value of

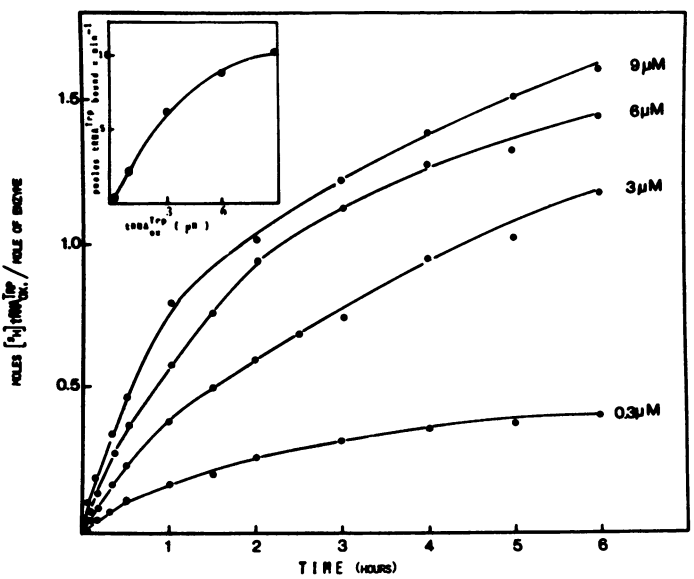

Figure 1. Kinetics of labeling by (^{3}H)$tRNA^{Trp}_{ox}$(beef) of AMV reverse transcriptase. The enzyme (0.6 μM) was incubated with labeled tRNA at the concentrations indicated in the Figure. Each point represents the value obtained by analyzing 10 μL of the incubation mixture for the radioactivity retained on nitrocellulose filters as described in Methods. Each value of the inset plot was obtained from the slope of the linear part of each curve. One pmole incorporated corresponds to 1,700 cpm counted in a toluene,PPO,POPOP scintillation mixture. The background level are lower than 0.05 pmoles (85 cpm).

1.7 moles of tRNA per mole of enzyme was obtained. Each point of the inset plot shown in the same Figure represents the slopeof each curve. The incubation time chosen for the inset plot was 20 min. These results are in good agreement with the binding stoichiometry we have found for the non covalent complexes formed between reverse transcriptase and tRNA analyzed on nitrocellulose filters under non dissociating conditions (12), and with the results obtained for the crosslinked complexes obtained after U.V. irradiation (12,13).

Once we had shown that $tRNA_{ox}^{Trp}$ can be covalently linked to the enzyme we tried to determine whether enzyme activity was affected by the label. As seen in Figure 2 there is a loss of activity concomitant with the fixation of $tRNA_{ox}$ to the enzyme. The incorporation of 1 mole of tRNA per mole of enzyme coincides with the complete loss of enzyme activity. Reverse transcriptase inactivation by affinity labeling followed with the synthetic template poly rC-oligo dG_{12} (Figure 2) is indistinguishible from the inactivation curve obtained with the natural 35 S RNA-$tRNA^{Trp}$ template-primer complex (not shown). This may suggest that the same binding site is occupied by

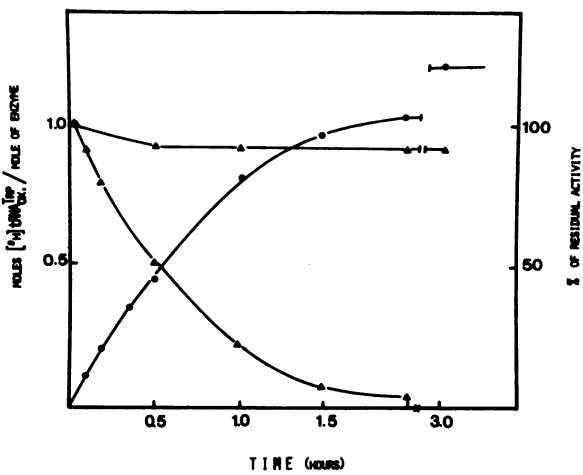

Figure 2. Kinetics of inactivation of AMV reverse transcriptase by $tRNA_{ox}^{Trp}$ (beef). The reaction mixture was as described in Figure 1 except that unlabeled $tRNA_{ox}$ was used when enzyme activity was determined. Two µL of the incubation mixture were assayed for DNA synthesis in 100 µL of a standard reaction mixture as previously described (14).One hundred % of activity represents 396 pmoles of dGMP (500 mCi/mmole) incorporated in 5 min at 37°C. The kinetics of labeling was performed in a separate experiment in which 9 µM of tritiated beef $tRNA_{ox}$ was present. The results were analyzed as described under Figure 1. (●) $tRNA_{ox}$ fixation; (△) % Residual activity; (▲) Control performed in the absence of $NaCNBH_3$.

synthetic and natural templates. No inhibition was observed in the absence of reducing agent, indicating that reversion of the Schiff's base may have ocurred prior to the transcriptase assay, since the $tRNA_{ox}$-enzyme complex is diluted 50 times. A similar case was observed for the PLP inhibition of reverse transcriptase as we have described previously (31).

The kinetics of $tRNA_{ox}^{Trp}$ fixation followed in the presence of other tRNAs is shown in Figure 3. Non-primer $tRNA_{ox}^{Val}$(beef) and $tRNA_{ox}^{Trp}$(yeast) were used as well as control $tRNA^{Trp}$(beef). The results shown in this Figure and in Table I indicate that $tRNA_{ox}^{Val}$ will not affect the rate nor the extent of $tRNA_{ox}^{Trp}$(beef) fixation, while $tRNA_{ox}^{Trp}$(yeast) and $tRNA^{Trp}$(beef) will affect the rate but not the plateau of labeling. We have previously shown that AMV reverse transcriptase will form stable complexes with non-primer tRNAs as determined with Sephadex gel filtration (12). Those experiments were performed in the presence of an excess tRNA, while in Figure 4 we shown complex formation

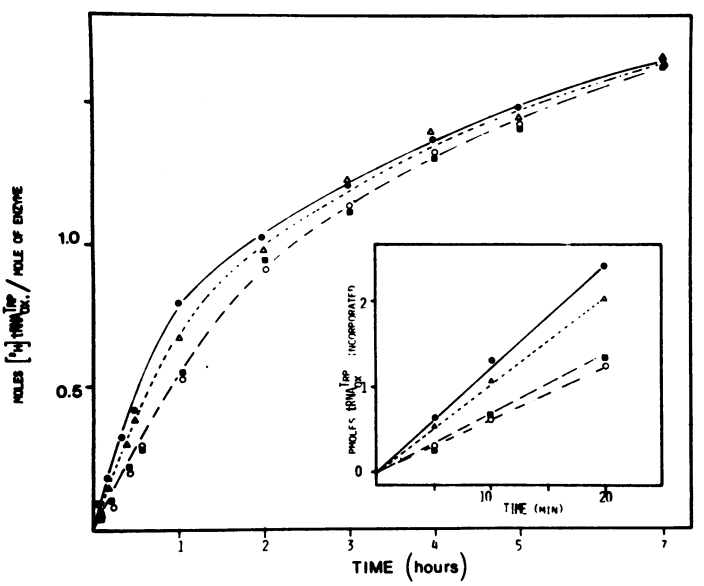

Figure 3. Competition of(^{3}H)$tRNA_{ox}^{Trp}$ fixation on AMV reverse transcriptase by non-primer oxidized tRNAs. In a final volume of 150 µL, the enzyme (0.6 µM) was incubated with 9 µM(^{3}H)$tRNA_{ox}^{Trp}$(beef) at 37°C in the presence of $NaCNBH_3$. At each indicated time, 10 µL of the incubation mixture were withdrawn and analyzed as described in Methods.
(●)(^{3}H)$tRNA_{ox}^{Trp}$(beef) alone ; (▲)(^{3}H)$tRNA_{ox}^{Trp}$(beef) plus 9 µM $tRNA_{ox}^{Val}$(beef) (■)(^{3}H)$tRNA_{ox}^{Trp}$(beef) plus 9 µM $tRNA_{ox}^{Trp}$(yeast);(^{3}H)$tRNA_{ox}^{Trp}$(beef) plus 9 µM $tRNA^{Trp}$(beef).(○)

TABLE I

	tRNA used	Initial Rate (pmoles/min)	Extent of the reaction (moles $tRNA_{ox}^{Trp}$/mole enzyme)
(1)	(^{3}H)$tRNA_{ox}^{Trp}$(beef)	0.100	1.48
(2)	(1) plus $tRNA_{ox}^{Val}$(beef)	0.094	1.42
(3)	(1) plus $tRNA_{ox}^{Trp}$(yeast)	0.062	1.46
(4)	(1) plus $tRNA^{Trp}$(beef)	0.064	1.50

Table I. Effect of non-primer tRNAs on the rate and extent of the labeling of AMV reverse transcriptase by (^{3}H)$tRNA_{ox}^{Trp}$(beef). The rate of fixation was obtained from the slopes of the kinetics shown in Figure 3, taken at 20 min of incubation. The extent of the reaction (plateau) was taken at 240 min of incubation. Enzyme concentration was 0.6 μM and tRNAs were 9 μM. The reaction was started by adding the labeled primer $tRNA_{ox}$ plus $NaCNBH_3$. The specific activity of tritiated tRNA was 1,700 cpm/pmole.

using a Sephadex G-100 column in the presence of limiting concentrations of tRNAs. Under these conditions $tRNA_{ox}^{Val}$(beef) did not form a complex with reverse transcriptase while $tRNA^{Trp}$(beef) was very efficiently bound to the enzyme and about one half of $tRNA^{Trp}$(yeast) formed a complex with the enzyme.

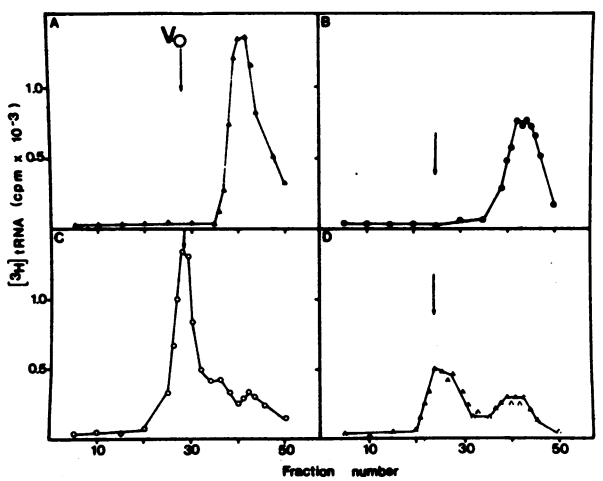

Figure 4. Sephadex G-100 chromatography of tRNA-reverse transcriptase complexes. (A) $tRNA^{Trp}$(beef) alone; (B)$tRNA^{Val}$(beef) plus reverse transcriptase; (C)$tRNA^{Trp}$(beef) plus reverse transcriptase; (D)$tRNA^{Trp}$(yeast) plus reverse transcriptase. Experimental details are given in the text.

These results confirm those of Figure 3 indicating that although $tRNA^{Trp}$ (yeast) is not a primer of reverse transcriptase (probably because the sequence of the last 16 nucelotides is quite different from that of primer tRNA) it may have some structural features in common with $tRNA^{Trp}$ purified from beef or chicken, allowing its partial recognition by the polymerase.The same common structural features are probably involved in the efficient aminoacylation of $tRNA^{Trp}$(yeast)by the beef synthetase and viceversa (27).

All the labeling experiments described in this work were performed with $tRNA^{Trp}_{ox}$ which had been made radioactive with (^{3}H)ATP and tRNA-nucleotidyl transferase. The tRNA-enzyme adduct was digested with pancreatic ribonuclease and the radioactive enzyme was analyzed by polyacrylamide gel electrophoresis in the presence of SDS. As seen in Figure 5 after 90 min of labeling with $tRNA^{Trp}_{ox}$ the radioactivity is found almost exclusively associated with the α subunit of reverse transcriptase. As seen in Figures 1 and 2 the stoichemistry of the adduct after 90 min is 1:1. At longer times of incubation Figure 5 shows that the β subunit is also labeled by the affinity probe. This experiment indicates that the specific labeling of α can account for the complete inactivation of the enzyme confirming the well stablished fact that the α subunit alone can carry out all the reactions of the holoenzyme. However an intriguing question remains open. As it has been

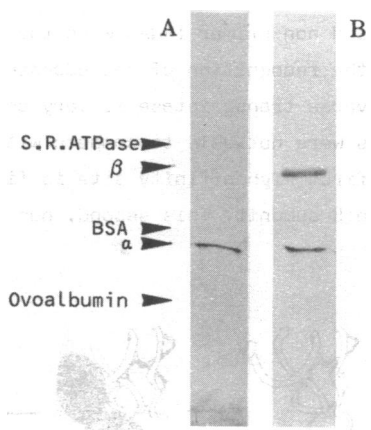

Figure 5. Polyacrylamide gel electrophoresis under denaturing conditions(SDS) of affinity labeled reverse transcriptase. The reaction mixtures and electrophoresis conditions are given in the text.
Lane A: The enzyme was labeled for 90 min (1.1 moles $tRNA^{Trp}$ per mole of enzyme).
Lane B: The enzyme was labeled for 360 min (1.7 moles $tRNA^{Trp}$ per mole of enzyme).Protein standards indicated in the text were detected by Coomassie Blue staining and the radioactive enzyme was detected by PPO fluorography.

shown that the β subunit carries all the sequences of the α subunit plus an extra polypeptide(28), we have to assume that the tRNA site in the β subunit is less accessible than in the α subunit. This difference may be ascribed to structural differences between the 2 subunits because of the extra piece of one of them. Another possibility is that the tRNA site of the β subunit is hidden in the intra-subunit junction. At longer times of incubation with the affinity label, and after the first site in the α subunit is saturated the β subunit site becomes accessible.

CONCLUSIONS.

The formation of a Schiff's base between AMV reverse transcriptase and periodate-treated primer $tRNA^{Trp}$ indicates that a lysine group plays an important role in the active catalytic site of the enzyme.Reverse transcriptase is strongly inhibited by pyridoxal-5'-phosphate (PLP); this inhibition is reversed by deoxynucleoside triphosphates (29,30). We have previously shown that PLP inhibition of reverse transcriptase is greatly enhanced by primer tRNA(31). It is tempting to think that the same lysine groups responsible for the inhibition by PLP may be involved in the enzyme-tRNA complex described in this article, since the terminal CCA binding site and the dNTP binding site in the polymerase molecule must be very near.

Several facts indicate the formation of stable complexes between reverse transcriptase and non-primer tRNAs when the latter are in excess. Our results show that the recognition of the acceptor stem of primer tRNA by the α subunit of reverse transcriptase is very specific since non-primer periodate-treated tRNAs were not able to compete with $tRNA^{Trp}_{ox}$. One can speculate that once this first high affinity site is filled, a second site becomes accessible in the β subunit. This second, non functional binding site,

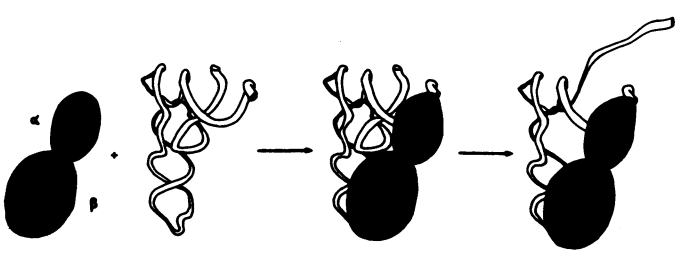

Figure 6. Schematic diagram illustrating the topography of the complex between reverse transcriptase and primer tRNA and the partial unwinding of the primer by the enzyme.

becomes evident at large molar excess of primer tRNA; this may reflect the molecular simmetry of the dimeric enzyme molecule since both subunits contain the same sequences.

The scheme shown in Figure 6 describes the topography and possible role in reverse transcription of the complex between reverse transcriptase and primer tRNA. This proposal combines our photochemical (12,13), unwinding (14) and affinity label data. The α subunit of the enzyme would be in close contact with the CCA end of primer tRNA while the β subunit would be near the anticodon region. Complex formation may lead to the partial unwinding of the acceptor stem thus favouring the annealing of primer tRNA to the viral genome.

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