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DNA polymerase activity in water-structured and confined environment of reverse micelles

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Abstract

DNA polymerases are the key enzymes of DNA replication and repair. These proteins in living cells are functioning not as isolated entities but in multiprotein complexes, and their work are carefully regulated. The main factors determining the enzyme activity are the structure and dynamics of 'biological' water. Reverse micelles (nano-sized water droplets dispersed in a continuous oil phase) are the simple model systems where the structure and dynamics of water are controlled. In this work, the activities and processivity of Klenow fragment of *E. coli* DNA polymerase I, thermostable *Tte* DNA polymerase, and HIV-1 reverse transcriptase are investigated as a function of the water pool size. Klenow fragment was more active on poly(rA)-oligo(dT) in reverse micelles compared with the water buffer with activity being increased upon increasing water content. *Tte* polymerase was more active at low water content. HIV-1 reverse transcriptase revealed comparable activity and processivity on poly(rA)-oligo(dT) in the water buffer and reverse micelles at water content of 15–40%. Thus, the polymerase activity appears in certain range of water concentration and depends on the local polarity determining the protein 'expansion', microviscosity inside nano-droplets determining the enzyme dynamics, and nucleic acid hydration degree.

Keywords: DNA polymerase; Enzymatic nucleic acids synthesis; Reverse micelles

1. Introduction

Biochemical assays are typically performed using very dilute solutions of macromolecular components. On the other hand, most of the enzymes in the living system are working in an environment, which is quite different from aqueous bulk solution. Water is an essential component in protein structure, function, and dynamics [1–3]. Biomembrane and bound water existing near the boundary surface must affect the catalytic activity of enzymes in cells more or less, resulting in regulation of the enzyme function. An experimental approach that allows the study of water–protein interactions with different, but low content of water is to entrap proteins in the interior of reverse micelles [4–7]. Reverse micelles, or water-in-oil microemulsions, are nano-meter-sized water droplets dispersed in an apolar solvent with the aid of a surfactant monolayer, forming a thermodynamically stable and optically transparent solution. The main feature of these systems is their ability to solubilize both hydrophilic and hydrophobic substances, which are localized within their different distinct microdomains. In these systems it is possible to vary the size of the micelles and, hence, the amount of water in contact with the protein. Reverse micelles have been used as a model system for biological studies ranging from basic biochemical research [4,8] to applied biotechnology [9,10].

Abbreviations: *Tte* pol, DNA polymerase from *Thermus thermophilus* B35; *Tth* pol, DNA polymerase from *Thermus thermophilus* HB8; *Taq* pol, DNA polymerase from *Thermus aquaticus* YT1; CTAB, cetyl trimethylammonium bromide; Brij58, polyoxyethylene 20 cetyl ether; Brij30, polyoxyethylene 4 lauryl ether; SDS, sodium dodecylsulfate; Triton X-100, polyoxyethylene 9.5 *p-tert*-octylphenyl ether

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Earlier, we have found that DNA polymerase α-primase complex and Klenow fragment of *E. coli* DNA polymerase I synthesize DNA on synthetic templates in reverse micelles. The enzyme activity was observed in micelles stabilized by non-ionic surfactants and their mixtures with other types of detergents. But DNA polymerases revealed a maximal activity in the system composed of cationic CTAB, anionic SDS, non-ionic Triton-X-114 and Brij 58 surfactants (concentration of 15, 25, 128, and 10 mM, respectively) in hexanol–decane (1:12 v/v). An increase in water content resulted in an increase of DNA polymerase processivity. The enzymes, however, synthesized more products in the water buffer than in reverse micelles [11].

In this work we significantly improved the above system by modifying composition of microemulsion and reaction conditions. In addition, we have investigated three different enzymes, namely Klenow fragment of *E. coli* DNA polymerase I, thermostable *Tte* DNA polymerase, and HIV-1 reverse transcriptase. Despite of different properties and peculiarities in DNA synthesis these enzymes have a similar three-dimensional structure, which assumes a hand-like structure [12–14]. The fingers, palm and thumb subdomains form the template-binding cleft and may influence the processivity of DNA synthesis [15–17]. Since the structure of the nucleic acid substrate depends on water content, we also studied the similarities and differences of catalytic activities of DNA polymerases on RNA and DNA templates. All the enzymes displayed high activity and processivity on these templates in reverse micelles at defined water content.

2. Materials and methods

2.1. Materials

Ammonium persulfate, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), acrylamide, *N*,*N*′-methylene-bis-acrylamide, poly(dT), poly(dA), poly(rA), dTTP, dATP, CTAB, Brij 30, Brij 58, Triton X-100 were purchased from Sigma (USA). $MgCl_2$ was from Merck (USA). Tris, bromophenol blue, dithiothreitol were obtained from Serva (Germany). [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Biosan (Russia). Klenow fragment of *E. coli* DNA polymerase I was from SibEnzyme (Russia). The oligonucleotides $(rA)_{15}$ and $(dT)_{16}$ were synthesized and kindly supplied by Dr. Abramova T.V. (Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences). Other chemicals were of chemical-grade purity or extra pure products.

Recombinant HIV-1 reverse transcriptase heterodimer (p66/p51) was isolated from *E. coli* DH5 strain harboring the plasmid pUC12N [18]. Recombinant *Tte* pol was purified from *E. coli* BL21 strain carrying plasmid pGT12 [19].

2.2. Separation of oligonucleotides by denaturing polyacrylamide gel electrophoresis

The nucleotide material was precipitated with 0.3 ml of 2% $LiClO_4$ in acetone at 0 °C. The precipitate was centrifuged at $10^4 \times g$ for 10 min, washed with cold acetone (0.3 ml), dried and dissolved in 5 μl of 7 M urea (in water) containing 0.5% bromophenol blue as a marker dye.

Electrophoresis was performed on vertical plates (15 cm × 25 cm × 0.03 cm). The polymerization mixture contained 50 mM Tris–borate buffer, pH 8.3, 20% acrylamide, 1% *N*,*N*′-methylene-bis-acrylamide, 7 M urea, 0.1% TEMED, and 0.05% ammonium persulfate. Electrophoresis was continued at 10 W until the marker dye migrated to the 3/4 of distance. The positions of radioactive oligonucleotides were located by autoradiography.

2.3. Preparation of reverse micelles

The microemulsion solutions were prepared by adding the measured volumes of hexanol and octane (1:6 v/v) to dried, pre-weighted amounts of Brij30, Triton X-100, SDS, CTAB, Brij 58 (concentration of 133, 77, 22, 11 and 7 mM, respectively), and further injecting the required volume of the water buffer. The mixture was shaken until being optically clear (1–5 s).

2.4. DNA polymerase and HIV-1 reverse transcriptase assays in the water buffer

Reaction mixtures (0.1 ml) contained 50 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM dithiothreitol, 25 μM dTTP, 10 μM (in all the cases the concentration of polynucleotide is as mononucleotide concentration) poly(dA) or poly(rA), 0.1 μM 5′-[^{32}P]$(dT)_{16}$ (specific activity of 1 Ci/μmol), and 5–10 nM DNA polymerase. Reactions were initiated by adding the enzyme. The assays were performed at 22 °C for 20 min and quenched with 10 volumes of 2% $LiClO_4$ in acetone. The samples were treated, and electrophoresis was performed as described above. Polymerization products were detected by autoradiography.

2.5. Klenow fragment assays in the water buffer on poly(dT) template

Reaction mixtures (0.1 ml) contained 50 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM dithiothreitol, 25 μM dATP, 10 μM poly(dT), 0.1 μM 5′-[^{32}P]$(rA)_{2–15}$ (specific activity of 1 Ci/μmol), and 5–10 nM DNA polymerase. Reactions were initiated by adding the enzyme. The assays were performed at 22 °C for 20 min and quenched with 10 volumes of 2% $LiClO_4$ in acetone. The samples were treated, and electrophoresis was performed as described above. Polymerization products were detected by autoradiography.

2.6. DNA polymerase and HIV-1 reverse transcriptase assays in reverse micelles

The final mixtures (0.1 ml) contained 20 mM Tris, 1 mM $MgCl_2$, 1 mM dithiothreitol, 25 μM dTTP, 10 μM poly(dA) or poly(rA), 0.1 μM 5′-[^{32}P](dT)$_{16}$ (specific activity of 1 Ci/μmol), and 5–10 nM DNA polymerase. The reaction mixtures containing the substrates were introduced into the organic system as water solutions. Micelles were formed by vigorous stirring in vortex. After formation of the micelles, the DNA polymerase in the water buffer was introduced to start the reaction. After being vigorously stirred, the mixtures were incubated at 22 °C during 20 min and quenched with 10 volumes of 2% $LiClO_4$ in acetone. The products were analyzed by electrophoresis as described above.

2.7. Klenow fragment in reverse micelles on poly(dT) template

The final mixtures (0.1 ml) contained 20 mM Tris, 1 mM $MgCl_2$, 1 mM dithiothreitol, 25 μM dATP, 10 μM poly(dT), 0.1 μM 5′-[^{32}P](rA)$_{2–15}$ (specific activity of 1 Ci/μmol), and 5–10 nM DNA polymerase. The preparation of reverse micelles and the product analysis by electrophoresis were described above.

3. Results

3.1. Conditions for DNA polymerases activity in reverse micelles

In the previous paper we have shown that DNA polymerases preferred non-ionic microenvironment and they were very sensitive to the ionic strength at a low water content [11]. We have also found the system composed from the Triton X-114, SDS, CTAB, and Brij 58 (concentration of 128, 25, 15 and 10 mM, respectively) in hexanol–decane (1:12 v/v) in which DNA polymerases revealed the higher activity, than in system containing only Brij 58 (single homogeneous surfactant).

Based on these findings we improved the above system by introducing Brij 30 and changing the composition of the micelles to: 133 mM Brij 30, 77 mM Triton X-100, 22 mM SDS, 11 mM CTAB, and 7 mM Brij 58 in hexanol–octane (1:6 v/v).

The main criteria for selection of this composition were following. Firstly, the system must be stable in a wide range of water content. It is well known that the solubilization in microemulsions and the stability of macroemulsions are considerably enhanced by mixing of surfactants, compared with a single homogeneous surfactant. Especially, a big difference of hydrophile–lipophile balance (HLB) between the used surfactants leads to a greater increase of solubilization of water in microemulsions [20–22]. We mix hydrophilic (Triton X-100, SDS, CTAB, Brij 58) and lipophilic (Brij 30) surfactants in respect to their HLBs to produce the microemulsions with maximal solubilization capacities. Secondly, the system must provide a high conformational flexibility of the solubilized macromolecules because experimental kinetic analyses for several DNA polymerases have suggested rate-limiting conformational changes in polymerases both before and after the chemical reaction of nucleotide incorporation in DNA synthesis [23–25]. It is evident that the protein dynamics and function are coupled through hydration shell to dynamics of reverse micelle. On the other hand, almost all aspects of the dynamics and thermodynamics of reverse micelles are affected by the flexibility of the oil–water micellar interface. The poly(ethylene glycol) non-ionic surfactants have their polar headgroups within the water pools, and tend to produce more flexible films as they try to curve the interface away from the water pool [26,27]. In addition, there is a general feeling that poly(ethylene glycol) is essentially an inert hydrophilic polymer—repelling and being repelled by proteins—thereby providing a protective coat for any surface to which it is attached [28].

3.2. Klenow fragment in reverse micelles

Fig. 1 shows DNA polymerase activity of Klenow fragment on poly(dT)-oligo(rA)$_{2–15}$ template primers in reverse micelles and in the water buffer. One can see that the DNA polymerase effectively elongates the primers of seven nucleotide length or longer in both systems. The processivity of

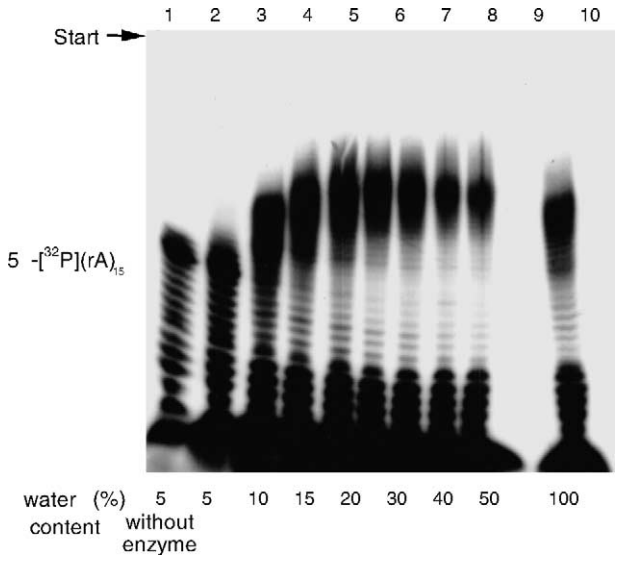

Fig. 1. DNA products synthesized on poly(dT)-oligo(rA)$_{2–15}$ template-primers by Klenow fragment in reverse micelles. Lane 1 shows the reaction mixture without the enzyme; lanes 2–9 represent DNA polymerase activity in reverse micelles that contained 20 mM Tris, 1 mM $MgCl_2$, 1 mM dithiothreitol, 25 μM dATP, 10 μM poly(dT), 0.1 μM 5′-[^{32}P](rA)$_{2–15}$ (specific activity of 1 Ci/μmol) and 5–10 nM DNA polymerase. Lane 10 represents DNA synthesis by Klenow fragment in the water buffer. Reaction mixture contained 50 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM dithiothreitol, 25 μM dATP, 10 μM poly(dT), 0.1 μM 5′-[^{32}P](rA)$_{2–15}$ (specific activity of 1 Ci/μmol) and 5–10 nM polymerase.

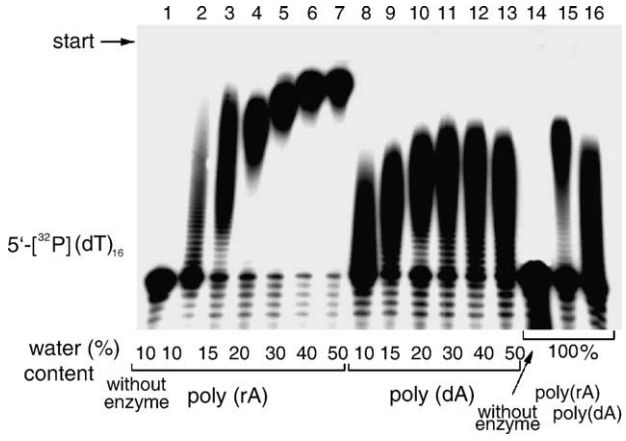

Fig. 2. DNA products synthesized on poly(rA)-oligo(dT)$_{16}$ and poly(dA)-oligo(dT)$_{16}$ templates-primer by Klenow fragment in reverse micelles. Lanes 1 and 14 show the reaction mixture without the enzyme; lanes 2–13 show DNA polymerase activity in reverse microemulsion that contained 20 mM Tris, 1 mM $MgCl_2$, 1 mM dithiothreitol, 25 μM dTTP, 10 μM poly(dA) or poly(rA), 0.1 μM 5′-[^{32}P](dT)$_{16}$ (specific activity of 1 Ci/μmol) and 5–10 nM DNA polymerase. Lanes 15 and 16 represent Klenow fragment activity in the water buffer contained 50 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM dithiothreitol, 25 μM dTTP, 10 μM poly(dA) or poly(rA), 0.1 μM 5′-[^{32}P](dT)$_{16}$ (specific activity of 1 Ci/μmol) and 5–10 polymerase.

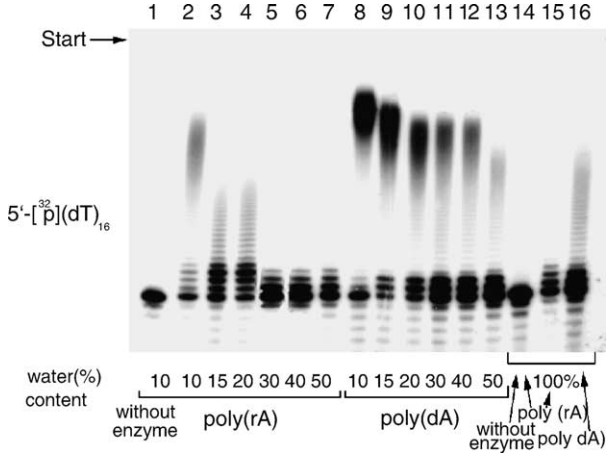

Fig. 3. DNA products synthesized on poly(rA)-oligo(dT)$_{16}$ and poly(dA)-oligo(dT)$_{16}$ templates-primer by *Tte* polymerase in reverse micelles. Lanes 1 and 14 show the reaction mixture without the enzyme; lanes 2–13 show DNA polymerase activity in reverse microemulsion that contained 50 mM Tris, 1 mM $MgCl_2$, 1 mM dithiothreitol, 25 μM dTTP, 10 μM poly(dA) or poly(rA), 0.1 μM 5′-[^{32}P](dT)$_{16}$ (specific activity of 1 Ci/μmol) and 5–10 DNA polymerase. Lanes 15 and 16 represent *Tte* polymerase activity in the water buffer contained 50 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM dithiothreitol, 25 μM dTTP, 10 μM poly(dA) or poly(rA), 0.1 μM 5′-[^{32}P](dT)$_{16}$ (specific activity of 1 Ci/μmol) and 5–10 nM polymerase.

the enzyme increases with an increase of water content from 5 to 20% and it is slightly higher in the micelles than in the water buffer.

Replacing the DNA template strand by RNA leads to an increase in processivity of Klenow fragment (Fig. 2). This reverse transcription on poly(rA) template strongly depends on water content and the enzyme reveals maximum activity at 20–50% water concentration. We can also note that the DNA polymerase activity on the RNA templates is significantly higher in the reverse micelles than in the water buffer. Processivity of Klenow fragment in micelles was higher on poly(rA) than on poly(dA).

3.3. *Tte* polymerase in reverse micelles

The thermostable *Tte* pol is an analog of *Tth* pol and has 87% homology to *Taq* pol used in amplification of DNA fragments by the polymerase chain reaction [19]. The C-terminal domain of the large fragment of *Taq* pol is identical in folding to the equivalent region of the Klenow fragment [14]. However, the thermostable DNA polymerase works at higher temperatures (75–85 °C) and has low activity at 20–25 °C.

To understand the mechanism of this behavior of *Tte* pol, we analyze the dependence of the enzyme activity on water content. As shown in Fig. 3, this dependence is exact antithesis to that for Klenow fragment. The activity of the thermostable DNA polymerase decreases with an increase of water content. In addition, the enzyme activity and processivity are significantly higher in the reverse micelles than in the water buffer. The thermostable DNA polymerase works also better on poly(dA) template than on poly(rA).

3.4. *HIV-1 reverse transcriptase in reverse micelles*

The above results show that Klenow fragment displays high activity on RNA template. In vivo, copying RNA into DNA is a prerogative of reverse transcriptase—a DNA polymerase able to use both RNA and DNA as a template in a series of reactions yielding a double-stranded DNA molecule. HIV-1 reverse transcriptase is required for conversion of the viral genomic RNA into a double-stranded proviral DNA precursor.

Fig. 4 shows that the DNA polymerase activity on poly(rA) template is comparable in the water buffer and in the reverse micelles at water content of 20–50%. However, the processivity of the reverse transcriptase on poly(dA) template is lower in the micelles than in the water buffer. We can also note that the enzyme activity on the DNA template does not significantly depend on water content higher than 10%.

4. Discussion

A common feature of enzymes in all non-conventional systems (organic solvents, reverse micelles or microemulsions) is that at very low water concentrations, enzymes exhibit activities that are much lower than in aqueous media; another common feature is that the increase of water content results in an increase of enzyme activity. The low activity that the enzymes reveal at low water concentration is not consequence of the enzyme denaturation at least irreversible, as an increase in water content restores the catalytic activity.

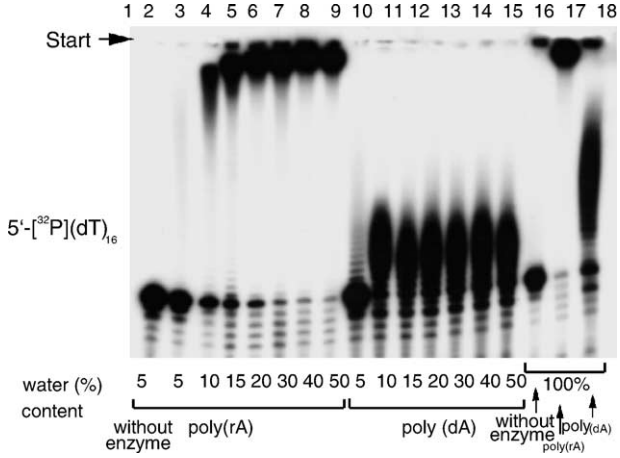

Fig. 4. DNA products synthesized on poly(rA)-oligo(dT)$_{16}$ and poly(dA)-oligo(dT)$_{16}$ templates-primer by HIV-1 reverse transcriptase in reverse micelles. Lanes 1 and 14 show the reaction mixture without the enzyme; lanes 2–13 show DNA polymerase activity in reverse microemulsion that contained 20 mM Tris, 1 mM $MgCl_2$, 1 mM dithiothreitol, 25 μM dTTP, 10 μM poly(dA) or poly(rA), 0.1 μM 5′-[^{32}P](dT)$_{16}$ (specific activity of 1 Ci/μmol) and 5–10 nM polymerase. Lanes 15 and 16 represent HIV-1 reverse transcriptase activity in the water buffer contained 50 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM dithiothreitol, 25 μM dTTP, 10 μM poly(dA) or poly(rA), 0.1 μM 5′-[^{32}P](dT)$_{16}$ (specific activity of 1 Ci/μmol), 5–10 nM polymerase.

This phenomenon can be ascribed to low flexibility of the enzymes in such conditions. At present the relationship between protein flexibility, activity and water, has been found for the lyophilized protein films, the enzymes dispersed in organic solvents, and the enzymes entrapped in reverse micelles [5,29–31].

On the basis of extensive kinetic measurements and a large body of crystallographic open and closed structures of several polymerases complexed with primer/template duplex DNA and/or incoming nucleotide structures [23–25,32–37], the overall pathway of incorporation of a correct 2′-deoxyribonucleoside-5′-triphosphate by a DNA polymerase has been proposed. Namely, a DNA polymerase binds DNA to form a binary complex in an open state, which further binds a correct 2′-deoxyribonucleoside 5′-triphosphate to form an open ternary complex. This open complex undergoes a conformational transition to generate a closed ternary complex, in which the chemical reaction of incorporating dNTP to the primer 3′-terminus occurs. Following the chemical reaction, the product complex undergoes a second conformational change from a closed to an open state prior to release of the product pyrophosphate. The conformational closing and opening during each catalytic cycle have been suggested to be key players in the faithful incorporation of nucleotides via an 'induced-fit' mechanism [36–38].

In the light of these data it is clear that DNA polymerase activities should depend on the enzyme conformational flexibility, which encompasses a wide range of hydration states. Equilibrium between these states will depend on the activity of the water within its microenvironment, i.e. the freedom that the water has to hydrate the protein. Our study exactly demonstrates the water activity dependence of DNA polymerase activity, and the increase of Klenow fragment and HIV-1 reverse transcriptase activities with an increase of water content can be explained by higher conformational mobility of these enzymes at higher water content.

In case of the thermostable DNA polymerase the increase of activity at low water content comes out from improvement of the protein dynamics by reducing of a hydrophobic effect in low polar environment. The thermostability of proteins corresponds to increase rigidity at low temperature, which is shifted to normal flexibility at the physiological temperature level [39]. The existing crystallographic models of Klenow fragment of *E. coli* DNA polymerase I and *Taq* pol revealed the dramatic differences in their structures. The N-terminal domain has undergone extensive sequence and structural rearrangements that resulted in an enhanced hydrophobic core [14]. Consequently, the conformational flexibility of this enzyme could be increased by weakening of hydrophobic effect in a low polar medium such as reverse micelles at low water content.

Next, our interesting finding is the high reverse transcriptase activities of Klenow fragment and *Tte* polymerase in reverse micelles. These results indicate that hydration is very important for the conformation and utility of nucleic acids. It is expected that the strength of these aqueous interactions is far greater than those for proteins due to their highly ionic character [40]. RNA has a greater extent of hydration than DNA due to its extra oxygen atoms (i.e. ribose 2′−OH). On the other hand, the binding of nucleic acid to polymerase active site requires its partial dehydration. So, the reduction of nucleic acid hydration in reverse micelle by lowering water activity could facilitate the utilization of RNA by DNA polymerase.

Overall, our data indicate that the micellar microenvironment accentuates the difference between the enzymes in terms of the relation structure/activity. It appears clear that water is instrumental in protein flexibility and substrate structure that participate in the expression of catalysis. The analysis and proof of this concept has led to the visualization of many novel and unsuspected characteristics of enzymes. Study of properties of DNA polymerases and their activities in reverse micelles seems very important to advance our understanding of mechanism of DNA replication in vivo. In addition, this knowledge should be very useful to operate by DNA synthesis in biotechnology, e.g. to improve conditions of DNA synthesis on RNA templates or stimulate activity of thermophilic enzymes at moderate temperatures.

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