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DNA polymerase β reveals enhanced stability in reverse microemulsions

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ABSTRACT

Water is essential for the stability and functions of proteins and DNA. Reverse microemulsions are model systems where the structure and dynamics of water are controlled. We have investigated the different hydration and confinement effects on the activity and the stability of mammalian DNA polymerase β in the complex reverse microemulsions, containing ionic and nonionic surfactants in decane/hexanol. The enzyme displays high processivity on primed single-stranded M13mp19 DNA with maximal activity at 10% of water content. DNA polymerase reveals the enhanced stability toward the thermal and the chemical denaturation. The enzyme is still active at 65 °C and in 4 M urea. The data provide direct evidence for strong influence of microenvironment on DNA polymerase activity and stability.

1. Introduction

Water is essential for the stability and functions of proteins and DNA [1–3]. Hydration plays a major role in the assembly of a protein structure and dynamics. In living systems, the interactions between water and biopolymer occur in restricted geometries in cells and organelles. Generally, the properties of purified macromolecules have been studied in dilute solutions. On the other hand, biological macromolecules have evolved over billions of years to function inside cells, so researchers studying the properties of such molecules in vitro systems, ignore factors that reflect the intracellular environment. There are several universal aspects of the cellular interior that is largely neglected—the macromolecules crowding [4–6], confinement, and the altered structure and properties of water.

Reverse microemulsions (hydrated reverse micelles) allow the study of water–protein interactions with different water content [7–10]. Reverse microemulsions are transparent, isotropic, and thermodynamically stable liquid media with nanosized water droplets dispersed in a continuous oil phase and stabilized by surfactant molecules at the water–oil interface. They are the model systems where the structure and dynamics of water and confinement are controlled by changing the molar ratio of water to surfactant.

DNA polymerase β is one of the smallest nuclear eukaryotic DNA polymerases (molecular mass of 39 kDa) with a host of biochemical properties that make this enzyme an ideal model for studying the detailed mechanism of enzymatic DNA polymerization. DNA polymerase β has been suggested to play a role in DNA repair, DNA replication, and recombination [11–13].

In our previous papers [14,15], we have investigated the effect of different compositions of reverse microemulsions on activity of DNA polymerases and have found the system optimal for polymerase activity. In the present work, we investigate the influence of confinement on DNA polymerase β activity and stability.

2. Experimental

2.1. Materials

Ammonium persulfate, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), acrylamide, *N*,*N*′-methylene-bis-acrylamide, bromophenol blue, dithiothreitol, Tris, dNTP, cetyl trimethylammonium bromide (CTAB), polyoxyethylene 4 lauryl ether (Brij30), polyoxyethylene 20 cetyl ether (Brij58), polyoxyethylene 9.5 *p*-*tert*-octylphenyl ether (Triton X-100), sodium dodecylsulfate (SDS), fluorescein were purchased from Sigma. $MgCl_2$ was from Merck. [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Biosan (Russian Federation). The oligonucleotide 5′-GGCGATTAAGTTGGG (primer), single-stranded M13mp19 DNA, and T4 polynucleotide kinase was purchased from SibEnzyme (Russian Federation). The primer was labeled with [γ-^{32}P]ATP using polynucleotide kinase according to

Abbreviations: 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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the standard protocol [16] and annealed to M13mp19 DNA at position 6338–6352. Recombinant DNA polymerase β was purified from *E. coli* BL21DE3 pLys S harboring the plasmid pRSET [17].

2.2. Preparation of reverse microemulsions

The reversed microemulsions were prepared by adding the measured volumes of decane and hexanol (6:1, v/v) to dried, preweighted amounts of Brij30, Triton X-100, SDS, CTAB, Brij58 (to give total concentration of 133, 77, 22, 11 and 7 mM, respectively), and further injecting the required volume of the water buffer. The mixtures were shaken until being optically clear (5–10 s).

2.3. Turbidity and absorption spectra measurements

The absorption spectra of reverse microemulsions and Flu-4-dUTP were recorded on a Hitachi U-0080D photodiode array UV–vis spectrophotometer by using 1-cm path length cells. The reverse micelle pseudo-absorbance signal, *A*, read in an absorption spectrophotometer is related to turbidity by: $\tau = 2.303A$.

2.4. DNA polymerase assays in reverse microemulsions

The final mixtures (0.1 ml) contained 20 mM Tris, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 10 μM dNTP, 0.5–3.0 nM M13mp19 ssDNA, and 0.5–3.0 nM 5′-[^{32}P]GGCGATTAAGTTGGG primer (specific activity of 1 Ci/μmol). The reaction mixtures containing the substrates were introduced into the organic system as water solutions. Microemulsions were formed by vigorous stirring in vortex. After formation of the microemulsions, the DNA polymerase (30–100 nM) in the water buffer was introduced to start the reaction. After being vigorously stirred, the mixtures were incubated at 0–65 °C for 30 min. The nucleotide material was precipitated with 1 ml of 2% $LiClO_4$ in acetone at 0 °C, washed with cold acetone (1 ml), dried, and dissolved in 7 μl of 95% formamide containing 0.5% bromophenol blue as a marker dye. Reaction products were separated by electrophoresis on 20% polyacrylamide gels in 7 M urea. The gels were dried and subjected to autoradiography and/or phosphoimaging for quantification using Molecular Imager FX (Bio-Rad) and software (Quantity One).

2.5. DNA polymerase assays in the water buffer

Reaction mixtures (10 μl) contained 50 mM Tris–HCl, pH 8.6, 10 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol, 100 μM dNTP, 5–30 nM M13mp19 ssDNA, 5–30 nM 5′-[^{32}P]GGCGATTAAGTTGGG primer (specific activity of 1 Ci/μmol). Reactions were initiated by adding the enzyme (0.1–1 μM). The mixtures were incubated at 0–65 °C for 30 min. The nucleotide material was precipitated with 1 ml of 2% $LiClO_4$ in acetone at 0 °C, washed with cold acetone (1 ml), dried, and dissolved in 7 μl of 95% formamide containing 0.5% bromophenol blue as a marker dye. Reaction products were separated and visualized as described above.

3. Results and discussion

3.1. The exploration of reverse micellar microenvironment

In our previous papers we have shown that DNA polymerases does not work in ionic reverse microemulsions, are slightly active in nonionic microenvironment and are the most active in the microemulsions composed of mixed ionic and nonionic surfactant [14,15]. The reverse microemulsion (also called water-in-oil microemulsion or reverse micelle) consists of an aqueous microdomain facing the polar heads of surfactant that surrounds this core interacting with the bulk organic solvent, through the hydrophobic

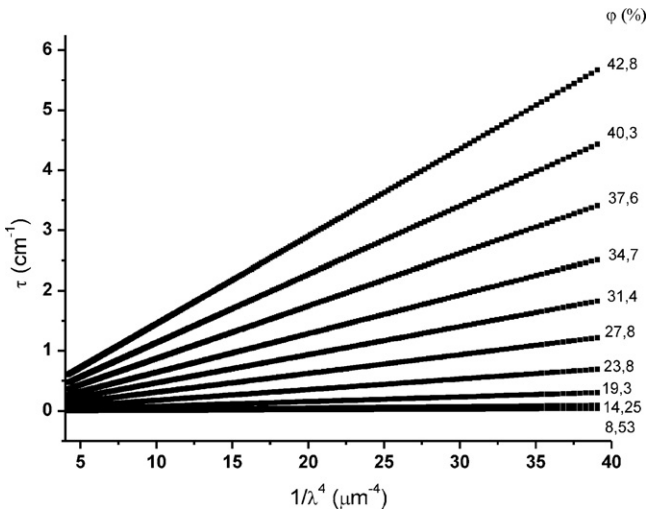

Fig. 1. The dependence of reverse micelle turbidity (τ) on wavelength (λ) of the light at various water content (φ).

chains. The reverse microemulsion definitions do not include some important properties, concerning the behavior of this system. One is the fact that the microemulsions are dynamical entities, which can exchange their constituents including water, surfactant, or other contents. The mixed multi-component systems provide an environment in which the different type of interaction can be realized, including hydrophobic, van der Waals, electrostatic and hydrogen bond interaction. The fluidity of the microemulsions is permanent since no covalent chemical bonds are formed, and, therefore, the structure and dynamics of reverse microemulsions is expected to be modified by solubilized proteins and nucleic acids. In fact, the enzyme and DNA can create cell-like environment by rearrangement of different types of surfactants at surface. On the other hand, the microemulsion system can change the structure of protein and DNA. Under the circumstances, the true criterion of native-like environment for given enzyme is its high-catalytic efficiency. Compared to reverse micelles created from ionic surfactants, there exists substantially less information about nonionic reverse micelles and, especially, about mixed multi-component systems. Therefore, we have investigated some physicochemical properties of the reversed microemulsions used in this work.

The turbidity measurements are the simple method to estimate the size of the scattering particles. Fig. 1 shows that the turbidity of reverse microemulsions is inversely proportional to the fourth power of the electromagnetic wavelength as predicted by classical light scattering theory (Rayleigh scattering). Rayleigh theory applies to small particles with radii less than 1/10 the wavelength of the radiation and predicts $\tau \sim r^6/\lambda^4$, where τ is the turbidity, *r* the radius of the scattering particles, and λ is the electromagnetic wavelength. Therefore, our results indicate that the reverse microemulsions are smaller that 25 nm, and their sizes are decreased with a decrease in the water content. The fitting of experimental points by a linear function of $1/\lambda^4$ in the range from 400 to 700 nm gave the correlation coefficient $R = 1$ (standard deviation S.D. = 10^{-15}), according to the Rayleigh light scattering theory. This result indicates the relatively low polydispersity of water droplet sizes as well.

The physical characteristics of the water in the reverse micellar medium are strongly depending on the molar ratio of water to surfactant and on the nature of the surfactant. Experimental studies of water pool confined in reverse micelles revealed a substantial decrease of polarity and a dramatic slowing down of the rotational relaxation of water molecules [18–23]. We investigate the microenvironment in our reverse microemulsions using fluorescein

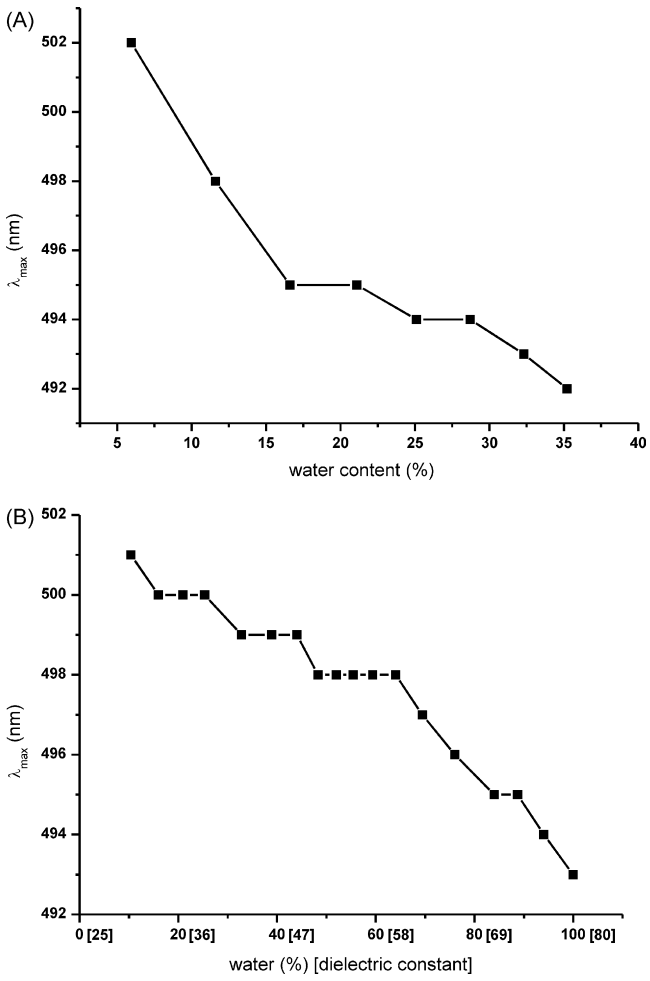

Fig. 2. The shift in the maximum absorption wavelength (λ_{max}) of fluorescein in reverse microemulsions (A) and in water–ethanol mixture (B). Dielectric constants (in square brackets) were calculated according to volume fractions of water and ethanol.

as molecular probe. The shift in the maximum absorption wavelength (λ_{max}) of a solvatochromic probes can sensitively reflect the local environment about the probe. Fluorescein is a useful probe molecule to investigate the micro-polar environment. When we place this probe in the reverse microemulsions, the absorption maximum is red-shifted with decreasing water content (Fig. 2A). We see that the absorption maximum of fluorescein is shifted from 492 to 502 with a decrease in water content from 35 to 6%. As shown in Fig. 2B, the same behavior of the probe is observed in a homogeneous mixture of water and ethanol. While the wavelength of absorption maximum in water (dielectric constant of 80) is 493 nm, that in 90% ethanol (dielectric constant of 30.5) reaches 501 nm. Therefore, a red shift of the absorption maximum suggests that the apparent polarity of fluorescein microenvironments in the microemulsion is less than the polarity of the bulk water when the water content in reverse microemulsions is below 25%.

3.2. Dependence of DNA polymerase activity on the water content in the reverse microemulsions

Decrease of polarity can lead to weakening the hydrophobic interactions, and, hence, to partial protein unfolding with the increase of its conformational mobility. However, confinement of proteins to water nanodroplets increases the free energy of the system by substantially attenuating the configurational entropy of the constituent macromolecules (excluded volume effect). Processes that reduce excluded volume will be entropically favored, such as processes, leading to macromolecular compaction. The size of the water droplets could determine the extent of protein compaction and, respectively, conformational mobility of the enzyme. These predictions are supported by theoretical and experimental studies of protein folding under confined, as well as crowded, conditions [24]. For example, studies of Förster resonance energy transfer of a dansyl probe covalently bound at the surface of cytochrome c to the heme group of the protein in reverse micelles shown the effect of the reverse micelle size on the folding compaction [25]. The most dramatic stabilizing effect of confinement was observed when a metastable protein that is largely unfolded in free solution was encapsulated in AOT reverse micelles. An unresolved ^{15}N heteronuclear single quantum correlation spectrum in a dilute solution was sharpened, indicating the formation of a three-helix bundle structure in the water core of a reverse micelle [26]. On the other hand, experimental kinetic analyses for DNA polymerase have suggested rate-limiting conformational changes in polymerases both before and after the chemical reaction of nucleotide incorporation in DNA synthesis [27–29]. Therefore, it is evident that DNA polymerase activity should be depend on the water content (the water droplet size).

Indeed, Fig. 3 shows strong dependence of DNA polymerase activity on water content in reverse microemulsions. The maximal activity was observed at 10% water content (Fig. 3, lane 1). At higher water content, the enzyme activity is decreased (Fig. 3, lanes 2–9). We can also see that DNA polymerase is more active in the reverse microemulsions than in the water buffer (Fig. 3, lane 10), i.e. the micellar environment is more favorable for the enzyme catalysis.

3.3. DNA polymerase is active in reverse microemulsions at high temperature

The theory predicts that confining a protein to a small inert space should stabilize the protein against reversible unfolding [24,30,31]. Confinement eliminates some expended configuration of the unfolded chain, shifting the equilibrium from the unfolded state toward the native state. Therefore, the reverse microemulsions can increase the folding stabilities of DNA polymerase. Indeed, in the reverse microemulsions we observe the enzyme activity even at 65 °C (Fig. 4, lane 6). The optimal temperature for the polymerase activity in this environment is 31 °C, below this temperature the enzymatic catalysis is likely decreased due to an overall slowing down of the system dynamics. DNA polymerase activity also decreases at higher temperature possibly due to the protein unfolding and (or) the template-primer duplex melting.

3.4. Stability of DNA polymerase in presence of urea

Urea is a well-known protein denaturant. It can affect the enzyme and DNA duplex structures by direct interaction with the macromolecules or by an indirect action through effects on the structure and properties of the surrounding solvent or by a combination of both of these mechanisms. As shown in Fig. 5, urea in concentration ranges up to 4.8 M reduces the enzyme activity both in aqueous solution and in the reverse microemulsions. However, a comparison of DNA polymerase activity in water buffer (Fig. 5, lanes 2–7) with those in micellar system (Fig. 5, lanes 14–19) indicates that the enzyme is more resistant to urea denaturation in the reverse microemulsions. In addition, when we preincubate the polymerase in water solution during 5 min with following determination of the enzyme activity in the reverse microemulsions (Fig. 5, lanes 8–13), we can see a decrease in the protein activity only at high-urea concentration (Fig. 5, lane 13). These results open the way to refolding the protein in the reverse microemulsions after the enzyme denaturation in water–urea solution. It must be noted that

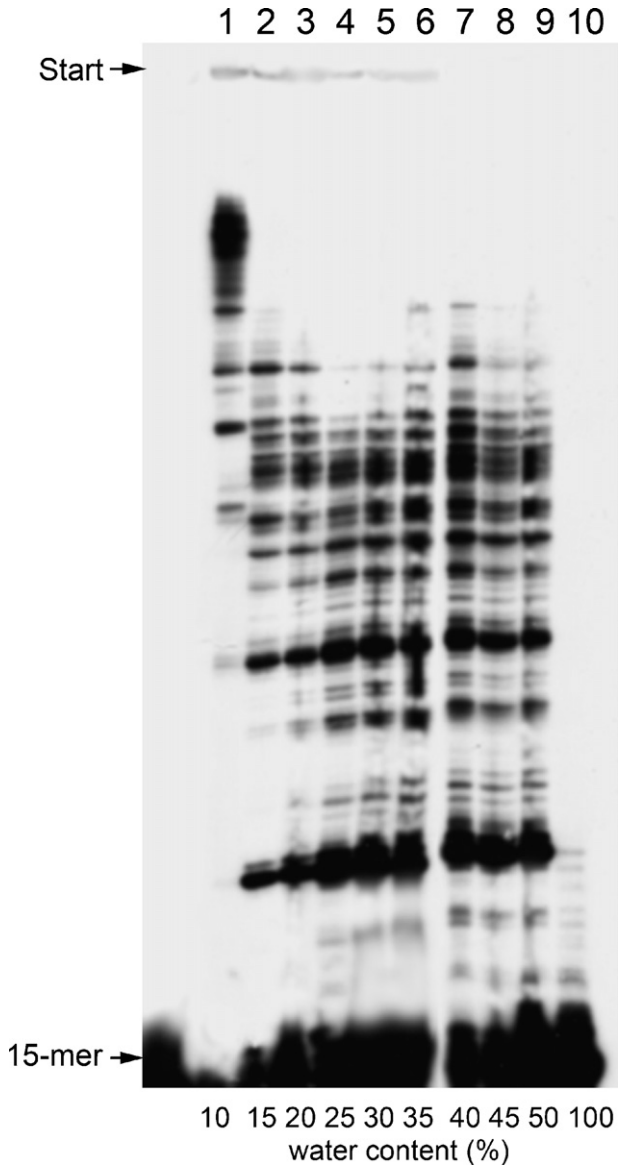

Fig. 3. The dependence of DNA polymerase β activity on water content in the reverse microemulsions. Lanes 1–9 represent DNA polymerase activity in the reverse microemulsions (100 μl) that contained 20 mM Tris, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 10 μM dNTP, 3 nM M13mp19 ssDNA, 3 nM 5′-[^{32}P] primer, and 30 nM DNA polymerase; lane 10 is the enzyme activity in water buffer (10 μl) that contained 50 mM Tris–HCl, pH 8.6, 10 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol, 100 μM dNTP, 30 nM M13mp19 ssDNA, 30 nM 5′-[^{32}P] primer, and 100 nM DNA polymerase. The mixtures were incubated at 22 °C for 30 min.

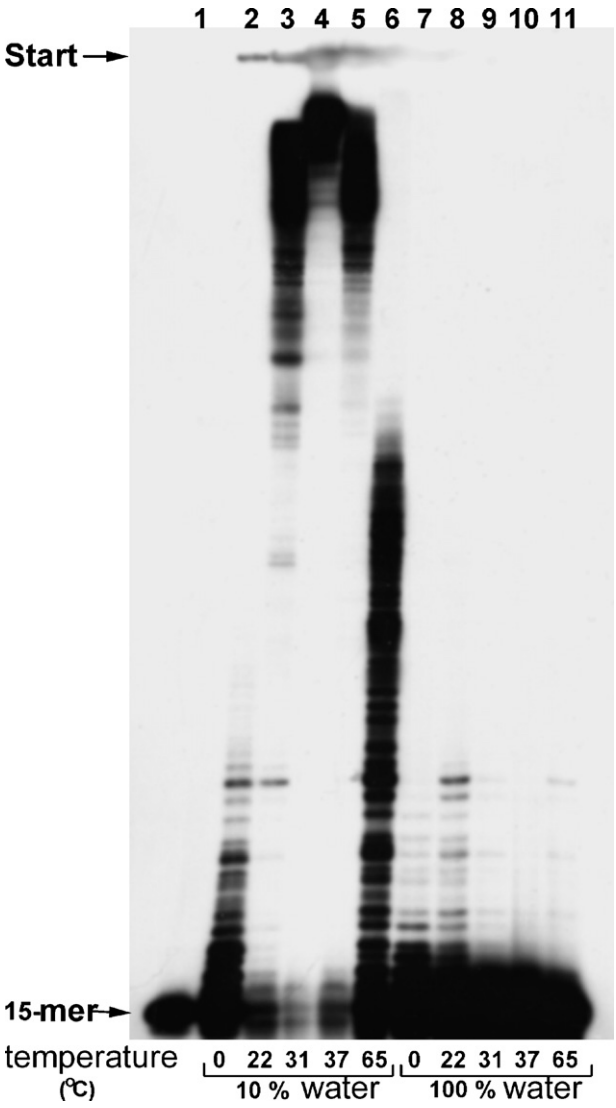

Fig. 4. The dependence of DNA polymerase β activity on temperature. Lane 1 shows the reaction mixture without the enzyme; lanes 2–6 represent DNA polymerase activity in the reverse microemulsions that contained 20 mM Tris, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 10 μM dNTP, 3 nM M13mp19 ssDNA, 3 nM 5′-[^{32}P] primer, and 30 nM DNA polymerase; lanes 7–11 show enzyme activity in water buffer (10 μl) that contained 50 mM Tris–HCl, pH 8.6, 10 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol, 100 μM dNTP, 30 nM M13mp19 ssDNA, 30 nM 5′-[^{32}P] primer, and 100 nM DNA polymerase. The mixtures were incubated at 0–65 °C for 30 min.

the polymerase activity in water solution is significantly increased at high-protein concentration in large excess toward the template-primer duplex (compare Fig. 3, lane 10 and Fig. 5, lane 2).

The similar results have been reported in case of a lipase encapsulated in AOT–heptane–water reverse micelles. In the micellar solution, the enzyme was more resistant to denaturation by urea than in aqueous solution. In addition, in the presence of urea, the Michaelis constant and the catalytic rate constant for the hydrolysis reaction of 2-naphthyl acetate catalyzed by lipase both increased [32].

Enzyme stability is thought to be dictated by its three-dimensional configuration, which is determined by genetic (primary structure) and environmental (interaction with the surroundings) factors. Most enzymatic reactions are performed in aqueous media, which favors inactivation. Water acts as a reactant in inactivation reactions and as a lubricant in conformational changes associated with protein unfolding [33]. An approach for enzyme stabilization is medium engineering, i.e. the manipulation of reaction medium [34]. Since water is involved in enzyme inactivation, partial or almost total substitution of water might be beneficial for biocatalyst stability [35]. In fact, numerous cases have been reported where remarkable enzyme stability has been obtained in such media [36]. Until recently, the use of enzymes in non-aqueous media seemed unfeasible because of the very low activities obtained. Reverse micelles (microemulsions) provide novel environments that allow to study the influence of confinement, hydration, and temperature in limits that are beyond the scope of other experimental approaches [37]. Our work demonstrates that the complex enzymatic reaction, such as the template-directed DNA synthesis catalyzed by DNA polymerase β, can be carried out in a low-water systems with efficiency higher than in conventional water solution.

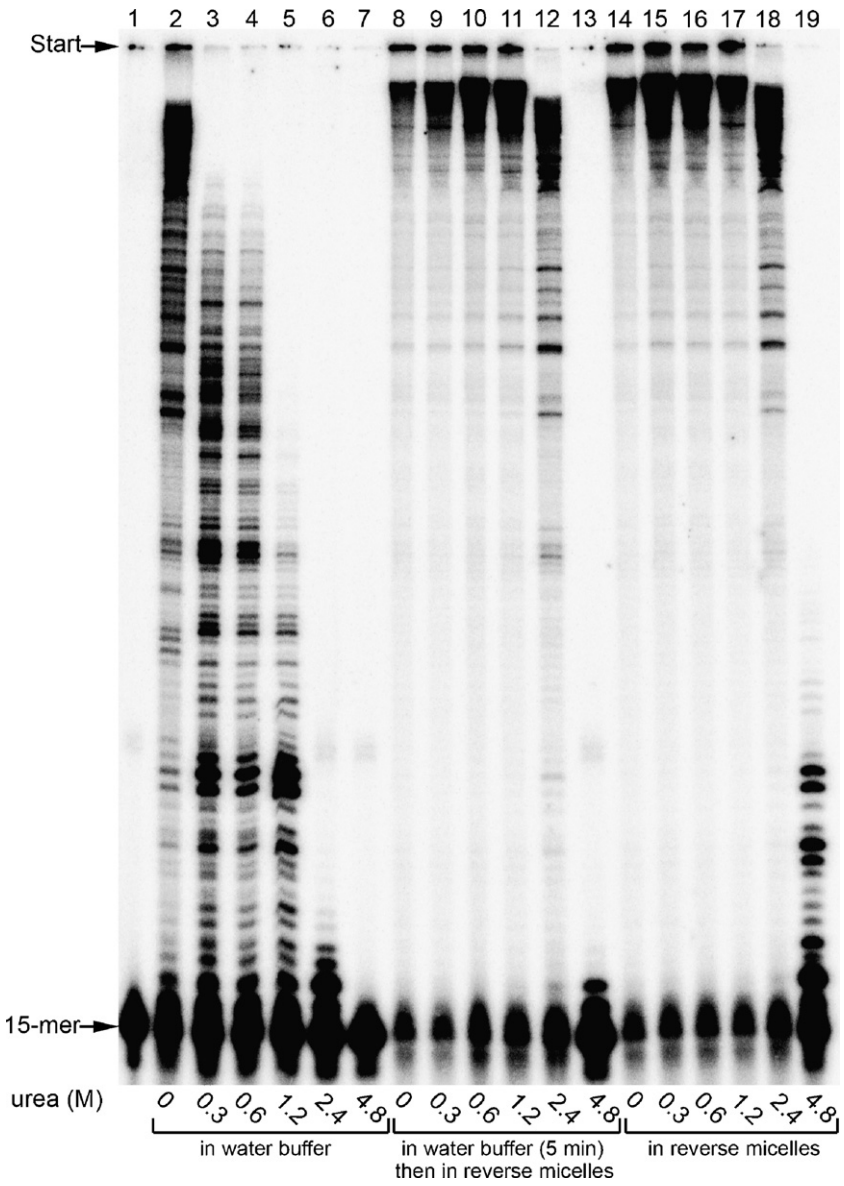

Fig. 5. DNA polymerase β activity in presence of urea. Lane 1 shows the reaction mixture without the enzyme; lanes 8–19 represent DNA polymerase activity in the reverse microemulsions (100 μl) that contained 20 mM Tris, 1 mM $MgCl_2$, 1 mM dithiothreitol, 10 μM dNTP, 0.5 nM M13mp19 ssDNA, 0.5 nM 5′-[^{32}P] primer, and 100 nM DNA polymerase; lane 2–7 is the enzyme activity in water buffer (10 μl) that contained 50 mM Tris–HCl, pH 8.6, 10 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol, 100 μM dNTP, 5 nM M13mp19 ssDNA, 5 nM 5′-[^{32}P] primer, and 1 μM DNA polymerase. The mixtures were incubated at 22 °C for 30 min.

4. Conclusion

We have explored the properties of complex reverse microemulsions and have discovered that the size and polarity of water droplets increases with an increase in the water content. We have investigated the different hydration and confinement effects on the activity and stability of DNA polymerase β in reverse microemulsions. We have found that the maximal enzyme activity is observed at 10% water content, and the protein is more resistant to thermal and urea denaturation in the reverse microemulsions. Our results suggest that DNA polymerase structure and stability are influenced in this microenvironments by two important factors that are not apparent in dilute solutions: (1) confinement (excluded volume) effects; and (2) a variable hydrophobic effect that depends on the surface chemistry of the surfactants and their interaction with water. In general, excluded volume effects due to molecular confinement should always favor protein stability, but changes in the hydrophobic effect may supplement or oppose the effect of excluded volume. In the case of the reverse microemulsion system, the observed enhancement in thermal and chemical stability of encapsulated protein and DNA is attributed to excluded volume effects, but this enhancement might be attenuated by the unfavorable influence of the microemulsion surface and confinement on bulk water structure. In vivo, both excluded volume effects and hydration effects due to biosurfaces and solutes may have an impact on the structure and stability of macromolecules.

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