ON THE MECHANISM OF NUCLEOTIDE INCORPORATION INTO DNA AND RNA

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1. Introduction

The discovery of the exonuclease activity associated to some DNA polymerases [1–3] gave support to the notion that accuracy of polymerization with these enzymes is related to the kinetic interlocking of the exonuclease to the polymerase activities [2,3] although no satisfactory model is available to date. Eukaryotic DNA polymerases have no associated exonuclease activity [4] and yet may replicate templates with high fidelity [5]. Our studies on the dynamics of recognition processes [6–8] led us to the suggestion of a new possibility: kinetic amplification of discrimination [8]. Very similar conclusions were independently reached by J. Hopfield [9]. Thus, reaction schemes were described which allowed a much better discrimination between correct and incorrect substrate than would be predicted from the simple comparison of the kinetic parameters of the reaction carried by an enzyme on the two substrates [8,9].

We have performed kinetic experiments with DNA polymerase I and RNA polymerase from *E. coli*. Both enzymes are able to convert nucleoside triphosphates into nucleoside diphosphates in the course of the polymerization reaction on synthetic templates. The qualitative and quantitative aspects of the phenomenon are suggestive of a mechanism of incorporation using kinetic proofreading in Hopfield's sense [9].

2. Materials and methods

E. coli DNA polymerase I was purified up to step 7 of the procedure of Jovin et al. [10]. For *E. coli* RNA polymerase we followed the method of Burgess [11] with an additional chromatography on a DNA-cellulose column [12]. Incubation conditions for these two enzymes are reported in the legends of the figures.

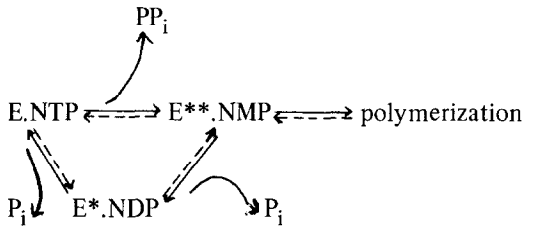
Mono-, di- and triphospho-nucleosides of the ribo- and deoxyribo-series were purchased from Boehringer, Mannheim. Labelled compounds were from the Radiochemical Center at Amersham (^{3}H) or from the C.E.A. at Saclay (^{32}P). Templates were provided by Miles Laboratories, Inc. and by Collaborative Research, Inc.

In every kinetic experiment, aliquots were withdrawn with a capillary tube and deposited on a P.E.I. - cellulose plastic sheet on a spot preloaded with the cold mono-, di- and triphosphate markers, and EDTA for stopping the reaction. Ascending thin-layer chromatography as in [2] with LiCl as solvant (1.2 M for purines, 1.0 M for pyrimidines) permitted a complete resolution of the polymerized material, remaining at the origin and the three kinds of monomers. After drying, the spots were cut and counted in a toluene-PPO-POPOP scintillation liquid.

Experiments with ^{32}P-labelled ATP involved high-voltage electrophoresis on DEAE paper with a 7% formic acid solution.

3. Incorporation scheme

The following scheme was found convenient to rationalize our results:



(For clarity, a number of side-reactions discussed in the text are omitted in this representation).

First the nucleoside triphosphate binds to the enzyme, the binding being more or less stabilized by the opposite base on the template. In that state, the nucleoside triphosphate (NTP) encounters a first probabilistic fork; (a) it may leave the enzyme, (b) a first phosphorus may be cleaved and the energy of cleavage used to bring the enzyme into the high-energy state E* such that the access of substrate to E* is much less easy than access through E, (c) hydrolysis of pyrophosphate bond may occur between the α and β phosphorus, bringing the enzyme into the high-energy state E**. Similarly, if the nucleoside triphosphate has been converted into nucleoside diphosphate, it encounters a second probabilistic fork, with a second opportunity to leave the complex in case its association with the opposite base is too weak. The use of energy to bring the enzyme into a less accessible state is an essential element of the picture since without it, the substrates could bypass the double-trial procedure [9].

A first consequence of such a scheme is that just prior to incorporation, the substrate is in the nucleoside monophosphate form (or, in a possible variant scheme, in the nucleoside diphosphate form). The data on pyrophosphorolysis and pyrophosphate exchange [13,14] are compatible with this possibility.

A second consequence is that nucleoside monophosphate release, nucleoside diphosphate release, and incorporation should remain in approximately constant proportions over a wide range of substrate concentrations. In contrast, when NMP production is attributable to an exonuclease activity, it should increase relatively to incorporation when substrate concentration decreases. As a consequence, misincorporations (i.e., escape from the exonucleolytic activity) should *increase* with increasing concentrations of the dNTP which is incorporated at the next position [8]. A third (plausible but not necessary) consequence is that the direct pathway E → E** with pyrophosphate release is likely to be less frequently used than the two-step pathway [15]. There is a fourth consequence. Let us define a 'well-behaving enzyme' as an enzyme with only one conformational state allowing the entry of the ligand and such that the kinetic parameters of the catalyzed reactions are rather insensitive to variations in ligand concentrations (these conditions exclude allosteric enzymes). Inasmuch as states E* and E** are hardly accessible, the scheme above corresponds to a well-behaving polymerase. Now, suppose that such an enzyme acts upon two competing substrates of concentrations a(t) and b(t), t being the time, and that the measured reactions are practically irreversible. The relationship log [a(t)/a(0)]/log [b(t)/b(0)] = constant should hold true throughout the reaction, whatever the detailed mechanisms and the particular rate-equations for each substrate taken individually.

4. Results

4.1. *DNA polymerase*

When Poly (dC) is used as a template, *E. coli* DNA polymerase I incorporates dGTP and (with a lag) dCTP; dATP and dTTP act as incorrect substrates. With Poly (dA)·dT_{12}, dTTP is incorporated, followed by dATP; dGTP and dCTP are the non-complementary substrates. These two reactions were studied extensively, both in the presence of magnesium and of manganese. A detailed report will be published elsewhere (F.B., in preparation). The following features were observed; (a) the rates of formation of dAMP, dTMP and dCMP were high only when the bases were complementary to the templates, the kinetics being suggesitive of the action of the exonuclease-associated activity of DNA polymerase I. There was little dGMP formation when dGTP was the complementary substrate, (b) there was practically no dADP, dGDP, dTDP or dCDP formation when the bases were used as complementary substrates. When the bases were the incorrect ones with respect to the template some dNDP was, in every case, observed. Of the four deoxynucleoside triphosphates, dATP gave the highest rate of conversion into nucleoside diphosphate, and it was the only one which could be substantially misincorporated.

Here, we focus on the quantitative relationships which are observed in one particular reaction. Poly (dC) was the template, in the presence of manganese and 10^{-4} M dGTP and $3 \cdot 10^{-7}$ M dATP as competing substrates. We followed in parallel experiments dGMP incorporation, dGMP release, dGDP formation, and also dAMP incorporation and dADP and dAMP release.

Misincorporations of dAMP increase with increasing dGTP concentrations (a similar effect was demonstrated by Hall and Lehman [16] on a T4 DNA polymerase, which also contains an exonuclease activity). Misincorporations of dAMP also require relatively high enzyme concentrations. This property, rather than reflecting allosteric interactions [17] may be related to the fact that after every incorporation, the enzyme leaves the template [18,19]. The longer it takes to return, the higher the chances that a base which was misincorporated in an abnormal tautomeric form or an abnormal ionic environment, has regained its usual conformation or environment and thus is detected as incorrect by the exonuclease function.

It can be seen on fig.1 that dGTP disappearance (mainly through incorporation) and dATP disappearance (mainly through convertion into dADP) parallel each other, and satisfy the log/log relationship to a very good approximation. At the early stages of the reaction (fig.2) there is a five-fold excess of dAMP incorporated over dAMP released. As the reaction goes on, and dGTP is consumed, the release of

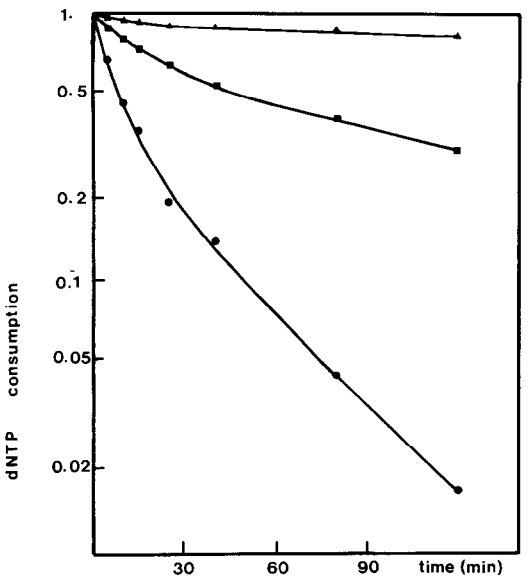


Fig.1. Consumption of correct and incorrect substrate by *E. coli* DNA polymerase I. Incubations were performed at 37°C in a final vol of 100 μl containing 0.75 unit of enzyme; 5 μmol of Tris, pH 7.4; 10 μmol of KCl; 10 pmol of $MnCl_2$; 0.06 ODU_{270} of Poly (dC); 10 pmol of dGTP; 1.5 pmol of dATP, the ^{3}H-label being either on G (●) or on A (■ and control without template: ▲). The fraction of the label remaining as nucleoside triphosphate is plotted as a function of time. In the presence of Poly(dC) dGTP consumption is 3.2 times faster than dATP consumption (the log/log ratio equals 3.2 ± 0.2).

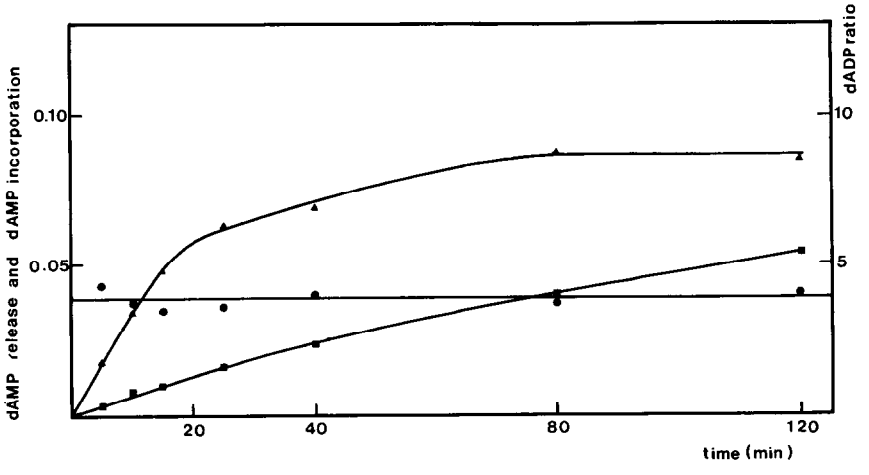


Fig.2. Relationships between dAMP misincorporation, dADP release and dAMP release. Same incubation conditions as for fig.1. (▲) fraction of initial dATP converted into polymerized dAMP; (■) fraction of initial dATP converted into free dAMP; (●) ratio of released dADP to (incorporated + released) dAMP.

dAMP becomes comparable to the incorporation of dAMP – exactly what we expect from the exonuclease activity. Now, in our incorporation scheme, after the interaction of dATP with the enzyme, relatively constant proportions should be found between dAMP incorporation, and dAMP and dADP release. Later on, some dAMP incorporated may be converted into free dAMP by the exonuclease activity. Then, the prediction of the model becomes that although the ratio dAMP incorporated/dAMP released could vary with time, we should have constancy in the ratio dADP released/(dAMP incorporated + dAMP released) and we show on fig.2 that this is indeed the case.

In that experiment, two constraints are revealed: the log/log relationship, and the dADP/(dAMP polymerized + dAMP released) constancy. In addition, the variations in the ratio dAMP incorporated/dAMP released are understood qualitatively [8] and the other constraints are verified in a trivial way (almost no dGDP or dGMP release).

4.2. *RNA polymerase*

Our preparation of RNA polymerase was able to convert all four nucleoside triphosphates into nucleoside diphosphates while the incorporation reaction was strictly dependant upon the presence of the complementary bases on the templates. The two activities co-migrated upon elution from the DNA-cellulose column. The kinetic data is not as convincing as that obtained with DNA polymerase I, and further studies on the polymerization reaction under stabilizing and destabilizing conditions will be necessary to reach totally secure conclusions. However, there are a number of evidence which are consistent with the proposed Hopfield mechanism.

In fig.3 we present an experiment with *E. coli* RNA polymerase. The template Poly (dG-dC) was transcribed with GTP 10^{-5} M and ATP 10^{-6} M, the label being either on G or on A. GTP is very efficiently incorporated. After 80 min of incubation, 86% of the GTP input was polymerized, and 4% was converted to GDP. In the parallel experiment, 80% of the ATP input was converted to ADP. One can see on the semi-log plot of fig.3 that the disappearance of ATP parallels that of GTP, suggesting that we are observing a competition on a well-behaving enzyme. It can also be seen that the presence of a template

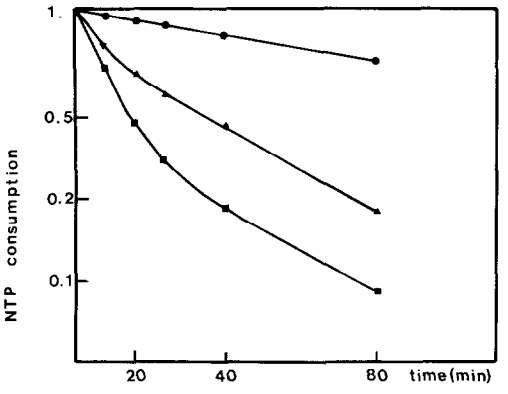


Fig.3. Consumption of correct and incorrect substrate by *E. coli* RNA polymerase. The incubation mixtures contained in a volume of 80 μl: 0.5 unit of enzyme; pH 7.9 buffer (2 μmol of Tris, 0.2 μmol of sodium phosphate); 15 μmol of KCl; 1 μmol of $MgCl_2$; 10 pmol of EDTA; 10 pmol of DTT; 0.025 ODU_{260} of Poly (dG-dC); 1 pmol of GTP; 0.1 pmol of ATP; 0.1 pmol of UTP, the ^3H label being either on G (■) or on A (▲ and control without template: ●). Incubations were carried at 37°C. The fraction of the label remaining as nucleoside triphosphate is plotted as a function of time. In the presence of Poly (dG-dC), GTP consumption is 1.6 times faster than ATP consumption.

produces a five-fold enhancement of ATP → ADP conversion. The contrast between the reaction with or without a template is enhanced by decreasing the enzyme concentration which is logical since in that case a higher proportion of the enzyme is bound to the template.

In the reported experiment we had an average of 4.2 molecules of GDP released for every 100 GMP incorporated, the ratio being nearly constant throughout the reaction. Increasing the enzyme concentration by a factor of 5 and decreasing that of GTP by a factor of thirty, we find 3.8 molecules of GDP formed for every 100 GMP incorporated.

The template dependance of ATP → ADP conversion is illustrated in fig.4 Poly d(A-T) is slightly more efficient than Poly (dG-dC) which in turn markedly stimulates dephosphorylation with respect to the control without a template.

If diphosphates were formed through a kinase reaction:

$$NTP + NMP \rightleftharpoons 2\ NDP$$

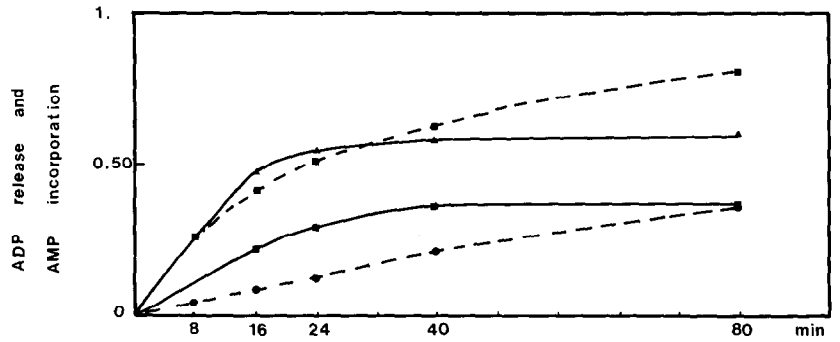


Fig.4. Template dependence of ATP → ADP conversion promoted by *E. coli* RNA polymerase. Incubation conditions were the same as for fig.3. (- -●- -) Without template; (- -■- -) With Poly (dG-dC); (—▲—) With Poly d(A-T); (—■—) AMP incorporation in the presence of Poly d(A-T). The fraction of the initial label either converted to ADP or incorporated into RNA is plotted as a function of time.

one should expect that using γ^{32}P-labelled ATP as a substrate in the presence of added AMP, some label would be transferred to ADP as the reaction goes on. Several such experiments were performed and this never occurred, even when the incubation was carried in the presence of ten times more AMP than ATP. Some of the label was transferred from ATP to a compound with an R_f value of 1.4 with respect to ATP (that of ADP being 3.3). Its nature was not investigated further. The compound did not show up in the experiments described below in which polymerization was carried in the presence of pyrophosphate. Otherwise, the radioactivity released from ATP was found almost exclusively in the orthophosphate spot.

Normally, one would expect to observe some ^{32}P-label co-migrating with pyrophosphate. In order to rule out the presence of a contaminating pyrophosphatase, the polymerization reactions were performed in the presence of pyrophosphate. Incubations were carried as described in the legend of fig.3, in the presence of Poly d(A-T) with added 10^{-5} M pyrophosphate. Three different labels were used in parallel experiments: [^{3}H]ATP in order to follow the polymerization, [γ-^{32}P]ATP in order to measure orthophosphate and pyrophosphate release, and [^{32}P]pyrophosphate in order to estimate the possible pyrophosphate activity in the course of polymerization.

The efficiency of incorporation was lower in these experiments (3 ADP released for one AMP incorporated). The label carried by [γ-^{32}P]ATP was transferred to orthophosphate and pyrophosphate in a ratio of ten to one. After 40 min of incubation, while incorporation was of 15%, less than 5% of the exogenous pyrophosphate had been cleaved into orthophosphate, showing that the enzyme preparation was practically free of pyrophosphatase activity. From these results, it is possible to deduce rather unequivocally that at least 54% of AMP incorporation is attributable to a pathway which involves orthophosphate and not pyrophosphate release.

A similar result was obtained in the presence of $MnCl_2$. While two molecules of AMP were incorporated for every molecule of ATP converted to ADP, the ratio PP_i/P_i was of 0.5 at the early stages of the reaction, suggesting that 50% of the incorporation involved orthophosphate and not pyrophosphate release.

5. Discussion

Our results strongly suggest that nucleotide incorporation and conversion of nucleoside triphosphate into nucleoside diphosphate are two alternative outcomes of the interaction of a NTP with the polymerase–template complex in the course of polymerization. We have shown for both enzymes that the nucleoside diphosphate is not formed from the nucleoside monophosphate. If the NDP or the dNDP was obtained from a Polynucleotide-phosphorylase-like attack on the synthesized polynucleotide,

one should expect to find an ADP/GDP or a dADP/dGDP ratio reflecting the composition of the synthesized chain. We observe an opposite pattern: the NDP or dNDP is mainly observed when the corresponding NTP or dNTP cannot be polymerized for lack of a complementary base on the template. Contamination of the DNA polymerase preparation with template-dependant dNTPases is highly unlikely. The kinetic data presented here and extended elsewhere (F.B., in preparation) is neat. In order to explain quantitatively the results, the putative contaminants would have to be endowed with such special properties that our data would be the first evidence for a novel kind of template-dependant dNTPases. The kinetic data obtained with RNA polymerase suggest a superposition of specific and non-specific effects. Further work with this enzyme is required in order to demonstrate unambiguously that the NDP formation is indeed a side-product of polymerization.

The relative frequencies of the upper and lower pathways in our scheme (corresponding to pyrophosphate and orthophosphate release respectively) may be highly dependant on the concentrations of the reactants, the substrates (purines or pyrimidines), and the incubation conditions. Further elucidation of the mechanism of the reaction will require establishing under various stabilizing and destabilizing conditions the quantitative relationships between incorporation, orthophosphate and pyrophosphate production, and NMP and NDP release.

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