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DNA polymerase β reveals enhanced activity and processivity in reverse micelles

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ABSTRACT

Water is essential for the stability and functions of proteins and DNA. Reverse micelles are simple model systems where the structure and dynamics of water are controlled. We have estimated the size of complex reverse micelles by light scattering technique and examined the local microenvironment using fluorescein as molecular probe. The micelle size and water polarity inside reverse micelles depend on water volume fraction. We have investigated the different hydration and confinement effects on activity, processivity, and stability of mammalian DNA polymerase β in reverse micelles. The enzyme displays high processivity on primed single-stranded M13mp19 DNA with maximal activity at 10% of water content. The processivity and activity of DNA polymerase strongly depend on the protein concentration. The enzyme reveals also the enhanced stability in the presence of template-primer and at high protein concentration. The data provide direct evidence for strong influence of microenvironment on DNA polymerase activity.

1. Introduction

Water is essential for the structure and functions of biological macromolecules [1–3]. Hydration plays a major role in the assembly of a protein structure and dynamics. In living systems, water–biopolymer interactions occur in restricted geometries in cells and organelles. Generally, the properties of purified macromolecules have been studied in dilute solutions due to ease and aggregation problems that often occur on at further concentration. 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 fact that it is highly crowded with macromolecules [4–6], confined due to compartmentalization, and with altered structure and properties of water.

An experimental approach that allows the study of water–protein interactions with different content of water is to entrap proteins in the interior of reverse micelles [7–9]. Reverse micelles 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 simple model systems where the structure and dynamics of water and confinement are controlled by changing water per surfactant molar ratio.

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 [10–12].

In the present work we study the activity, processivity, and stability of DNA polymerase β in reverse micelles composed of mixed ionic and nonionic surfactant in decane/hexanol. We revealed significantly higher activity and processivity of DNA polymerase in reverse micelles than in aqueous buffer, which is strongly dependent on water content. The data provide direct evidence for strong influence of microenvironment (local polarity and excluded volume effect) on DNA polymerase activity.

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), BSA (bovine serum albumin), fluorescein were purchased from Sigma. $MgCl_2$ was from Merck. [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Biosan (Russia). The oligonucleotides 5′-GGCGATTAAGTTGGG (primer), single-stranded M13mp19 DNA, and T4 polynucleotide kinase was purchased from SibEnzyme (Russia). The primer was labeled with [γ-^{32}P]ATP using polynucleotide kinase according to the standard protocol [13] and

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annealed to M13mp19 DNA at position 6338–6352. Recombinant DNA polymerase β was purified from *E. coli* BL21DE3 pLys S harboring the plasmid pRSET [14]. The concentration of the protein was determined using the extinction coefficient, ε_{280}=23,380 cm^{-1} M^{-1}, computed by ProtParam tool (the Swiss Institute of Bioinformatics), assuming no Cys residues appear as half cystines [15]. The absorption spectra of reverse micelles and fluorescein were recorded on a Hitachi U-0080D Photodiode Array UV–Vis spectrophotometer by using 1-cm path length cells. The pH values of the probe aqueous solutions were measured at 22 °C on an Oakton pH/Ion 510 m (Eutech Instruments Pte Ltd/ Oakton Instruments) calibrated with three USA standard buffers of pH 4.01, 7.00, and 10.01.

2.2. Preparation of reverse micelles

The reverse micelle solutions were prepared by adding the measured volumes of decane and hexanol (6:1 v/v) to dried, pre-weighted amounts of Brij 30, Triton X-100, SDS, CTAB, Brij 58 (to give total concentration of 133, 77, 22, 11 and 7 mM, respectively), and further injecting the required volume of the aqueous buffer solution. The mixtures were shaken until being optically clear (5–10 s). The stock solution with minimum 1.5% (v/v) water content was stable at least one year.

2.3. DNA polymerase assays in reverse micelles

The final mixtures (0.1 ml) contained 20 mM Tris, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 10–15 μM dNTP, 2–3 nM M13mp19 ssDNA, and 2–3 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. Micelles were formed by vigorous stirring in vortex. After formation of the micelles, the DNA polymerase (final concentration of 0.1–100 nM) in the aqueous buffer solution was introduced to start the reaction. After being vigorously stirred, the mixtures were incubated at 22 °C for 3–30 min. The nucleotide material was precipitated with 1 ml of 2% (w/v) $LiClO_4$ in acetone at 0 °C, washed with cold acetone (1 ml), dried, and dissolved in 7 μl of 95% (v/v) formamide containing 0.5% (w/v) bromophenol blue as a marker dye. Reaction products were separated by electrophoresis on 20% (w/v) 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).

2.4. DNA polymerase assays in the aqueous buffer solution

Reaction mixtures (10 μl) contained 50 mM Tris–HCl, pH 8.6, 10 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol, 150 μM dNTP, 20 nM M13mp19 ssDNA, 20 nM 5′-[^{32}P]GGCGATTAAGTTGGG primer (specific activity of 1 Ci/μmol), and 1–1000 nM DNA polymerase β. Reactions were initiated by adding the enzyme. The mixtures were incubated at 22 °C for 3–30 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

3.1. The structure of reverse micelles

Fig. 1A shows the chemical structures of surfactants, oil (decane), and cosurfactant (hexanol) used in this work. Surfactants are amphiphilic molecules containing non-polar (hydrophobic tail) and polar (hydrophilic head) parts capable of interacting with interface. Above the critical micelle concentration (CMC), the surfactant molecules readily associate to form supramolecular structure, such as micelles, vesicles, bilayer or other. According with the nature of the head groups the surfactants are classified into anionic, cationic, or zwitterionic. In this work, we used three types of surfactant: anionic sodium dodecylsulfate (SDS), cationic cetyl trimethylammonium bromide (CTAB), and non-ionic polyoxyethylene 4 lauryl ether (Brij 30), polyoxyethylene 20 cetyl ether (Brij 58), polyoxyethylene 9.5 p-tert-octylphenyl ether (Triton X-100). The type of surfactant used to form the reverse micelles can largely influence the enzyme activity. In our previous papers we have shown that DNA polymerases does not work in ionic reverse micelles, are slightly active in nonionic microenvironment and are the most active in the micelles composed of mixed ionic and nonionic surfactant: 133 mM Brij 30, 77 mM Triton X-100, 22 mM SDS, 11 mM CTAB, and 7 mM Brij 58 in hexanol-decane (1:6 v/v) [16,17].

A hypothetical structure of reverse micelle with incorporated DNA polymerase β is shown in Fig. 1B. The structure of DNA polymerase was taken from Protein Data Bank (http://www.pdb.org/pdb/explore/explore.do?structureId=2FMS, primary citation [18]). The reverse micelle (also called water-in-oil or reverse microemulsion) consists of an aqueous micro-domain facing the polar heads of surfactant that surrounds this core interacting with the bulk organic solvent, through the hydrophobic chains. In fact, the surfactant capped water droplets that are dispersed in bulk oil serve as a nano-reactor for the enzymatic reaction. The reverse micelle definitions do not include some important properties, concerning the behavior of this system. One is the fact that reverse micelles are dynamical entities, which can exchange their constituents including water, surfactant, or other contents. The forces that keep these structures together include hydrophobic, van der Waals, electrostatic and hydrogen bond interaction, whence the fluidity of the micelles is permanent since no covalent chemical bonds are formed. Upon collision of two micelles their coalescence occurs through a transient dimmer, that permits a rapid exchange of material [8]. 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 micelles used in this work.

3.2. The estimation of the size of water droplets

Light scattering techniques are very easy to perform and are very useful in studies about the structure of reverse micelles. They can be used to determine average sizes of water droplets provided that essentially all of the droplets are either much smaller than the wavelength of the incident light. The turbidity of reverse micelles is a measure of the reduction in intensity of the transmitted beam due to scattering and is defined by:

$$\tau = \frac{1}{l}\ln\frac{I_0}{I} \tag{1}$$

where l is the length of the light path in the dispersion and I_0 and I are the intensities of the incident and transmitted beams, respectively. Therefore, the pseudo absorbance signal, A, read in absorption spectrophotometer is related to turbidity by:

$$\tau = A\ln 10 \tag{2}$$

τ can be regarded as an extinction coefficient that attenuates the beam passing through the sample cell. The formalism is similar to the one used to derive Lambert–Beer's law. Only the physical phenomena are different: in one case light is absorbed while in the other it is scattered.

According to the Rayleigh light scattering theory, the turbidity of a dilute suspension of size-homogeneous spherical particles is inversely proportional to the 4th power of wavelength and is given by:

$$\tau = 24\pi^3\left(\frac{n^2-n_0^2}{n^2+2n_0^2}\right)\frac{n_0^4 NV^2}{\lambda^4} \tag{3}$$

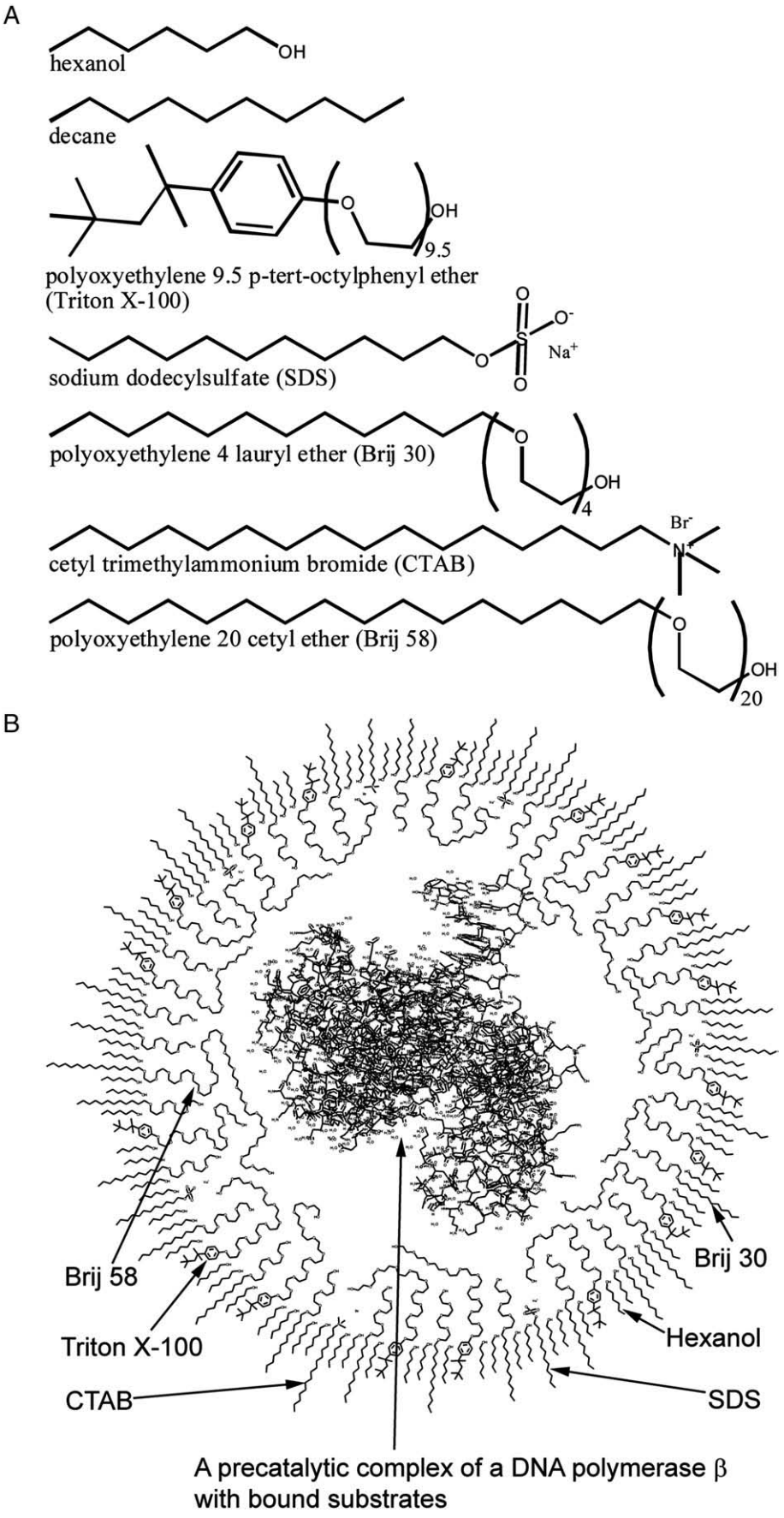

Fig. 1. Chemical structure of the organic solvents and surfactants mentioned in this article (A), and a hypothetical model of reverse micelle with incorporated DNA polymerase β (B).

where N is the number concentration of scattering particles, V is the volume of the particle, n is the refractive index of the particle and n_0 is the refractive index of the solvent. The value of the volume fraction of the dispersed phase $\varphi = NV$, and the volume of droplet is related to its radius r, as $V = 4/3\pi r^3$. Therefore, the above equation is written as

$$\tau = 32\pi^4 \left(\frac{n^2 - n_0^2}{n^2 + 2n_0^2} \right) \frac{n_0^4 \varphi r^3}{\lambda^4} \quad (4)$$

Fig. 2A shows the turbidity spectrum of the reverse micelles. It is seen that the turbidity of the micelles increases non-linearly with the wavelength in the region where the dispersion does not absorb light. The refractive index of solvent (86% decane, 14% hexanol) is $n_0 = 1.4090 \times 0.86 + 1.4178 \times 0.14 = 1.4102$ (refer to a wavelength of 589 nm, 20 °C [19]), when the presence of surfactants in oil phase and the hexanol incorporated into the micelles are optically negligible. The reverse micelle has a complex structure: the first shell of the hydrophobic tails of surfactants and hexanol molecules as cosurfactants, the second shell of polar heads, and the water core. The first shell has the refractive index near the same as the refractive index of solvent. The refractive index of the second shell is about 1.4577 (tetraethylene glycol) in dry micelles. However, in the hydrated reverse micelles the refractive index of this shell also becomes equal to the refractive index of solvent due to hydration of the oxyethylene chains. The refractive index of water is 1.3333. Evidently, the turbidity is caused by the light scattering on the water droplets. As shown in Fig. 2B the turbidity is a linear function of $1/\lambda^4$, the fitting of experimental points by a linear function in the range from 400 to 700 nm gave the correlation coefficient $R = 1$ (standard deviation SD = 10^{-15}), according to the Rayleigh light scattering theory. This result indicates the relatively low polydispersity of water droplet sizes as well. From the turbidity data at 589 nm and using the Eq. (4), we have calculated the size of water droplets as well as their concentration in the disperse system (Table 1). As shown in Table 1 and Fig. 2C, the radius of spherical reverse micelles (r) depends linearly on water volume fraction (φ):

$$r(nm) = (27.3 \pm 0.5) \times \varphi + (0.98 \pm 0.16) \quad (5)$$

For many reverse micellar systems the radius of water core is a linear function of water to surfactant molar ratio w_0 [8]. Indeed, for a spherical droplet containing a core of water phase (volume per molecule is equal to V_{water}) and containing N_s surfactant molecules having an interfacial area A_s (assuming that all of them are interfacially bound), droplet surface area and droplet core volume is given by

$$\text{surface} = 4\pi r^2 = N_s A_s \quad (6)$$

and

$$\text{volume} = \frac{4}{3}\pi r^3 = V_{water} N_s w_0 \quad (7)$$

Combining Eqs. (6) and (7) yields

$$r = \frac{3 V_{water}}{A_s} w_0 \quad (8)$$

However, this equation is valid if the surface area per surfactant is constant. In case of long polyoxyethylene chains, the surface area depends on a degree of hydration and is not constant upon varying in water content. It may be emphasized that the model of reverse micellar system used is very simple and ignores any possibility of polydispersity, shape fluctuation and interdroplets interaction in the systems, as well as multiple scattering. Though the above model is very simplified, the turbidity measurement of our concentrated system gives reasonable results for the micelle size. It may be due to self-compensatory of different factors, including the interference of scattered waves in systems with optical inhomogeneities. However, our results should be verified by a different technique.

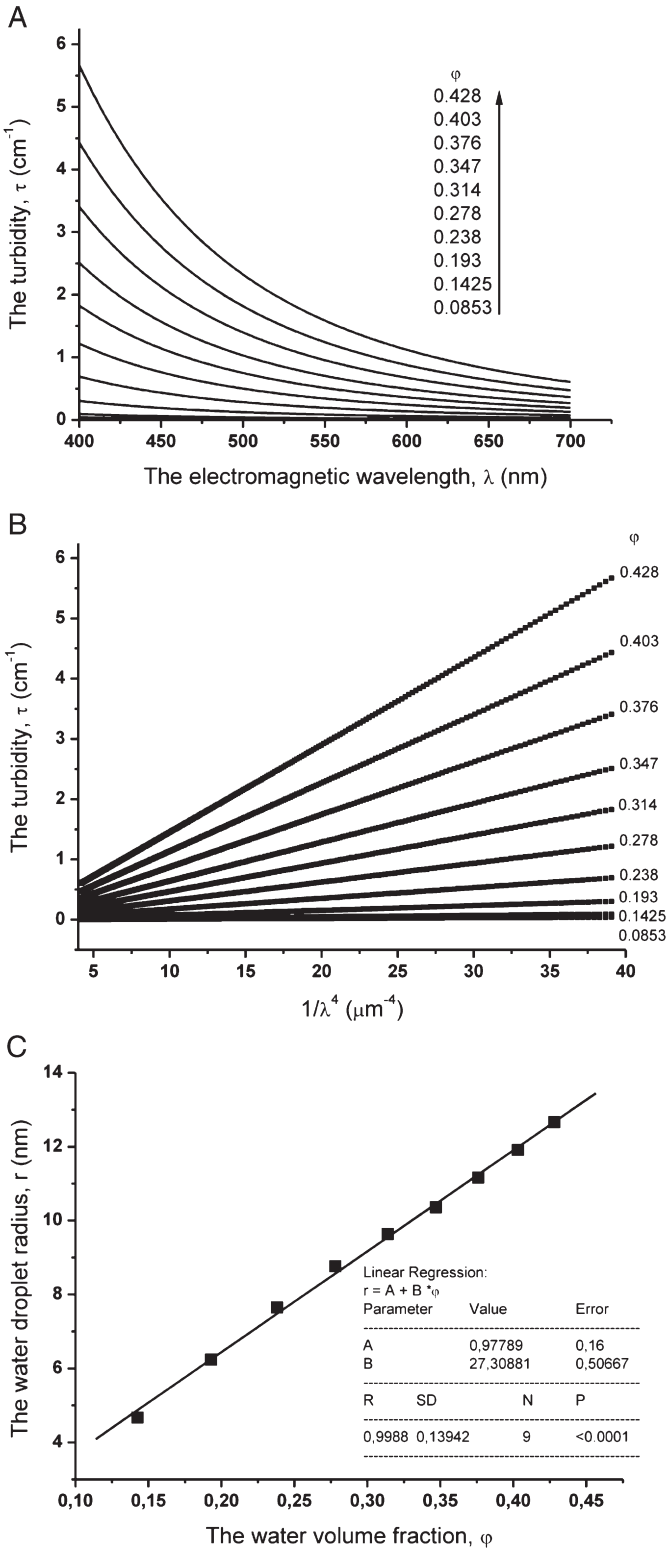

Fig. 2. The turbidity spectrum of reverse micelles (A), the dependence of turbidity spectrum on inverse forth power of electromagnetic wavelength (B), and the dependence of water droplet radius on water volume fraction (C).

3.3. The exploration of microenvironment of reverse micelles

The physical parameters of the water in the reverse micelles are strongly depending on the size of water droplet and on the nature of the head group of surfactant. Experimental studies of water pool confined in reverse micelles revealed a substantial decrease of polarity

Table 1
The experimental and calculated data from the light scattering measurements

Water volume fraction, φ	Turbidity, τ_{589} (cm^{-1})	Water droplet radius, r (nm)	Water droplet concentration, C (mM)
0.1425	0.020±0.001	4.68±0.14	0.551±0.028
0.193	0.065±0.002	6.25±0.19	0.314±0.016
0.238	0.148±0.004	7.67±0.23	0.209±0.010
0.278	0.259±0.008	8.79±0.26	0.163±0.008
0.314	0.389±0.012	9.66±0.29	0.138±0.007
0.347	0.534±0.016	10.38±0.31	0.123±0.006
0.376	0.725±0.022	11.19±0.34	0.106±0.005
0.403	0.943±0.028	11.94±0.36	0.094±0.005
0.428	1.206±0.036	12.70±0.38	0.083±0.004

and a dramatic slowing down of the rotational relaxation of water molecules [20–25]. We have investigated the microenvironment in our reverse micelles using fluorescein as molecular probe. Fluorescein is one of the most commonly used fluorescent probes in the biosciences. The shift in the maximum absorption wavelength (λ_{max}) of a solvatochromic probes can sensitively reflect the local environment about the probe [26]. When we place this probe in reverse micelles, the absorption maximum is red-shifted with decreasing water content (Fig. 3B). We see that the absorption maximum of fluorescein is shifted from 492 to 502 with a decrease in water content from 35 to 6% (v/v). The spectra of fluorescein in reverse micelles shift continuously toward the bulk water spectra with the increasing water content but never overlap the spectrum in bulk water at neutral pH. A red shift of the absorption maximum suggests that the polarity of the micellar environments is less than the polarity of the bulk water.

The chromophore can exist in two different prototropic forms (Fig. 3A) with distinct spectral properties. The dianion form is usually observed under neutral conditions and possesses a large extinction. The pKa of the monoanion–dianion transition is substantially increased when the chromophore is present in media of lower polarity (Fig. 3C, 86% ethanol). However, the prototropic equilibrium is sensitive to the hydrogen-bonding environment and this complicates the interpretation of the pKa shifts in the micellar environment. Therefore, the pKa shifts may be used as indicators of a change in environment, but they cannot be unambiguously related to the polarity of the microenvironment. This can account for the inability to correlate the pKa shifts of the fluorescein transitions in reverse micelles with those in a single solvent–water mixture of defined polarity.

When Tris-base/acetic acid is used for pH adjustment in aqueous buffer solution, even if an acidic or alkaline aqueous solution is solubilized into the micelles, the solubilized probe reports unexpectedly an almost linear absorbance over a wide pH range (Fig. 3C). This result suggests that the water pool of reverse micelles has buffer-like action. Such buffer-like action is likely related to a considerably high concentration of the polar groups localizing on the water/oil interface.

Results reported here show that the interactions of water with the polyoxyethylene ether, as well as the micellar confinement inside the reverse micelles, effectively modify the water properties in the micellar interiors, which can reflect on the enzymatic reactivity.

3.4. Excluded volume (confinement) effect

Steric repulsion is the most fundamental of all interactions between macromolecules or between macromolecule and surface in solution and is always present at finite concentration, independent of the magnitude of additional electrostatic or hydrophobic interactions. The volume that cannot be occupied by the center of mass of a particular solute species at a particular instant is called the excluded volume, and the volume that may be occupied is called the available volume (Fig. 4A). In a highly volume-occupied solution, the solute reactivity is determined by the number of molecules of that solute per unit of available volume (the effective concentration or the thermodynamic activity) [4].

When macromolecule interacts with surface exclusively via steric repulsion there is an extremely simple relationship between the effective (a) and actual concentration (C) of solute [4]

$$\gamma = \frac{a}{C} = \frac{V_{\text{total}}}{V_{\text{available}}} \tag{9}$$

The thermodynamic activity of the protein represented by the macromolecule concentration in the available volume is reasonably accurate only at low protein concentrations. It is assumed that no interaction potentials exist between macromolecules or between a

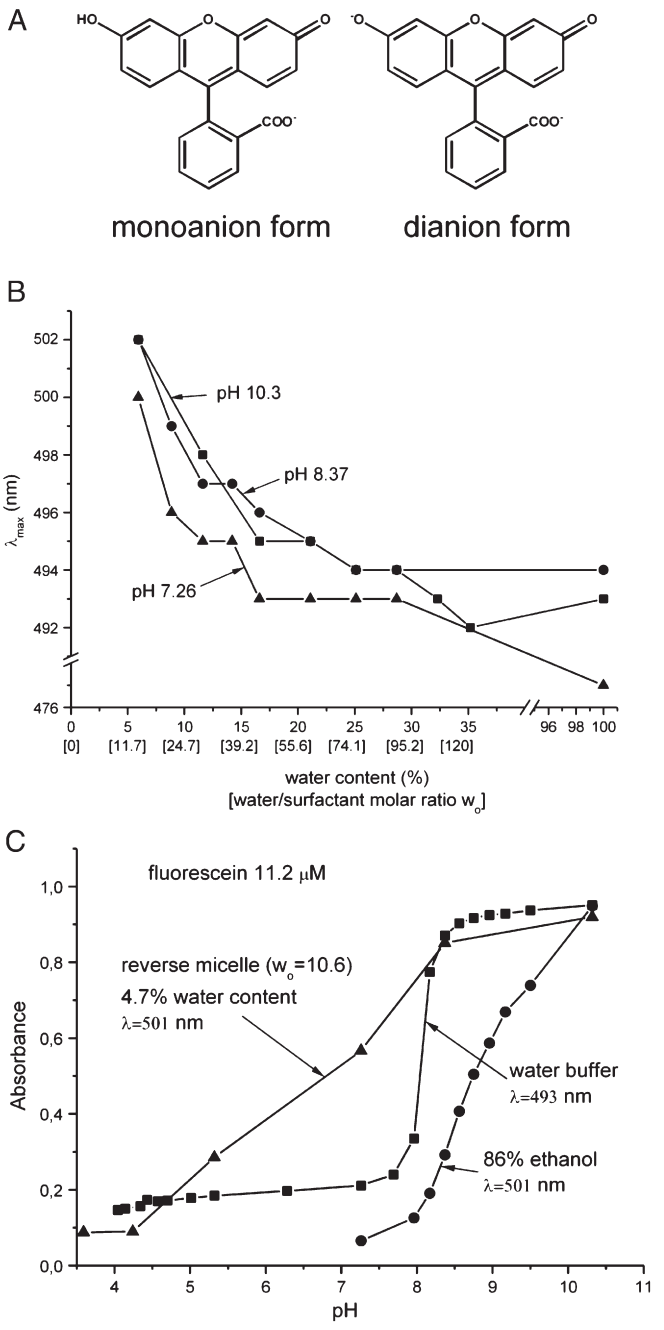

Fig. 3. Chemical structure of fluorescein monoanion and dianion forms (A), the dependence of the maximum absorption wavelength of fluorescein on water content (B), and the absorbance of fluorescein in reverse micelles, in water, and in ethanol at various pH of added buffer (C).

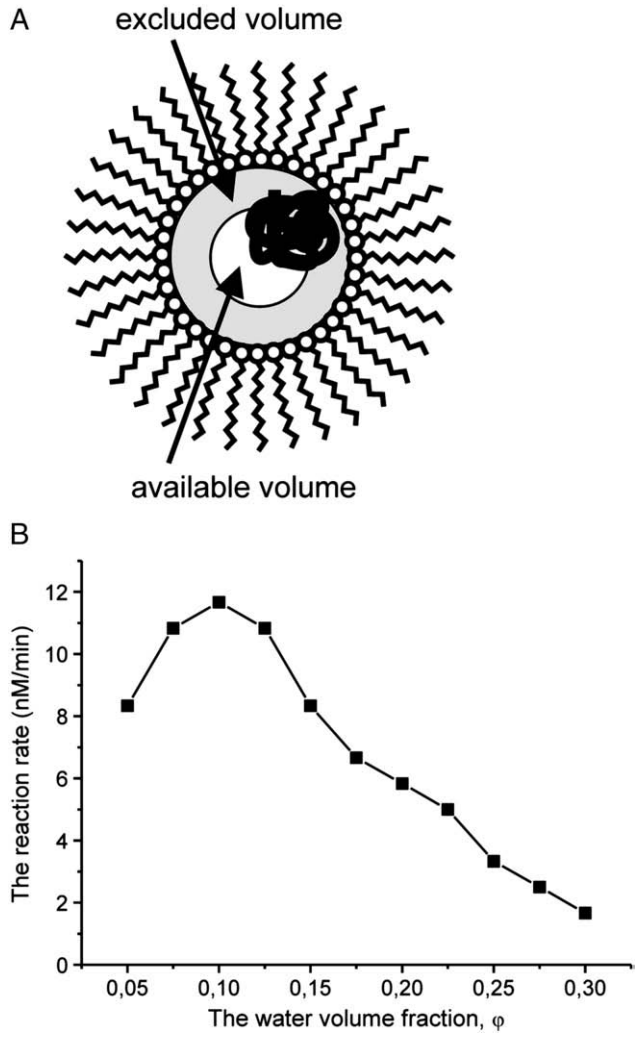

Fig. 4. The excluded and the available volumes in reverse micelles (A), and the dependence of DNA polymerase β activity on water content in the reverse micelles. The reverse micelles contain 20 mM Tris, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 10 μM dNTP, 3 nM M13mp19 ssDNA, 3 nM 5′-[^{32}P] primer, and 30 nM DNA polymerase β. The mixtures were incubated at 22 °C for 3–30 min. The reaction rate is expressed in mononucleotides incorporated into primer by DNA polymerase.

macromolecule and a surface, the effects are entropic in origin, i.e., deriving from the relative reduction in configurational entropy of solute due to confinement.

Available volume is given by

$$V_{\text{available}} = \frac{4}{3}\pi(r_w - r_p)^3 N_w \quad (10)$$

where r_w is the radius of water droplets, r_p is the radius of protein (macromolecule), N_w is the number of water droplets. The volume of water phase is defined as

$$V_w = \frac{4}{3}\pi r_w^3 N_w \quad (11)$$

Combining Eqs. (9)–(11) and using $V_w/V_{\text{total}} = \varphi$ yields

$$\gamma = \frac{r_w^3}{(r_w - r_p)^3 \varphi} \quad (12)$$

From this equation it is clear that when the size of water droplets approaches to the size of macromolecule, the activity (effective concentration) of this molecule significantly increases. Confinement by the reverse micellar structures can in principle affect the equilibrium and kinetics of any macromolecular reaction, such as protein folding/unfolding, biopolymer self- or hetero-association and isomerization, in which there exists a significant difference between the volume excluded to reactants and the volume excluded to products. Confinement may also affect enzyme catalyzed reactions involved significant conformational change of the enzyme.

3.5. The dependence of DNA polymerase activity on water content

As shown above, the size of reverse micelle can influence on water properties and on confinement of macromolecules. Decrease of water polarity can lead to weakening the hydrophobic interactions, and, hence, to partial protein expansion with the increase of its conformational mobility. However, confinement of proteins to water nanodroplets leads to macromolecular compaction due to excluded volume. 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 depend on water content (water droplet size).

Indeed, Fig. 4B shows strong dependence of DNA polymerase activity on water content in reverse micelles. The maximal activity was observed at 10% (v/v) water content (φ=0.1) that according to Eq. (5) correspond to the radius of water droplets r=3.7±0.2 nm. The experimental small-angle X-ray scattering (SAXS) data indicates that free DNA polymerase β has an elongated shape (the radius of gyration R_g=2.9 nm, the maximum particle dimension D_{max}=10.5 nm), whereas the binary complex (protein-DNA) is in a more globular conformation (R_g=2.4 nm, D_{max}=7.0 nm). Subsequent binding of matched dNTP to the binary complex further decreases R_g and D_{max} values, indicating formation of a more compact conformation in the matched ternary complex relative to the binary complex. Similar trend is also observed in contrast variation by small-angle neutron scattering (SANS), where R_g values for the free form, binary complex and ternary complex of DNA polymerase in 70% D_2O buffer, in which only protein signal is visible, are 2.90±0.02, 2.47±0.07 and 2.35±0.04 nm, respectively [30]. Thus, the optimum activity takes place around a value of water volume fraction at which the size of water droplet is similar to the size of the enzyme. At lower water content the decrease enzyme activity can be because of high confinement and slow solvation dynamics of water in small-sized reverse micelles, increasing protein compaction and slow downing its conformational mobility and activity.

3.6. The dependence of DNA polymerase activity on the protein concentration

Processive DNA synthesis by a DNA polymerase can be described by the repetitive steps of nucleotide binding, an induced conformational transition from the open to closed state, phosphodiester bond formation, a transition to an open conformation accompanied by the release of pyrophosphate, and, finally, movement along the DNA to the next position on the template. 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 [31,32].

It has been shown that DNA synthesis by DNA polymerase β is distributive on DNA substrates, in which primer is annealed to a single-stranded template, while the enzyme conducts processive synthesis on short gapped DNA templates with a 5′-phosphate at the end of the gap. With gaps of 2–6 nucleotides the polymerase exhibits increased catalytic efficiency compared to primed single-stranded DNA [33–35].

As shown in Fig. 5 the dependence of DNA polymerase efficiency on the protein concentration is unusual. When the protein concentration is greater than the concentration of primer, we observed significant increasing in the enzyme processivity and efficiency (especially in water solution). The polymerase, known as the distributive enzyme (dissociates from template-primer after each cycle of dNMP incorporation), becomes highly processive (like replicative polymerase). At molar ratio of enzyme to primer greater than 25, we see two-order increase in the enzyme efficiency (Fig. 5, lane 18). In reverse micelles, this effect is observed at threefold excess of enzyme (Fig. 5, lane 11), and the polymerase efficiency increases at higher protein concentration (Fig. 5, lanes 5–7, 11–13). Because only one protein can participates in catalysis, it seems that DNA polymerase stimulates itself. The presence of more than one protein molecules in water droplet can reinforce the excluded volume effect and, therefore, increase stability of the enzyme–template complex. However, the increased polymerase processivity is also observed in dilute water solution, where the excluded volume effect is negligible. It is clear that the increased efficiency and processivity is associated with the enzyme dimerization or oligomerization. It has been reported that a 2:1 polymerase–DNA complex is formed with two different binding modes, where one polymerase molecule has larger DNA-binding interface and the other has smaller contact [36]. These studies suggested that two DNA polymerase molecules bind to the DNA substrate at different positions with the same conformation, and the two polymerase molecules may or may not interact with each other. The other structural model of the 2:1 complex generated by SAXS and sedimentation velocity indicates formation of the asymmetric 2:1 polymerase–DNA complex that enhances the function of DNA polymerase β [37].

Fig. 6A shows that the dependencies of the enzymatic activity on the protein concentration are different at various water contents.

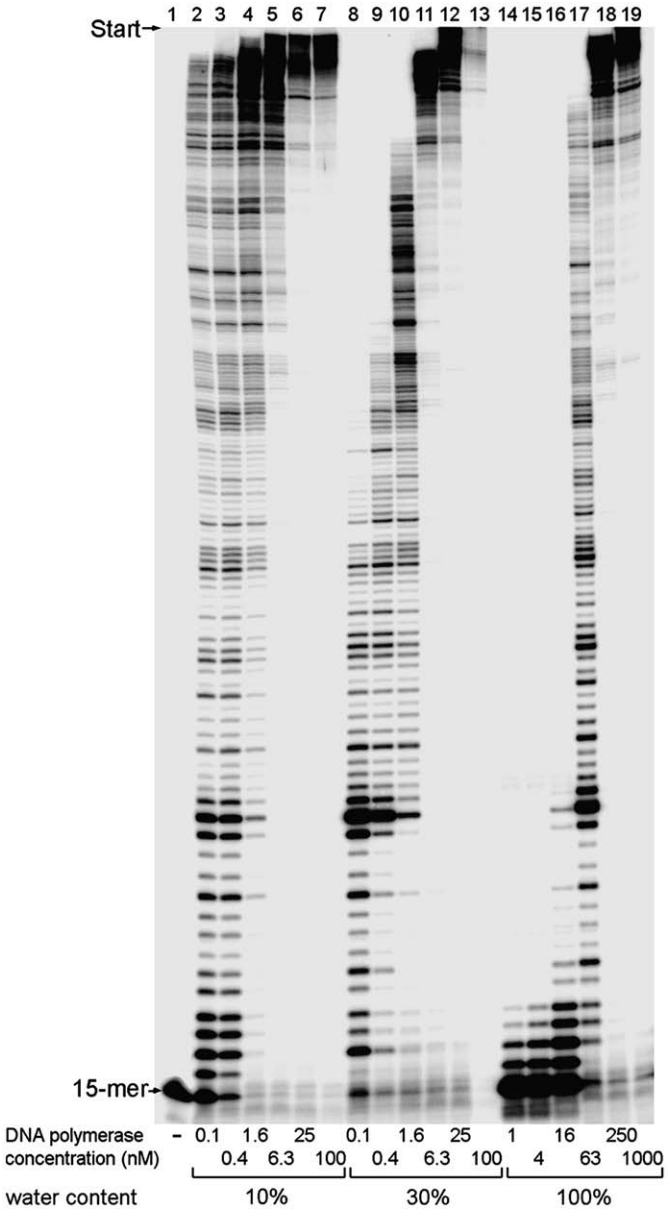

Fig. 5. Effect of increasing protein concentration on distribution of the product synthesized by DNA polymerase β. Lanes 2–13 represent DNA polymerase assay in the reverse micelles (100 μl) that contained 20 mM Tris, 1 mM $MgCl_2$, 1 mM dithiothreitol, 15 μM dNTP, 2 nM M13mp19 ssDNA, 2 nM 5′-[^{32}P] primer, and 0.1–100 nM DNA polymerase β. Lanes 14–19 represent DNA polymerase assay in the aqueous buffer solution (10 μl) that contained 50 mM Tris–HCl, pH 8.6, 10 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol, 150 μM dNTP, 20 nM M13mp19 ssDNA, 20 nM 5′-[^{32}P] primer, and 1–1000 nM DNA polymerase β. The mixtures were incubated at 22 °C for 30 min.

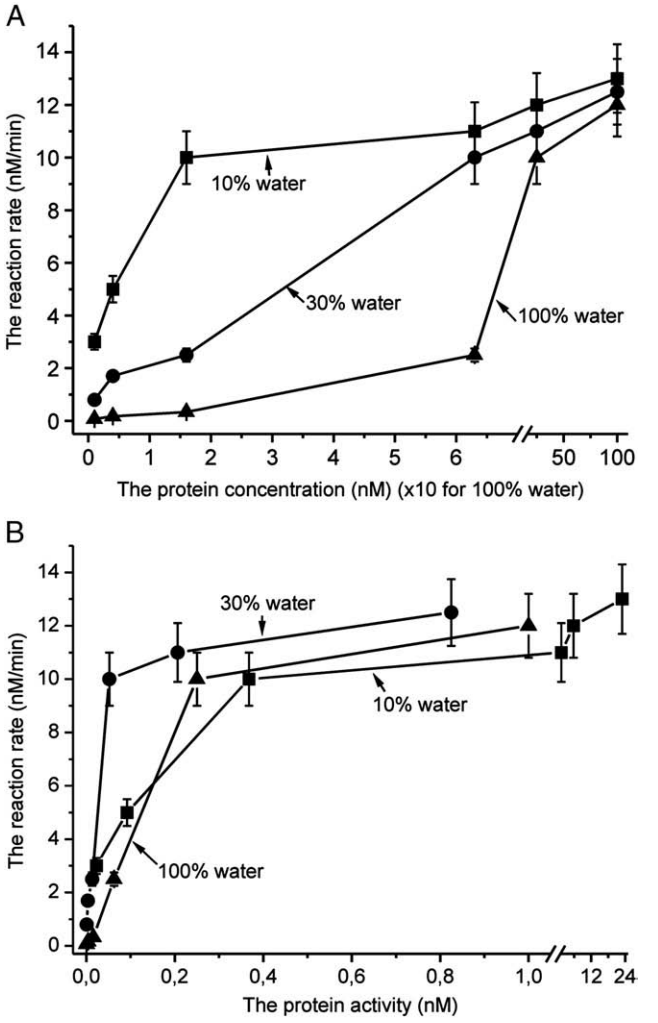

Fig. 6. The dependence of DNA polymerase reaction rate on the protein concentration (A) and on the protein thermodynamic activity (B). The reverse micelles (100 μl) contain 20 mM Tris, 1 mM $MgCl_2$, 1 mM dithiothreitol, 15 μM dNTP, 2 nM M13mp19 ssDNA, 2 nM 5′-[^{32}P] primer, and 0.1–100 nM DNA polymerase β. The aqueous buffer solution (10 μl) contain 50 mM Tris–HCl, pH 8.6, 10 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol, 150 μM dNTP, 20 nM M13mp19 ssDNA, 20 nM 5′-[^{32}P] primer, and 1–1000 nM DNA polymerase β. The mixtures were incubated at 22 °C for 3–30 min.

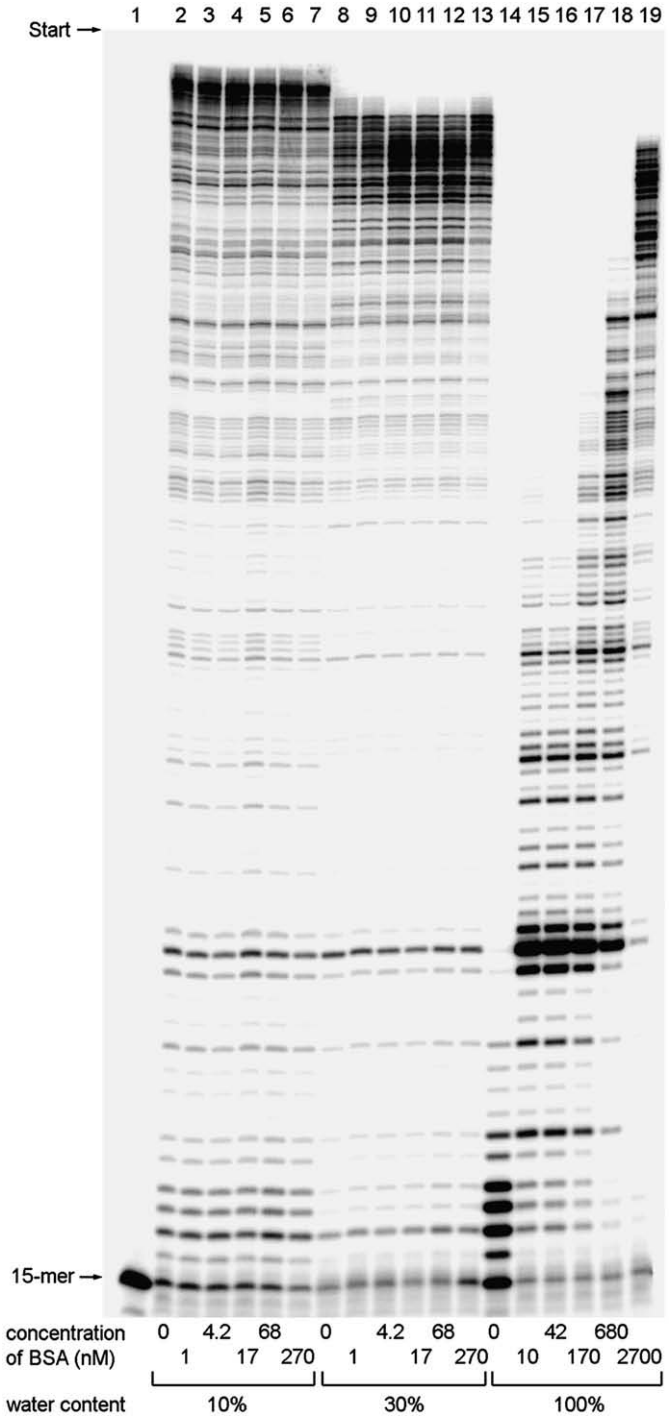

Fig. 7. Effect of BSA concentration on distribution of the product synthesized by DNA polymerase β. Lanes 2–13 represent DNA polymerase assay in the reverse micelles (100 μl) that contained 20 mM Tris, 1 mM $MgCl_2$, 1 mM dithiothreitol, 15 μM dNTP, 2 nM M13mp19 ssDNA, 2 nM 5′-[^{32}P] primer, 2 nM DNA polymerase β, and 0–270 nM BSA. Lanes 14–19 represent DNA polymerase assay in the aqueous buffer solution (10 μl) that contained 50 mM Tris–HCl, pH 8.6, 10 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol, 150 μM dNTP, 20 nM M13mp19 ssDNA, 20 nM 5′-[^{32}P] primer, 20 nM DNA polymerase β, and 0–2700 nM BSA. The mixtures were incubated at 22 °C for 30 min.

However, when the curves are replotted according to effective protein concentration (the thermodynamic activity), the functional dependencies become similar (Fig. 6B). The thermodynamic activities were calculated using Eqs. (5) and (12). If the protein radius r_p=2.4 nm, the activity coefficient γ_{30}=8.3 at 30% water and γ_{10}=230 at 10% water (γ_{100}=1 for 100% water).

3.7. BSA has no influence on DNA polymerase β activity in reverse micelles

There are two popular explanation of increased enzyme activity with increasing protein concentration: this occurs due to the increased number of active sites or to protection against dilutional inactivation (the loss of enzyme activity after dilution). To check the second explanation we investigate the influence of bovine serum albumin (BSA), stabilizing agent in enzymatic reactions and storage buffers, on the polymerase activity when the concentration of enzyme is low. As shown in Fig. 7, the BSA has no influence on DNA polymerase efficiency in reverse micelles (lanes 2–13) but stimulate the enzyme in dilute aqueous buffer solution (lanes 14–19). The stimulation of the polymerase by BSA is much smaller than the activation of enzyme by itself (compare with Fig. 5, lanes 14–19). From this it follows that BSA can non-specifically interact with DNA polymerase β and, in that way, stimulate the enzyme efficiency. At high concentration of BSA the polymerase activity is approached to whose in the reverse micelles. Thus, DNA polymerase efficiency increases in the reverse micelles, as well as in the presence of BSA.

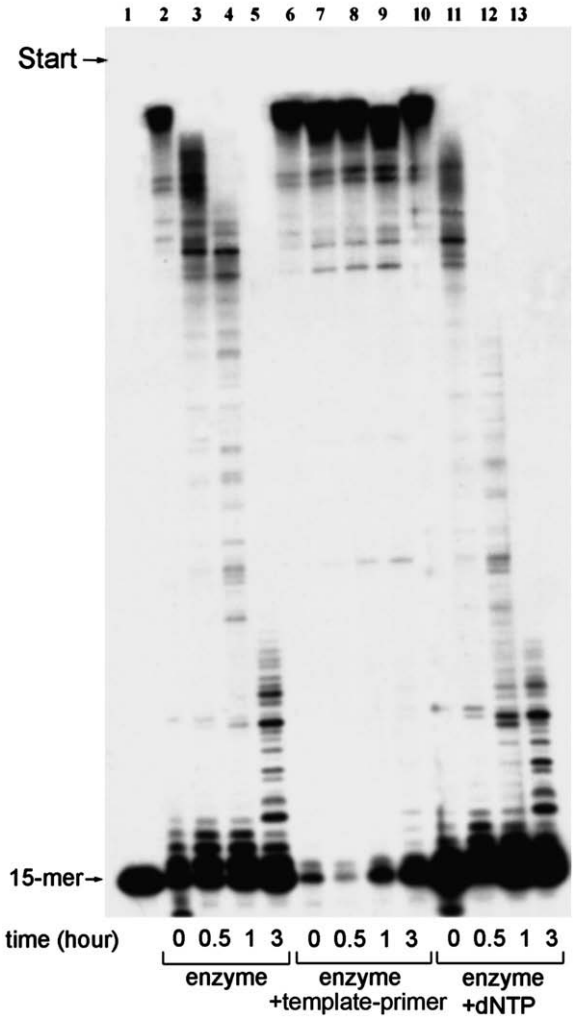

Fig. 8. The stability of DNA polymerase β in presence of template-primer and dNTP. After preincubation of 30 nM DNA polymerase alone (lanes 2–5), or with template-primer (lanes 6–9), or with dNTP (lanes 10–13) for 0–3 h in reverse micelles (10% water, 20 mM Tris, 2.5 mM $MgCl_2$, 1 mM dithiothreitol) the other necessary components (10 μM dNTP, 3 nM M13mp19 ssDNA, and 3 nM 5′-[^{32}P] primer) were added to start the reaction. Then the mixtures were incubated at 30 °C for 30 min.

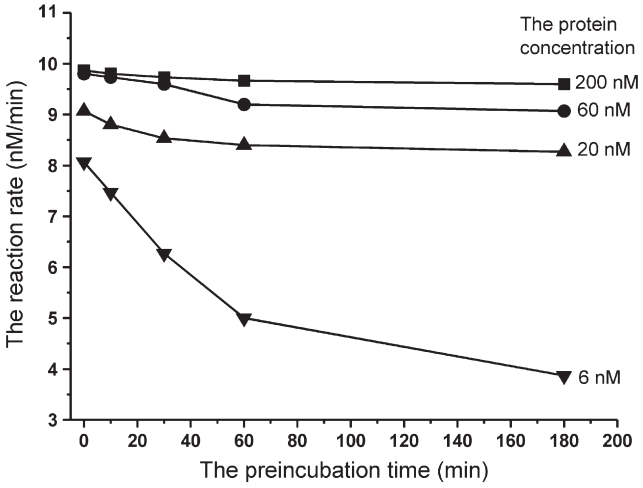

Fig. 9. The dependence of DNA polymerase β stability on the protein concentration in reverse micelles. After preincubation of 6–200 nM DNA polymerase alone for 0–180 min in reverse micelles (10% water, 20 mM Tris, 2.5 mM $MgCl_2$, 1 mM dithiothreitol) the other necessary components (10 μM dNTP, 2 nM M13mp19 ssDNA, and 2 nM 5′-[^{32}P] primer) were added to start the reaction. Then the mixtures were incubated at 22 °C for 3–30 min.

3.8. DNA polymerase β is stable in presence of template-primer

It is supposed that enzyme stability is determined by primary structure and interaction with the microenvironment. In aqueous media, enzyme is inactivated in the course of time, where water participates in inactivation reactions and conformational changes associated with protein unfolding [38]. An approach for enzyme stabilization is the reaction medium engineering [39], for example, by partial or almost total substitution of water [40]. In numerous cases reported, the remarkable enzyme stability has been obtained in such media [41].

The current general paradigm relating dynamics to activity suggest that enzymes need to be sufficiently flexible to be catalytically active, but not so flexible that they ready denature. How can we obtain a more stable and active enzyme?

From our data described above follows that we can use the reverse micelles and (or) high protein concentration to increase enzyme activity, but what is about DNA polymerase stability? Fig. 8 shows that DNA polymerase β is not stable when it is alone (lanes 2–5) or in presence of dNTP (lanes 10–13). On the other hand, we can see significant stabilization of the enzyme in presence of template-primer (lanes 6–9). The same stabilization is observed upon increase in the protein concentration (Fig. 9). The enzyme stabilization by DNA or by other proteins can be explained by decreasing flexibility of DNA polymerase upon template-primer binding or other protein molecule binding. However, in such conditions the enzyme is very active. Thus, there may be contradictions in the observed data and the existing theoretical conceptions.

4. Discussion

In the living cells, enzymes carry out their function in microheterogeneous environments interacting with membranes or other macromolecules. Water plays structural and functional roles, besides being the principal component. In vitro systems of self-assembly of surfactants in organic solvents are used to mimic “in vivo” conditions. The enzyme catalysis in reverse micelles is gaining increasing importance, both for academical studies and industrial applications [42–45]. From an enzymologist point of view, the reverse micelles have an array of unique properties that are important for enzymatic catalysis.

Firstly, the confinement (excluded volume effect) can significantly influence on the structure of macromolecules, increasing protein compaction and slowing down its conformational flexibility. There is a growing body of evidence that the molecular motions within the protein could have influence on the enzyme catalysis [46]. The protein dynamics can directly affect the reaction coordinate potential, and motions within the protein itself actually speed the rate of a chemical reaction [47]. Modeling studies revealed highly cooperative dynamics and critical roles of key residues in the DNA polymerase β function [48]. The other aspect of confinement is the effective concentration (thermodynamic activity) of macromolecules that is increased with decrease in water droplet size. As has been shown in this work, the value of activity coefficient can be two orders of magnitude. This is favoring specific macromolecular associations at low protein concentration, leading to the formation of oligomeric species of DNA polymerase, which have high catalytic efficiency. It must be noted that the effects of volume exclusion in reverse micelles will always be present and play an important role in influencing macromolecular structure and function, independent of and in addition to the influences of other types of interactions. Unfortunately, this effect has not discussed in literature on micellar enzymology.

Secondly, the structure and dynamics of water can be controlled in reverse micelles. Water has unique properties that are intimately related to its molecular level structure. Water's properties are strongly influenced by its dynamic hydrogen bond network. The hydrogen bond network and, therefore, the properties of water are sensitive to structural and chemical perturbations. Recent theories and molecular dynamic simulations [20–25,49,50] indicate that at least two types of water molecules are present in complex systems, free and bound. Free suggests water molecules with essentially bulk characteristics separated from the interface in the core of the system. Bound water molecules are associated with particular groups in proteins, the interface in reverse micelles, etc. and can reside in pockets separated from the bulk water phase. The long time components observed in dielectric relaxation and solvation dynamics experiments have been attributed to exchange between the associated and core populations. However, as shown by THz spectroscopic studies, the ‘free’ water is not structurally equivalent to the bulk water [24,51]. Our experiments with fluorescein as molecular probe also indicate that the properties of water in complex reverse micelles are different from bulk water. The shift in absorption maximum and in monoanion–dianion transition of fluorescein indicates that the polarity and the pH of the initial buffer solution are changed upon micelle formation. This effect is strongly dependent on the size of water droplets.

The dependence of DNA polymerase β activity on water content indicates that the confinement and changes in water properties have significant influence on the enzyme activity. The protein has enhanced activity and processivity in the reverse micelles when the size of water droplets is comparable with the size of the enzyme. The polymerase efficiency is significantly increased at high protein concentration. The enzyme reveals also the enhanced stability at high protein concentration and in presence of template-primer. This indicates that the protein clusters is more convenient environment for DNA polymerase, and this is not surprising because the enzyme evolved to work in highly crowded and dynamically ordered environment. In living cells, DNA polymerase clusters have been found within replication factories [52].

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