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Evidence for Allosterism in *In Vitro* DNA Synthesis on RNA Templates

(DNA polymerase/reverse transcriptase)

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ABSTRACT **Hemoglobin mRNA and $(rA)_n \cdot (dT)_{10}$ have been used as primer-templates in a kinetic study of DNA synthesis with *Escherichia coli* DNA polymerase I (DNA nucleotidyl transferase, EC 2.7.7.7) and Mason-Pfizer monkey virus reverse transcriptase (RNA-directed DNA polymerase). The rate *versus* enzyme concentration curve is sigmoidal and is consistent with a cooperative phenomenon. The results could be interpreted in terms of the formation of an active complex containing enzyme dimers (or oligomers) on the primer-template. We have also observed sigmoidal kinetics in rate *versus* deoxynucleotide triphosphate concentration. These results are consistent with an allosteric mechanism in which the triphosphates act as both modifiers and DNA precursors. In the critical range, a 6- to 8-fold increase in both enzyme and triphosphate concentrations can lead to a 1500-fold increase in the rate of synthesis on an RNA template. Thus, small changes in enzyme and precursor concentrations could play a regulatory role *in vivo*.**

The complexity of the process of polymerization by various DNA polymerases (DNA nucleotidyl transferases, EC 2.7.-7.7) is only partially understood. There are at least five components to the system, namely, primer, template, enzyme, deoxynucleoside triphosphates, and metal ions. Studies of physical interactions between the enzyme and other individual components of the reaction, such as the work of Loeb and coworkers (1) on *Escherichia coli* DNA polymerase I-Mn^{++} complexes, and of Kornberg and coworkers (2–4) on the binding sites of *E. coli* DNA polymerase I, have contributed to an understanding of the system. Such studies are necessarily limited in their bearing on the synthetic process as a whole. In order to learn more about the nature of the interactions which take place during DNA synthesis, all components of the reaction must be present. We have therefore undertaken a kinetic study of DNA synthesis using DNA and RNA templates with *E. coli* DNA polymerase I. We report in this paper the effect of both polymerase and deoxynucleoside triphosphate concentrations on the rate of DNA synthesis. Rather than simple saturation kinetics we have observed sigmoid curves with RNA primer-templates (as opposed to DNA templates), suggesting a cooperative mechanism.

MATERIALS AND METHODS

Unlabeled deoxynucleoside-5′-triphosphates, $(rA)_n \cdot (dT)_{10}$, $(dA)_n \cdot (dT)_{10}$, and $(dC)_n \cdot (dG)_{12}$ were purchased from P-L Biochemical Laboratories; labeled triphosphates (18 Ci/mmol) were purchased from Schwarz-Mann. The homopolymer pairs were reannealed by heating at 55–60° for $(rA)_n \cdot (dT)_{10}$ and $(dA)_n \cdot (dT)_{10}$ and at 80° for $(dC)_n \cdot (dG)_{12}$ for 15 min, in 0.05 M Tris, pH 7.8. The mixtures were then allowed to cool slowly to room temperature. The preparation of calf-thymus DNA has been described (5). Activated DNA was prepared according to the procedure of Aposhian and Kornberg (6). Hemoglobin messenger RNA was a generous gift of Dr. Arthur Bank. A homogeneous sample of Mason–Pfizer monkey virus polymerase was kindly donated by Dr. R. C. Gallo. *E. coli* DNA polymerase I was prepared from the ammonium sulfate fraction described previously (7). About 0.5 mg of this fraction in 0.2 ml of assay buffer [0.05 M Tris (pH 7.8), 2 mM $MgCl_2$, and 10 mM mercaptoethanol] were applied to 5% acrylamide gels (5 × 80 mm). After the protein entered the gel, a current of 4.5 mA per gel was applied for 2 hr at 4°. One-millimeter slices were eluted with a relative migration of 0.36–0.39 with 0.4 ml of 0.05 M Tris-10% glycerine. Ten μl were used for each assay (0.1 ml) to locate the activity. Recovery of activity varied between 50 and 75%. Using $(dA)_n \cdot (dT)_{12}$ as primer-template, the specific activity was about 50,000 nmol of dTMP per mg of protein per 1 hr. (This was with [^{3}H]dTMP from Amersham/Searle which seemed to be a better substrate than that obtained from Schwarz/Mann.) However, as discussed below, the specific activity is strongly dependent on enzyme concentration. A second sample of polymerase I was used for comparative purposes. This second sample was a generous gift of Dr. L. Loeb and was reported to be homogeneous with a specific activity of 240,000 nmol/mg (1). Both enzyme preparations behaved quite similarly in all types of experiments reported here.

Polymerase assays: standard assays were carried out in a 0.1 ml volume of buffer containing 50 mM Tris, (pH 7.8), 2 mM $MgCl_2$, and 10 mM mercaptoethanol. Ten μl of the eluted enzyme were used per assay. The substrate (deoxynucleoside triphosphate) concentrations were varied as indicated. The primer-template nucleotide concentration was 4 μM unless otherwise indicated. When more than one substrate was used, the individual concentrations were equal. All assays contained 50 μg/ml of bovine serum albumin. Incubations were for 30 min at 37° except in the case of the $(dA)_n \cdot (dT)_{10}$ reaction where the time of incubation was 5 min. Under these conditions, the rates of incorporation are linear and can be taken as initial velocities. The specific activities were adjusted to 400 cpm/pmol in all cases. For the hemoglobin mRNA assay, 0.20 μg of mRNA were first incubated with 0.01 μg of $(dT)_{10}$ for 5 min at 37° in 0.05 M Tris (pH 7.8). When $(dT)_{10}$ was not used as a primer the incorporation decreased by 95%.

Reactions were stopped with 5% trichloroacetic acid–0.01 M pyrophosphate. Samples were collected on Whatman GF/B filters and counted in a Beckman LS-350 scintillation counter.

RESULTS

Enzyme concentration and cooperativity

The effect of increasing *E. coli* polymerase I concentration on the rate of DNA synthesis is shown in Fig. 1. In the experiments reported here, the rate of deoxynucleoside triphosphate incorporation is linear with time and may, therefore, be assumed to be the initial rate. In simple enzyme kinetics, a hyperbolic saturation curve is obtained as the enzyme concentration is increased. This is what we observed with activated DNA (Fig. 1), $(dA)_n \cdot (dT)_{10}$ and $(dC)_n \cdot (dG)_{12}$ (data not shown) in the enzyme range shown; for these enzyme concentrations the rates of synthesis are of the same order of magnitude. On the other hand, Fig. 1 shows marked sigmoidicity on increasing the enzyme concentration when $(rA)_n \cdot (dT)_{10}$ or hemoglobin mRNA are the primer-templates. This was true whether Mn^{++} or Mg^{++} was used in the reaction. Because sigmoid kinetics imply cooperativity, it was essential to rule out possible artifacts. First, we have shown by appropriate preincubation controls that the lag in rate at low enzyme concentrations is not due to destruction of the primer-template or of the deoxynucleoside triphosphates. Secondly, we have shown that the lag still exists when the assay mixture contains 0.5 mg/ml of bovine-serum albumin, ruling out artifacts due to low protein concentration in the enzyme solution. All assays have been carried out with bovine-serum albumin. When it was omitted, the rates were about 25% lower. The same results were obtained with samples of polymerase I prepared by different procedures in our laboratory and that of L. A. Loeb, and with DNA polymerase from Mason–Pfizer monkey tumor virus; this makes it unlikely that there are artifacts due to impurities in the enzyme. The question arises as to whether other mechanisms or artifacts might lead to the kinetics observed. If there are sites on a template which bind polymerase tightly but which do not lead to synthesis, these sites would be saturated first; then the polymerase would be able to bind at the active sites. Such a situation would yield a sigmoid curve. We have ruled out this case by studying the kinetics at several primer-template concentrations; two [2 μM and 10 μM $(rA)_n \cdot (dT)_{10}$] are shown in Fig. 1. The third (data not shown) was an intermediate concentration (4 μM) and fell between the two curves shown. If tightly binding inactive sites were present, the lag portion of the curve would increase with template concentration, followed by a rise which would be steeper than that for the lower template concentration. This was not observed with $(rA)_n \cdot (dT)_{10}$ (Fig. 1). Instead, the lag decreased when the template concentration increased. This is consistent with the proposed mechanism of cooperativity. To further substantiate our interpretation, we have done the following experiment. We added $(dC)_n \cdot (dG)_{12}$, which we know to bind polymerase and to produce $(dC)_n \cdot (dG)_n$. In the present experiment, however, there was no dGTP to act as the substrate, only dTTP, and the $(dC)_n \cdot (dG)_{12}$ is, therefore, a source of inactive sites since it will bind enzyme. It can be seen from Fig. 1 (curves *3* and *4*) that, as expected, the lag portion of the curve increases as more $(dC)_n \cdot (dG)_{12}$ is added.

Deoxynucleoside triphosphate concentration and cooperativity

Kinetics with Various Primer-Templates. Fig. 2 shows the rate of DNA synthesis by polymerase I as a function of concentration of the appropriate deoxynucleoside triphosphates.

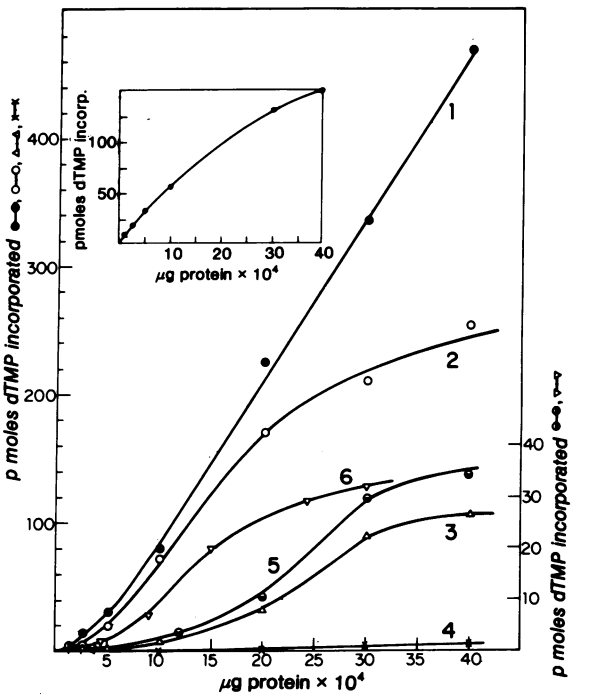

FIG. 1. Effect of enzyme concentration on rate of incorporation. Curves *1* and *2* are for $(rA)_n \cdot (dT)_{10}$ at 10 and 2 μM, respectively, calculated as adenylate monomer. Curves *3* and *4* are each for 2 μM $(rA)_n \cdot (dT)_{10}$ and contain in addition 2 and 10 μM respectively of $(dC)_n \cdot (dG)_{12}$ calculated as cytidylate monomer. Curve *5* is for hemoglobin messenger RNA $\cdot (dT)_{10}$ (see *Materials and Methods*); in terms of mononucleotides, the concentration is 6 μM. Curve *6* is for $(rA)_n \cdot (dT)_{10}$ (4 μM) using the polymerase from Mason–Pfizer monkey virus. The [^{3}H]dTTP concentration was 16 μM in all cases (specific activity 400 cpm/pmol). For curves *1–4*, and *6*, the buffer was 50 mM Tris, pH 7.8, 10 mM mercaptoethanol and 2 mM $MgCl_2$; for hemoglobin mRNA (curve *5*) the assay buffer was 50 mM Tris, pH 7.8, 0.5 mM $MnCl_2$, 25 mM KCl, and 10 mM mercaptoethanol. The inset shows the incorporation of dTMP using activated calf thymus (100 μM) as the template; the buffer was the same as for curves *1–4*, and *6*. The assay volume was 0.1 ml in all cases; assays were carried out at 37° for 30 min with the polymerase obtained from Dr. L. Loeb. The same assays were carried out with the enzyme isolated as described in *Materials and Methods*, except that four times more was used for each assay. The abscissae in the figure are for the Loeb enzyme. All assay mixtures contained 50 μg of bovine-serum albumin per ml. Samples were processed as described in *Materials and Methods*. Using hemoglobin mRNA as the template, sigmoid kinetics were also obtained using [^{3}H]dATP and dTTP as substrates resulting in $(dA,dT)_n$ synthesis and for [^{3}H]dGTP plus the other three unlabeled deoxynucleoside triphosphates for heteropolymeric synthesis (8).

Sigmoid kinetics are readily evident with $(rA)_n \cdot (dT)_{10}$, or hemoglobin mRNA as template. It is of interest that the acceleration in rate of synthesis is observed with these primer-templates at approximately the same total substrate concentration (about 3 μM). The kinetics are not sigmoid, at least in the deoxynucleoside triphosphate concentration range studied (0.08–16.0 μM), when calf-thymus DNA or $(dA)_n \cdot (dT)_{10}$ (Fig. 2), or $(dC)_n \cdot (dG)_{12}$ (not shown) is the template. These kinetics are now being investigated at lower concentrations of the deoxynucleoside triphosphates.

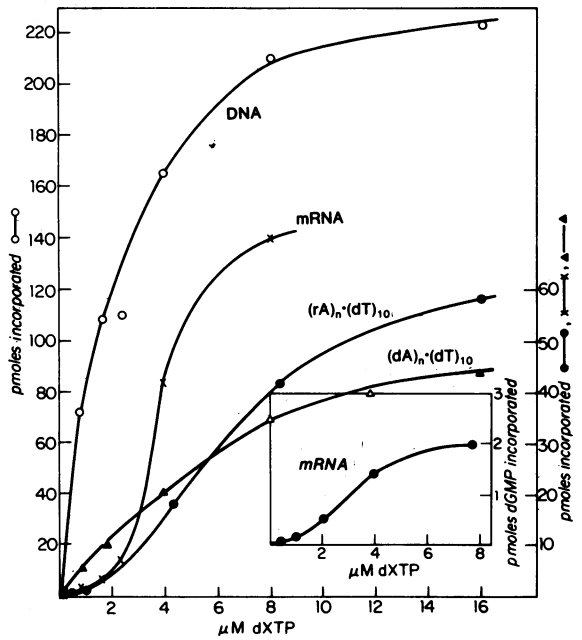

FIG. 2. Effect of substrate concentration on rate of synthesis. Standard assay conditions were used with 0.1 μg of enzyme prepared as described in *Materials and Methods*. All assays were also carried out with the *E. coli* polymerase I obtained from L. Loeb with similar results. The concentration (as nucleotide monomer) of the templates was: DNA, 100 μM; mRNA 6 μM; $(rA)_n \cdot (dT)_{10}$, 4 μM; $(dA)_n \cdot (dT)_{10}$, 4 μM. For DNA the label was [^{3}H]dGTP; for mRNA, $(rA)_n \cdot (dT)_{10}$, and $(dA)_n \cdot (dT)_{10}$ the label was [^{3}H]dTTP. The specific activity in all cases was 400 cpm/pmol. In the case of DNA all four triphosphates were present. For the others, only dTTP was present. The inset shows the kinetics for mRNA using [^{3}H]dGTP as label together with the other three unlabeled triphosphates. Other assay conditions as for the main part of the figure.

Authenticity of the Sigmoid Character of the Deoxynucleoside Triphosphate Curve. Since the *E. coli* polymerase I samples used here were isolated and purified by two entirely different procedures but, nevertheless, give similar sigmoid curves, it is highly unlikely that adventitious impurities in the enzyme could yield these results, especially since they vary with the template. Nevertheless, we tested and ruled out the possibility that abnormal kinetics might be produced by destruction of precursors or primer-template by some impurity in the enzyme. The precursor was exposed for 30 min to the enzyme under assay conditions in the absence of a primer-template, without affecting the rate of synthesis when either $(dA)_n \cdot (dT)_{10}$ or $(rA)_n \cdot (dT)_{10}$ was added. Likewise, preincubation of either of these primer-templates did not affect the subsequent rate of synthesis on adding the precursors. Further evidence that the sigmoid kinetics are not an artifact is the lack of sigmoidicity in the case of $(dA)_n \cdot (dT)_{10}$, under conditions which are otherwise identical to those which yield sigmoid kinetics with $(rA)_n \cdot (dT)_{10}$. It thus seems most likely that the deoxynucleoside triphosphates are not only involved as precursors in polymerization but are also acting at another, allosteric site or subsite on the polymerase I oligomer.

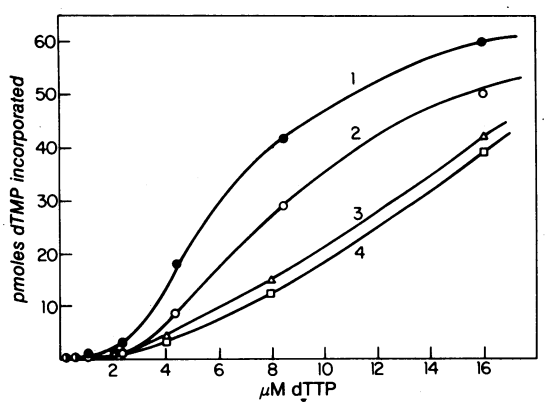

FIG. 3. Curve 1 shows incorporation of dTTP using $(rA)_n \cdot (dT)_{10}$ (8 μM) as the primer-template under standard conditions using the enzyme prepared as described in *Materials and Methods*. Curves *2*, *3*, and *4* show the effects of dCTP, dATP, and dGTP, respectively, (8 μM in each case) on the incorporation of dTTP.

Efficacy of Nonprecursor Deoxynucleoside Triphosphates as Modifiers. It has been shown (2) that polymerase I has one binding site for deoxynucleoside triphosphates in the absence of the primer-template, for which the four compete in the order G>A>T>C. Since we now have evidence for two deoxynucleoside triphosphate sites on the active enzyme–primer-template complex, it was of interest to examine the competition between the deoxynucleoside triphosphates for these sites. This was made possible by use of $(rA)_n \cdot (dT)_{10}$ as primer-template, for which the sole precursor is dTTP. Fig. 3 shows the effect of the other deoxynucleoside triphosphates on the sigmoid character of the rate *versus* concentration curve. The rate of the reaction is decreased on the addition of the other deoxynucleoside triphosphates but not in the order predicted above. Either the binding constants at the deoxyribonucleoside triphosphate sites are altered by the presence of the primer-template and the dimerization of the enzyme, and/or the binding of each deoxynucleoside triphosphate at the modifier site compensates to a different extent for its competitive effect at the precursor site.

Independent effects of enzyme and deoxynucleoside triphosphate concentration on the sigmoid kinetics

In order to determine whether the polymerase concentration alters the modifier effect of the deoxynucleoside triphosphates, a kinetic study was carried out at low and high enzyme concentration. The results are shown in Fig. 4; the sigmoid character is retained at both concentrations and the rapid rate of increase occurs at approximately the same deoxynucleoside triphosphate concentration, i.e., between 1 and 3 μM. Therefore, the modifier effect of deoxynucleoside triphosphates occurs when the enzyme concentration is both below and above the sigmoid transition value. In a companion experiment we examined the cooperative binding of the enzyme at two deoxynucleoside triphosphate concentrations. The results are shown in Fig. 5, where the dependence of the rate of synthesis on enzyme concentration is the same at two levels of deoxynucleoside triphosphate, 2.2 and 8.2 μM. Therefore, we deduce that the modifier role of the deoxynucleoside triphosphate is not related to enzyme oligomer formation on the primer-template. Rather, the modifier seems to interact with the preformed enzyme-oligomer–primer-template complex. The effect of increasing the concentrations simultaneously is dramatic. Thus, in Fig. 4 the rate of incorporation at 1 μM dTTP at 0.1 μg of protein is 0.2 pmol/30 min; at 8 μM dTTP

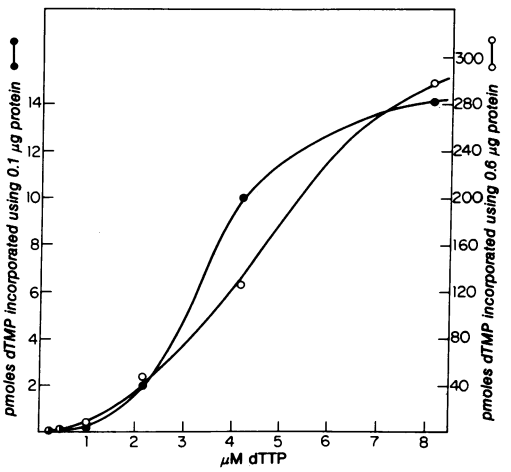

FIG. 4. Enzyme concentration and the allosteric effect of dTTP. 0.1 μg of protein (●——●) and 0.6 μg of protein (O——O) prepared as described in *Materials and Methods* were used in the standard assay with $(rA)_n \cdot (dT)_{10}$ (8 μM) as primer-template. Note the different scales for the two enzyme concentrations.

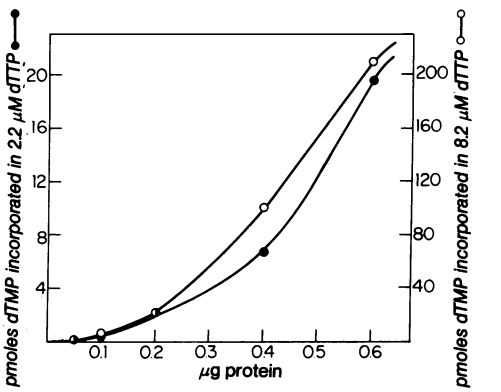

FIG. 5. Dependence of synthesis on enzyme concentration at different substrate concentrations using $(rA)_n \cdot (dT)_{10}$ (8 μM) as primer-template. The curve indicated by ●——● was determined in the presence of 2.2 μM dTTP; that shown by O——O, in the presence of 8.2 μM dTTP. Other conditions as in standard assay. Enzyme prepared as described in *Materials and Methods*.

and at 0.6 μg of protein, the rate is about 300. The combined effect therefore leads to a 1500-fold increase in rate, which is 30 times greater than that predicted from simple kinetics.

DISCUSSION

We have shown using *E. coli* polymerase I and the polymerase from Mason–Pfizer monkey virus with both synthetic and natural RNAs as templates, that the rate of DNA synthesis displays sigmoid kinetics as a function of enzyme concentration. Faras *et al.* (9) also reported, without interpretation, a sigmoid curve using Rous sarcoma virus reverse transcriptase with Rous sarcoma virus RNA, as did Leis and Hurwitz (10) using avian myeloblastosis virus reverse transcriptase with avian myeloblastosis virus RNA (the latter authors also mentioned that similar kinetics had been obtained with various DNAs as templates).* The sigmoid nature of these curves suggests a cooperative phenomenon (in the absence of artifacts); since we feel that we have ruled out the probable artifacts, we feel justified in this interpretation.

The fact that DNA polymerases from varying sources exhibit cooperativity with both natural and synthetic primer-templates in the presence of either Mn^{++} or Mg^{++} suggests the generality of the phenomenon with RNA templates. However, cooperative activation occurs at different enzyme concentrations with different primer-templates, presumably reflecting different polymerase binding constants of the primer-templates. The data can be interpreted in terms of an initial binding of one protein monomer to the primer-template, followed rapidly by the more facile binding of a second (and perhaps a third or fourth) monomer. Either the first monomer bound is altered allosterically by the primer-template to favor subsequent protein:protein association, or the second monomer interacts with the template as well as with the first monomer, the double interaction resulting in stronger binding. There is precedence for cooperative binding of protein to nucleic acid, as shown by Alberts and Frey (11) with gene-32 protein on DNA. A somewhat similar but less likely mechanism is as follows. The primer-template might bind an enzyme molecule to form an inactive complex which, nonetheless, alters the binding of a second enzyme molecule at some other site not near the first, to form the active complex.

With templates of the DNA type we have observed only hyperbolic kinetics when the enzyme concentration is varied in the same range which shows sigmoidicity with RNA templates. The curves pass through the origin, indicating that if there is a lag it would have to occur only at extremely low enzyme concentration. Nevertheless, because of experimental difficulties it is impossible to rigorously rule out that a short lag might exist with DNA templates at very low enzyme concentrations. Thus, we cannot eliminate kinetically the possibility that an enzyme dimer (or higher oligomer) is the active species on DNA templates; but if so, the binding constants of the first and subsequent monomers are very close. Moreover, the absence of cooperative binding of deoxynucleoside triphosphates with DNA templates suggests that there may be only one dXTP binding site (i.e., one enzyme monomer). Finally, Griffith *et al.* (12) have shown by electron microscopy that only one molecule of polymerase binds at each site on a DNA template $[(dA)_n \cdot (dT)_n]$.

In addition to the apparent cooperative binding of the enzyme, we have shown that with RNA templates the rate of DNA synthesis versus deoxynucleoside triphosphate concentration also yields sigmoid saturation kinetics, which cannot be attributed to destruction of primer-template or precursors. These results suggest the cooperative binding of two or more deoxynucleoside triphosphates to the polymerase-oligomer–primer-template complex, the first triphosphate acting as a modifier, the second as the true substrate. A similar suggestion has been made by Loeb *et al.* (1). However, it has been shown by Englund *et al.* and Huberman *et al.* (2–4) that the enzyme has only one triphosphate binding site for all four triphosphates; that is, once a site is occupied by one triphosphate, it can only be occupied by another which possesses a

* Using NP-40 (0.3%) lysates of Rauscher leukemia virus, avian myeloblastosis virus, and Moloney murine sarcoma virus, it has been observed that upon increasing the protein concentration in the lysates from 1 μg/ml to 2 μg/ml there is a 10-fold increase in the endogenous reverse transcriptase activity (personal communication from Dr. J. Huppert).

higher binding constant, with the concomitant displacement of the first. Thus, the two or more triphosphate sites required by our data may exist on different enzyme monomers, their differences probably being induced by interaction with the primer-template. The deoxynucleoside triphosphates do not affect enzyme dimerization, as shown by the fact that the sigmoid polymerase concentration curve is independent of the deoxynucleoside triphosphate concentration.

The initial steps in RNA-dependent DNA synthesis now appear to be dimerization (or oligomerization) of the enzyme on the primer-template, followed by the binding of one deoxynucleoside triphosphate molecule which modifies the complex allosterically so that a second deoxynucleoside triphosphate molecule is bound more tightly at the precursor site.

Whatever the exact mechanism, the polymerase and deoxynucleotide triphosphate concentration effects provide a basis for a possible regulatory mechanism *in vivo*. Thus, a relatively small increase in enzyme concentration accompanied by a similar increase in deoxynucleoside triphosphate concentration can increase the rate of RNA-dependent DNA synthesis dramatically. Such a change is tantamount to an on–off switch for synthesis and may be of importance in *in vivo* control. The fact that only RNA templates show these cooperative effects implies that such a control mechanism applies only to reverse transcription.

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