Conformational transitions provoked by organic solvents in β-lactoglobulin: can a molten globule like intermediate be induced by the decrease in dielectric constant?

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Background: It is known that nonnative states of protein molecules can exist in living cells and can be involved in a number of physiological processes. It has also been established that the membrane surface can be responsible for the partial denaturation of proteins due to negative charges on it. The local decrease in the effective dielectric constant of water near the organic surface has been suggested to be an additional driving force for protein denaturation in the membrane field, but data to confirm this suggestion were lacking.

Results: Conformational transitions induced in β-lactoglobulin by methanol, ethanol, isopropanol, dimethylformamide and dioxane were studied by near and far UV circular dichroism, steady-state tryptophan fluorescence and fluorescence decay of 8-anilinonaphthalene-1-sulfonate (8-ANS). The existence of at least two noncoinciding cooperative transitions has been established in all solvent systems studied. The first of these transitions describes the disruption of rigid tertiary structure in protein molecules, while the second reflects the formation of an expanded helical conformation typical of proteins in concentrated organic solvents. This means that the organic solvents provoke the formation of a denatured intermediate state with pronounced secondary structure and native-like compactness. We show that the positions of maxima in f_I versus dielectric constant dependence virtually coincide for all five solvent systems studied.

Conclusions: The decrease in the dielectric constant of the solvent induces in β-lactoglobulin an equilibrium intermediate state. This state, being denatured, is relatively compact and has pronounced secondary structure and high affinity for the hydrophobic fluorescent probe 8-ANS, i.e. possesses all the properties of the molten globule intermediate state.

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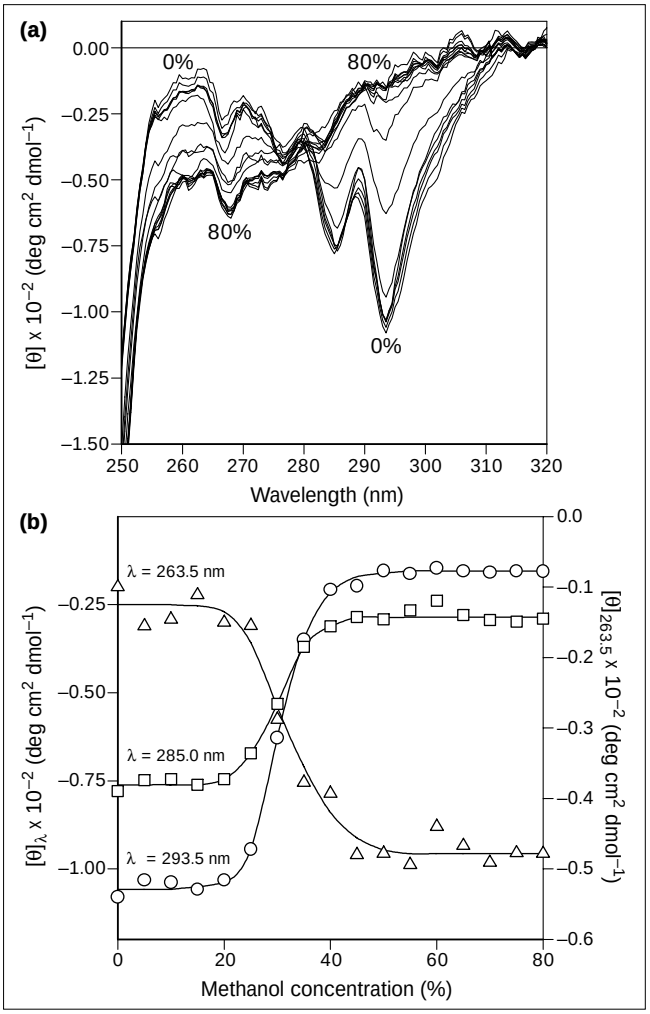
Introduction

The molten globule is a thermodynamically stable intermediate protein state stabilized by mild denaturing conditions such as acid or alkaline pH values and/or moderate concentrations of strong denaturants. The general properties of this intermediate are the presence of pronounced secondary structure and high compactness without rigid packing inside a molecule, and a substantial increase in fluctuations of sidechains as well as of larger parts of a molecule (for reviews, see [1–8] and references therein). The molten globule state accumulates during the *in vitro* renaturation of globular proteins from the completely unfolded state [1–4] and may, therefore, play a universal role in protein folding [9,10]. It has been shown that the molten globule, being separated from the native and completely unfolded states by all-or-none transitions, represents a third thermodynamic state of protein molecules [11–14].

It was suggested that the molten globule state (and other nonnative states of protein molecule) can exist in living cells and can be involved in a number of physiological processes [15]. Although this suggestion was confirmed by a number of observations (see [3,16] for reviews), the question arises: what are the driving forces for protein denaturation in living cells? It is obvious that in some cases the nonnative conformations of protein molecules may appear as the result of trapping of folding intermediates by chaperones or as the result of mutations preventing proteins from complete folding. At the same time, some protein molecules, being originally native, become denatured near the membrane surface (e.g. toxins on penetration into membranes or transport proteins during the release of ligands to the target cell). This means that the membrane is one of the possible denaturing agents of living cells. Indeed, it is known that the conditions at the membrane surface can cause partial denaturation of

Figure 1



(a) Near UV CD spectral changes of β-lactoglobulin at pH 2.0 and 23°C in the methanol concentration range 0 to 80% (in steps of 5%). The protein concentration was 0.82 mg ml^{-1} throughout the experiments. (b) Methanol concentration dependences of ellipticity at 263.5 nm (triangles), 285.0 nm (squares) and 293.5 nm (circles) at pH 2.0 and 23°C.

proteins due to negative charges on it [17]. The reason is that the negative electrostatic potential of the membrane surface can cause attraction of protons from the bulk solution leading to a local decrease in pH [18,19]. However, even in salt-free solutions this local decrease in pH is less than 2 pH units [19]. This value is usually insufficient for acid denaturation of protein molecules. On the other hand, it is known that the effective dielectric constant of water near an organic surface should be twice as small as its 'bulk' dielectric constant [20]. It was proposed that such a local decrease in the dielectric constant near the membrane surface could be considered as an additional denaturation agent of the membrane surface [16,21]. Water/alcohol mixtures at moderately low pH have been suggested as a model system for the investigation of such a 'concerted' action of the local decrease of pH and dielectric constant on protein structure [21,22]. In agreement with this suggestion, the existence of a methanol-induced molten globule state of cytochrome *c* has been established recently [22,23]. But the issue remained open as to whether this observation was a result of the change of the averaged dielectric constant of the solvent or whether it was nothing more than the simple result of specific interactions of alcohol molecules with the polypeptide chain.

We have investigated the influence of organic solvents of different classes on the structural properties of bovine β-lactoglobulin. To this end, the effects of alcohols with different lengths of the aliphatic part (methanol, ethanol and isopropanol) on β-lactoglobulin near and far UV CD spectra, tryptophan fluorescence and decay of 8-anilinonaphthalene-1-sulfonate (8-ANS) fluorescence were compared with the effects of amides (dimethylformamide [DMF]) and cyclic ethers (dioxane). Analyses of the results presented in our paper lead us to conclude that the decrease in the dielectric constant of a solution can provoke the transformation of protein molecules into a denatured intermediate state that possesses all the properties of the molten globule state.

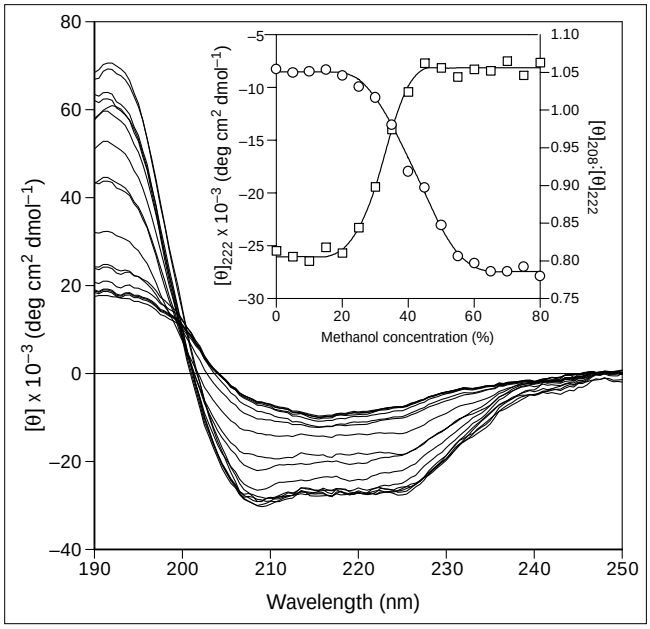
Results and discussion

Methanol-induced conformational changes in β-lactoglobulin

Denaturation (near UV CD)

Figure 1a represents near UV CD spectra of β-lactoglobulin measured at different methanol concentrations in solution at pH 2.0 and 23°C. This shows that the increase in alcohol content results in considerable changes in the near UV CD shape and intensity. In particular, the longer wavelength part of the spectrum ($\lambda \geq 280$ nm) decreases drastically in intensity, while the shorter wavelength part increases. It is seen also that on increasing methanol concentration, the peculiarities of the spectrum vanish; in the native state, the near UV CD spectrum of β-lactoglobulin shows sharp minima at 293.3 and 285 nm, while in the presence of more than 40% methanol, the near UV CD spectrum of protein is relatively monotonous and does not contain fine peaks (Fig. 1a). The same near UV CD spectra were described earlier for β-lactoglobulin in the presence of 40% trifluoroethanol [24] or 50% ethanol [25–27]. Methanol-induced changes in intensity of different bands of the near UV CD spectrum are presented in Figure 1b. One can see that the cooperative changes in intensity occur simultaneously for all three bands analyzed within 20–40% methanol. Considerable reductions in intensity and simplification of spectra reflect drastic changes in the environmental asymmetry of aromatic amino acid residues, i.e. describe the process of methanol-induced denaturation of β-lactoglobulin. Most of all, simultaneous transformation of all the bands in the near UV CD spectrum and the

Figure 2



Far UV CD spectral changes of β-lactoglobulin at pH 2.0 and 23°C in the methanol concentration range 0 to 80% (in steps of 5%). The protein concentration was 0.82 mg ml^{-1} throughout the experiments. The inset presents the methanol concentration dependence of ellipticity at 222 nm (circles) and changes in far UV CD spectral shape (squares), measured as $[\Theta]_{208} : [\Theta]_{222}$ ratio.

presence of an isobestic point (λ ~280 nm; see Fig. 1a) allow us to assume that the methanol-induced denaturation of β-lactoglobulin follows a two-state mechanism. Finally, it is necessary to emphasize that the process of methanol-induced denaturation of β-lactoglobulin was completely reversible, which is in a good agreement with the previous results of Dufour *et al.* [26].

Secondary structure (far UV CD)

Figure 2 collects far UV CD spectra of β-lactoglobulin measured at pH 2.0 and 23°C in solution with different methanol concentrations. One can see that the increase in organic component content leads to a considerable increase in far UV ellipticity (e.g. the value of $[\Theta]_{222}$ changes from ~–7000 to ~–27 000 deg cm^2 dmol^{-1} in the absence of alcohol and in the presence of 60% methanol, respectively; see inset to Fig. 2), shift of the point of intersection with the zero line (from 204 to 201 nm) and changes in the spectrum shape (cf. [26]). Indeed, in the absence of methanol, the CD spectrum of β-lactoglobulin is typical of β-structural proteins: it shows a minimum at 218 nm that reflects the high content of β-structure. The addition of alcohol transforms the far UV spectrum to one with minima at 208 and 222 nm, i.e. to the spectrum of a protein with a high content of α-helical structure. Such behaviour was observed earlier for several proteins, including β-lactoglobulin [24–28], and was interpreted in terms of alcohol-induced stabilization of helical structure. The processes of methanol-induced increases in intensity and changes in spectral shape, monitored as $[\Theta]_{208} : [\Theta]_{222}$ ratio, are presented in the inset to Figure 2. One can see that the methanol-provoked changes in the shape of the far UV CD spectrum of β-lactoglobulin occur at smaller alcohol contents — from 20 to 40% — as compared with the increase in spectral intensity — from 25 to 60% methanol.

Compactness (tryptophan fluorescence)

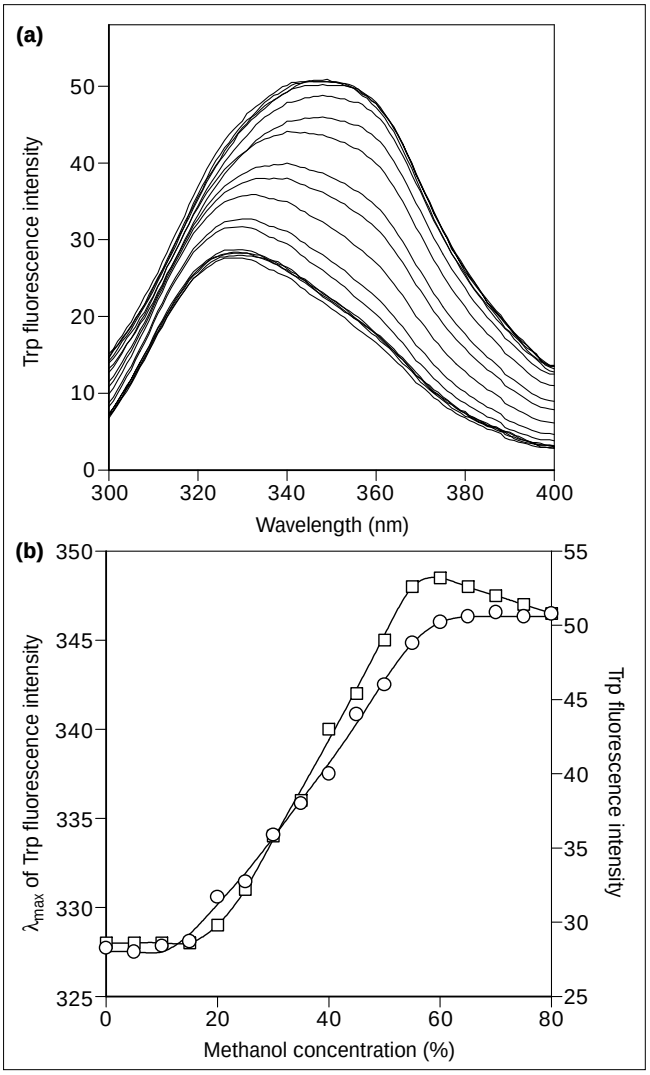
It is known that solvated tryptophan residues in aqueous solutions have a fluorescence maximum at 350 nm. Embedding of tryptophan in the nonpolar core of globular proteins results in the characteristic blue shift of its fluorescence spectrum. In other words, the position of the tryptophan fluorescence spectrum, being sensitive to the polarity of the environment, allows us to study protein unfolding [29] or other conformational transitions accompanied by the change of tryptophan solvatation, i.e. processes leading to considerable changes in the compactness of the protein molecule. Figure 3a shows that an increase in methanol concentration in solution at pH 2.0 and 23°C results in a considerable increase of fluorescence intensity and essential (from 328 to 348 nm) red shift of the position of the tryptophan fluorescence spectrum (these processes are visualized in Fig. 3b as dependence of the tryptophan fluorescence λ_{max} and intensity on methanol concentration). One can see that the cooperative changes in tryptophan fluorescence parameters of β-lactoglobulin occur within 20–60% methanol. The methanol-induced red shift of the tryptophan fluorescence spectrum reflects the considerable increase of the accessibility of these residues to the solvent, i.e. increase of the protein molecule hydrodynamic volume. This observation is in good agreement with the conclusion made by Kamatari *et al.* [23] that high concentration of alcohols can induce in a protein molecule the expanded highly helical denatured state (H).

Interaction with ANS (8-ANS fluorescence decay)

It is well known that the high affinity of a protein molecule to a hydrophobic fluorescence probe (such as 8-ANS) is a characteristic property of the molten globule state and other nonnative partially folded conformations [2,3,9,30–34]. Recently, we have also established that the transformation of the protein molecule into the molten globule state is accompanied not only by the increase in 8-ANS fluorescence intensity, but also by a considerable increase in the fluorescence decay of protein-embedded 8-ANS molecules [35].

It is necessary to emphasize here that ANS binding data can also be used as a complementary line of evidence on the compactness of the protein molecule. Indeed, it has been established that only compact protein molecules show high affinity to the hydrophobic fluorescent probe (e.g. 8-ANS),

Figure 3



(a) Methanol-induced tryptophan fluorescence spectral changes of β-lactoglobulin at pH 2.0 and 23°C in 0 to 80% methanol (in steps of 5%). The protein concentration was 0.82 mg ml^{-1} for all the experiments. (b) Dependence of tryptophan fluorescence intensity (circles) and position of spectral maximum (squares) on methanol concentration.

whereas there is no visible interaction between ANS and an expanded unfolded polypeptide chain [32–35]. More importantly, it has been established that the more structured (and, as a consequence, more compact) molten globule like equilibrium intermediate state has a much greater affinity to 8-ANS than has the less structured and less compact pre-molten globule state [21,36,37].

Figure 4a shows that methanol-induced overall changes in protein conformation are accompanied by considerable changes in the 8-ANS fluorescence lifetimes. The figure clearly shows that the dependence of the longest lifetime component on the methanol concentration passes through the maximum at ~40% alcohol. This observation reflects the fact that under studied conditions the formation of a relatively compact intermediate state with high affinity to the hydrophobic fluorescent probe is observed. Interestingly, the position of the maximum in this dependence practically coincides with the point of maximum population of an intermediate state determined from tryptophan fluorescence and CD data (cf. Figs 4a and 5a).

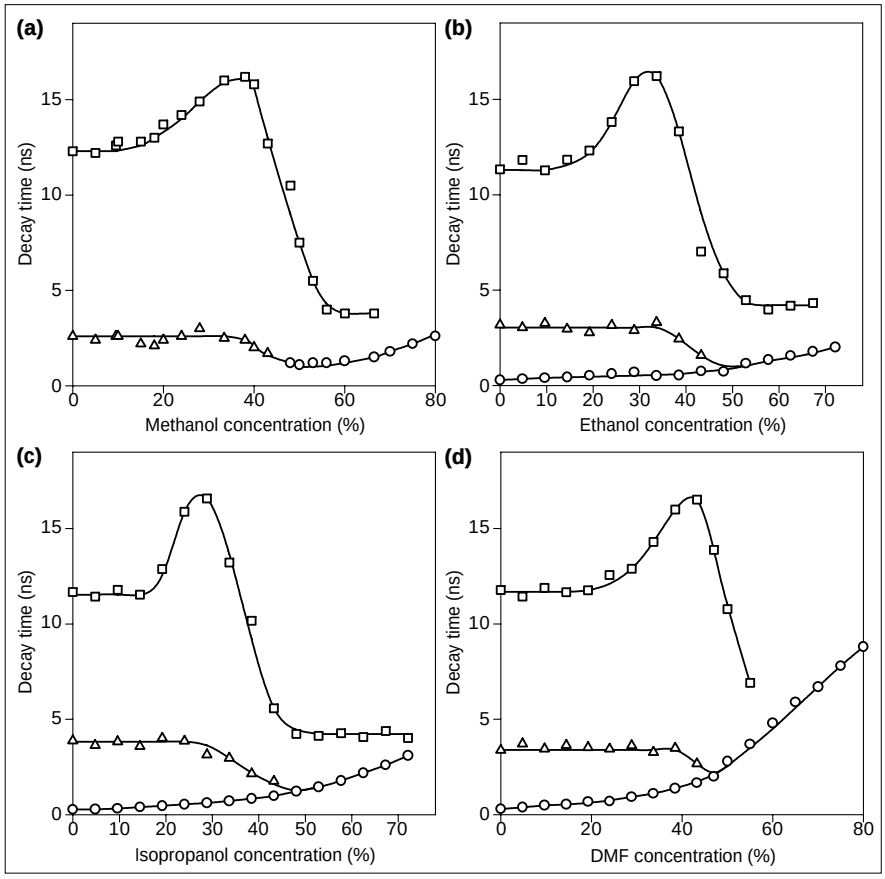
Methanol-induced intermediate state

Analyses of the data presented in Figures 1, 2 and 3 allow us to conclude that the methanol-induced denaturation of β-lactoglobulin is accompanied by a change in far UV CD spectrum shape ($[\Theta]_{208} : [\Theta]_{222}$ ratio increases from ~0.8 to ~1.06 in the absence and in the presence of alcohol, respectively) and precedes the process of protein secondary structure transformation and changes in solvatation of tryptophan residues (these conclusions are illustrated by Fig. 5b, which collects all the data and represents them in terms of relative units). It is obvious that if there are two cooperative transitions, we can expect the existence of at least three different conformational states separated by these transitions. The first of these states is observed at low alcohol concentrations. This is definitely the native (N) state of β-lactoglobulin, as the protein molecule is compact and has a specific near UV CD spectrum. The second state exists at high methanol concentrations and is characterized by a high content of α-helical structure and considerable expansion. This is the highly helical expanded state (H) typical of globular proteins in high concentrations of alcohols [22,23,26–28,38–46]. The third state is the denatured intermediate state (I) with pronounced secondary structure, high affinity to 8-ANS and native-like compactness. We cannot exclude the possibility that this intermediate is the molten globule state.

Figure 5a presents these processes as the dependence of relative numbers of native (f_N), intermediate (f_I) and helical (f_H) molecules of β-lactoglobulin on methanol concentration. These functions were calculated from two basic curves: the denaturation ($f_D = 1 - f_N$), monitored by near UV ellipticity and changes in shape of the far UV CD spectrum (see below), and the transformation into the helical expanded state (f_H), monitored by the increase in intensity of the far UV CD spectra and red shift of the tryptophan fluorescence spectrum. The relative population of an intermediate state (f_I) can then be calculate as $f_I = f_D - f_H$. Figure 5a shows how the intermediate state appears on the increase of methanol concentration and then transforms into the highly helical expanded state. Though these two transitions partly overlap, it is quite clear that the molecules of β-lactoglobulin can be transformed into the compact intermediate conformation by the methanol.

It is necessary to emphasize here that Figure 5b clearly shows that the process of protein denaturation (transi-

Figure 4



Changes in the fluorescence decay parameters of the 8-ANS–β-lactoglobulin complexes connected with the conformational transformations of this protein induced by (a) methanol, (b) ethanol, (c) isopropanol and (d) DMF. Here, squares are the longest lifetime component (attributed to the protein-embedded 8-ANS molecules), triangles are the intermediate lifetime component (attributed to 8-ANS molecules that interact with the protein surface), and circles are the shortest lifetime component (attributed to free 8-ANS molecules). In the case of methanol, measurements were carried out using a SLM 48 000 MHF Fourier transform spectrofluorimeter. In all other cases, the time domain fluorescence lifetime method was used.

tion from the native to the intermediate state) can be monitored easily not only by changes in the near UV CD spectrum, but also by changes in the shape of the far UV CD spectrum. This observation is in a good agreement with the recent work of Vassilenko *et al.* (KS Vassilenko *et al.*, unpublished data), where it has been established that a protein molecule in the molten globule state has a specific 'holster-like' far UV CD spectrum with the characteristic ratio between ellipticity values in minima at 208 nm and 222 nm $[\Theta]_{208} : [\Theta]_{222} = 1.12 \pm 0.08$.

Effect of other organic solvents on structural properties of β-lactoglobulin

To understand the driving forces of organic solvent induced conformational transformations, we have investigated the influence of alcohols with chains of different length (methanol, ethanol and isopropanol) and solvents of other classes (DMF and dioxane) on such structural properties of β-lactoglobulin as near and far UV CD spectrum, tryptophan fluorescence and fluorescence decay parameters of 8-ANS–protein complexes. All measurements were carried out at pH 2.0 and 23°C and results are summarized in Figures 4, 6 and 7.

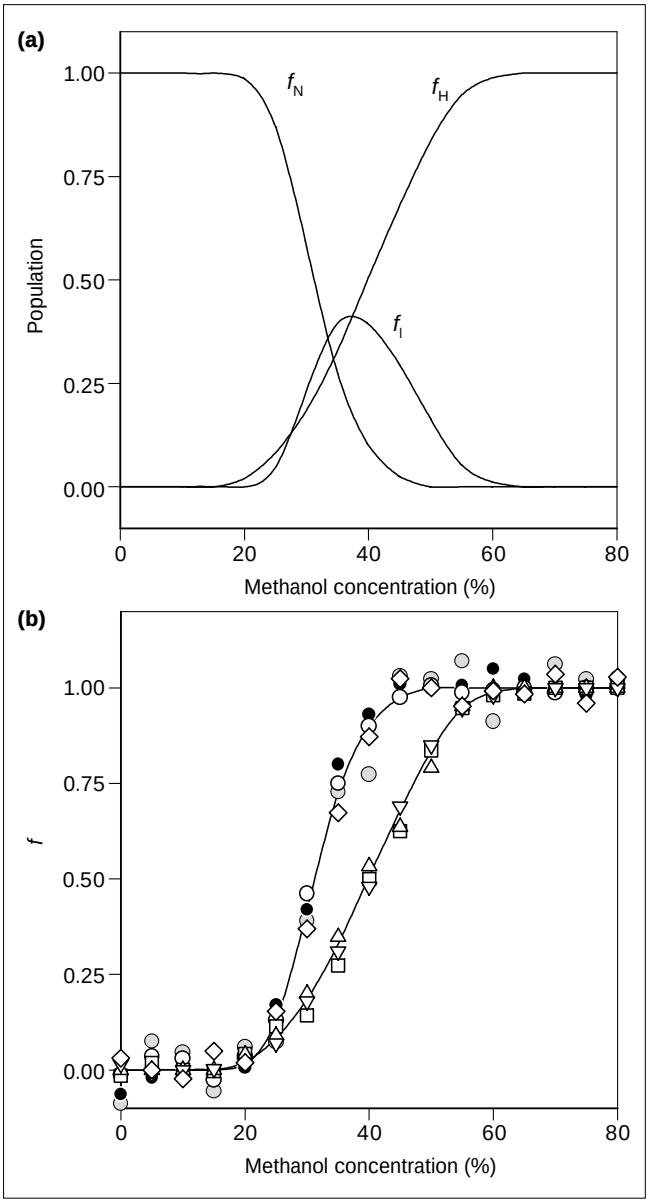
Denaturation (near UV CD)

The increasing content of any organic solvent in solution transforms the near UV CD spectra of β-lactoglobulin in the same manner as described above for methanol (see Fig. 1). In particular, a simultaneous change in the intensity of all the bands in the spectrum is observed. As already mentioned, this means that the process of organic solvent induced denaturation of β-lactoglobulin follows a two-state mechanism. Denaturation curves resulting from the normalization of $[\Theta]_{293.5}$ versus organic solvent concentration dependence are presented in Figure 6.

Secondary structure (far UV CD)

The effect of ethanol, isopropanol and DMF on the β-lactoglobulin far UV CD spectrum (Fig. 6a–c) is relatively close to that of methanol. That is why we present here only the normalized changes in the spectral shape and intensity induced by different organic solvents and do not show the corresponding far UV CD spectra of the protein. It is necessary to emphasize that in the case of DMF we have not been able to measure far UV CD spectra at wavelengths lower than 225 nm due to the high level of solvent absorption, so the corresponding plot (Fig. 6c) presents only data for the increase in far UV CD spectrum intensity

Figure 5



(a) Dependence of the relative numbers of native (f_N), molten globule intermediate (f_I) and helical (f_H) molecules of β-lactoglobulin on methanol concentration. These functions were calculated from two basic curves: denaturation ($f_D = 1 - f_N$), monitored by near UV ellipticity and changes in $[\Theta]_{208} : [\Theta]_{222}$ ratio, and transition into the helical expanded state (f_H), monitored by the increase in intensity of far UV CD spectra and red shift of tryptophan fluorescence spectrum. The relative population of the molten globule intermediate state (f_I) was calculated as $f_I = f_D - f_H$. (b) The normalized transition curves obtained from the ellipticity at 293.5 nm (open circles), 285 nm (black circles), 263.5 nm (grey circles), 222 nm (open squares), tryptophan fluorescence intensity (reversed open triangles), position of tryptophan fluorescence maximum (open triangles) and $[\Theta]_{208} : [\Theta]_{222}$ ratio (diamonds).

resulting from the normalization of $[\Theta]_{230}$ versus DMF concentration dependence.

Compactness (tryptophan fluorescence)

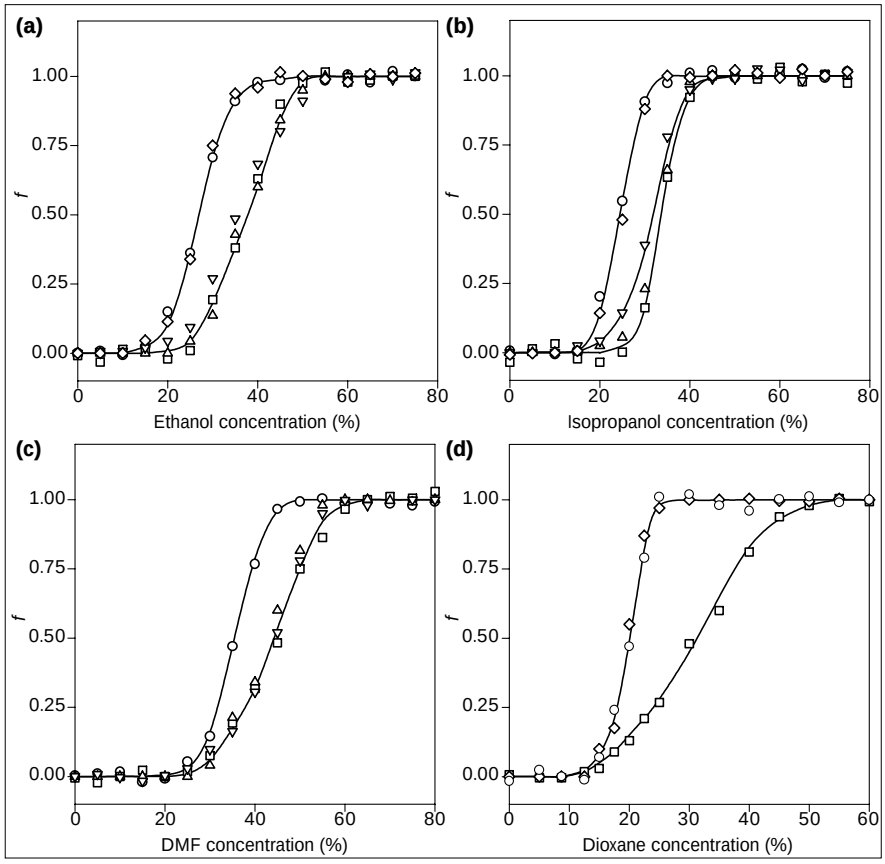
The effect of ethanol, isopropanol and dioxane on the tryptophan fluorescence spectrum of β-lactoglobulin is shown in Figure 6a,b,d. One can see that the organic solvent induced red shift in the tryptophan fluorescence maximum occurs simultaneously with the rearrangement of protein secondary structure. Figure 5 shows also that in the case where N→I and I→H transitions become more separated, the fluorescence intensity increases in two steps (see data for isopropanol in Fig. 6b), while in the case of overlapped transitions (methanol, ethanol and DMF; Figs 5b,6a,c) changes in tryptophan fluorescence intensity are described well by a sigmoidal curve that coincides with the I→H transition monitored by far UV ellipticity. Such behaviour can easily be explained by taking into account the fact that the N→I transition leads to only ~10% changes in tryptophan fluorescence intensity, while the main changes occur under the I→N transition (see Fig. 6b).

Interaction with ANS

Figure 4 summarizes the data on organic solvent effects on fluorescence decay times of 8-ANS in the complexes of this hydrophobic fluorescent probe with β-lactoglobulin. In the case of ethanol, isopropanol and DMF, the time-resolved fluorescence measurements were carried out using the time domain fluorescence lifetime method (see below), which allows us to measure much shorter fluorescence lifetimes as compared with the SLM 48000 MHF Fourier transform spectrofluorimeter used for the analogous investigations in the case of methanol (see above). As a result, three 8-ANS fluorescence decay times were measured (see Fig. 4b–d). The shortest lifetime corresponds to the free dye molecules, while two longer lifetimes describe 8-ANS–protein complexes. It has been established that the fluorescence decay time of free 8-ANS has a uni-exponential character ([47]; VN Uversky *et al.*, unpublished data). On the other hand, we have shown recently that the interaction of 8-ANS with the protein molecules yields a double-exponential fluorescence decay and both decay components in this case considerably exceed the value of the 8-ANS decay time in water [35], reflecting the preferably hydrophobic environment of the dye molecules attached to the proteins. We have also established that the shorter lifetime component of 8-ANS fluorescence decay is characteristic for the dye molecules that interact with the surface of the protein molecule, while a longer lifetime component refers to the protein-embedded 8-ANS molecules [35].

Figure 4 shows that the lifetime of free 8-ANS molecules increases monotonously on the increase of the organic solvent content. The intermediate decay time (which corresponds to the 8-ANS molecules attached to the surface of protein molecules) is independent of low concentrations of organic solvent and disappears at high organic contents, reflecting the disruption of the above

Figure 6



Conformational changes induced in β-lactoglobulin by different organic solvents at pH 2.0 and 23°C. (a) Ethanol, (b) isopropanol, (c) DMF, and (d) dioxane. Transition curves were obtained from the ellipticity at 293.5 nm (circles) and 222 nm (squares), tryptophan fluorescence intensity (reversed open triangles), position of tryptophan fluorescence maximum (open triangles) and $[\Theta]_{208} : [\Theta]_{222}$ ratio (diamonds). In the case of DMF, squares correspond to the changes in $[\Theta]_{230}$.

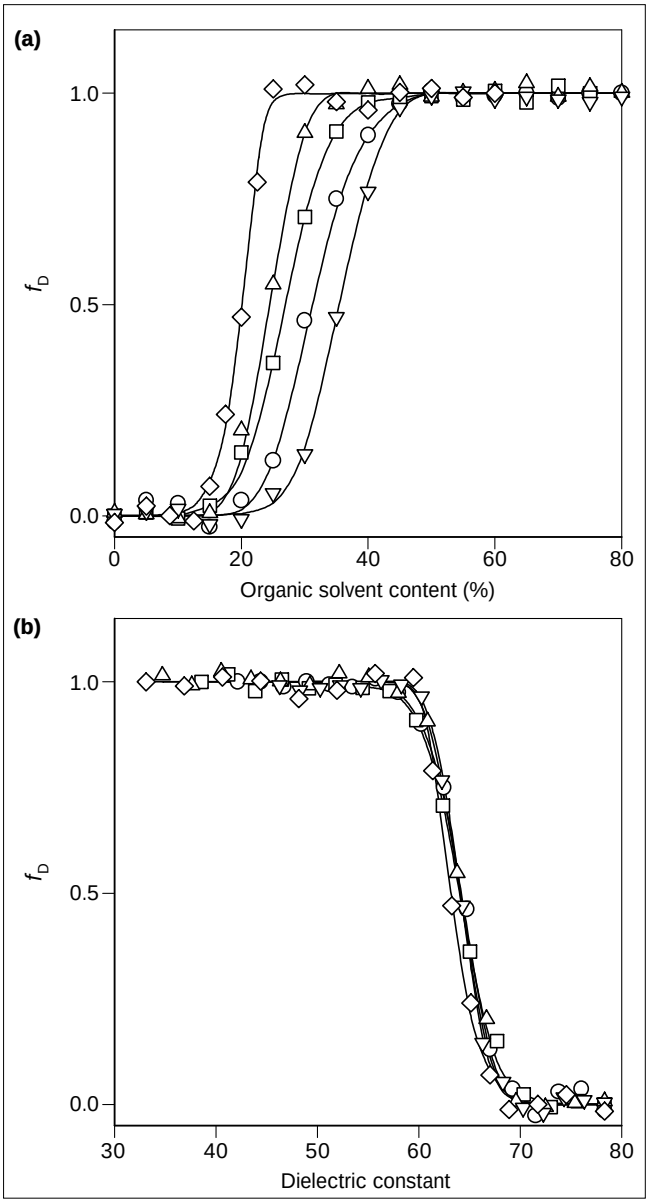
8-ANS–protein complexes. The dependence of the longest 8-ANS lifetime (which corresponds to the protein-embedded 8-ANS molecules) on the concentration of organic solvents passes through the maximum. The same behaviour was observed earlier for the guanidine hydrochloride induced unfolding of several proteins and was interpreted in terms of the formation of a compact intermediate (molten globule like) state ([35]; VN Uversky *et al.*, unpublished data).

Dielectric constant induced molten globule state

Results presented above leave no doubts that the increase in concentration of organic solvents leads to the transformation of β-lactoglobulin molecules into the compact denatured intermediate state with pronounced secondary structure (see Figs 5,6), which, in addition, has large affinity to the hydrophobic fluorescent probe 8-ANS (see Fig. 4). We cannot exclude the possibility that this intermediate state does not necessarily have native-like secondary structure and it may indeed have an increased helicity as compared to the native protein. On the other hand, the kinetic studies show that the refolding of β-lactoglobulin passes through the nonnative α-helical intermediate state [48,49]. This means that the formation of an intermediate state with an increased helicity is a specific property of β-lactoglobulin. All this allows us to assume that the decrease in the dielectric constant of the solution provokes the transformation of β-lactoglobulin into the molten globule like intermediate state. Processes of these conformational transformations are presented in Figures 7a, 8a and 9a in terms of dependence of relative populations of denatured (f_D), helical (f_H) and molten globule intermediate (f_I) molecules, respectively, on the concentration of the organic component. It is necessary to emphasize that the compact intermediate state in β-lactoglobulin can be induced by organic solvents of different classes — alcohols, amides and cyclic ethers. This means that some sort of general property of the solvent plays a major role in structural transformations of the protein molecule. We suggest that this general property of the solvent is its hydrophobicity. In other words, we assume that an intermediate state in β-lactoglobulin can be induced by changes in solvent hydrophobicity.

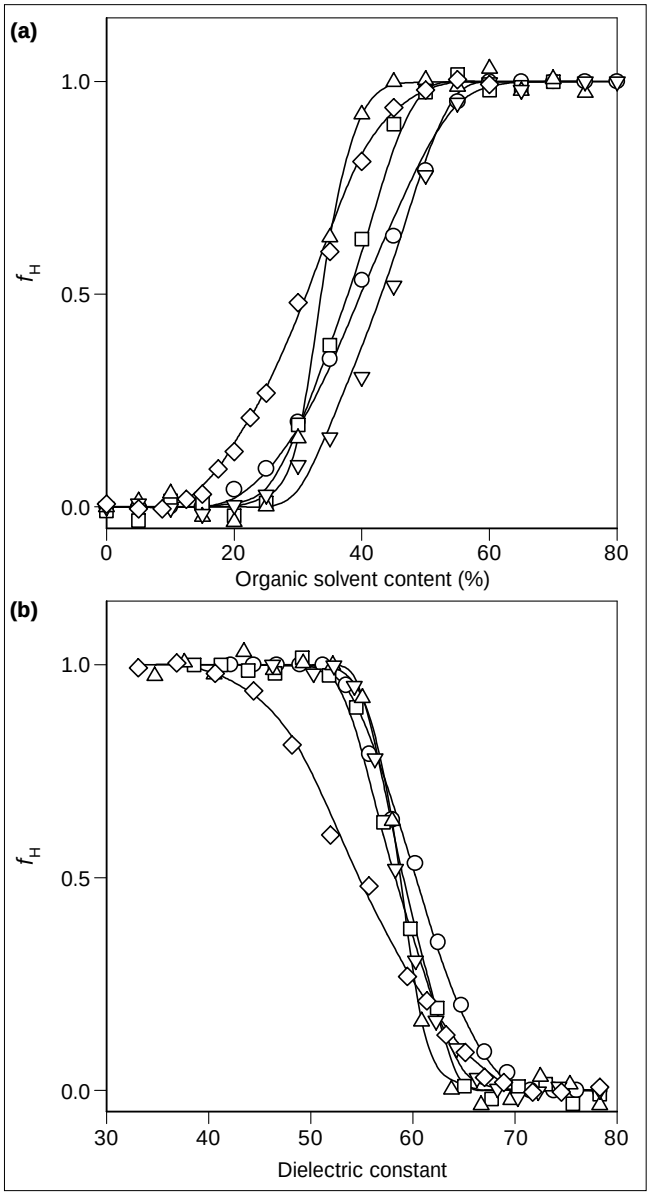
Figures 7–9 represent the data that confirm this suggestion. Indeed, Figures 7a, 8a and 9a collect the dependence of the relative numbers of the denatured, helical and molten globule molecules, respectively, on the organic solvent content measured for five different solvents — methanol, ethanol, isopropanol, DMF and dioxane. These

Figure 7



Dependence of the relative number of denatured molecules (f_D) on (a) concentration of different organic solvents and (b) dielectric constant of solution. Organic solvents: methanol (circles), ethanol (triangles), isopropanol (reversed triangles), DMF (squares) and dioxane (diamonds).

Figure 8

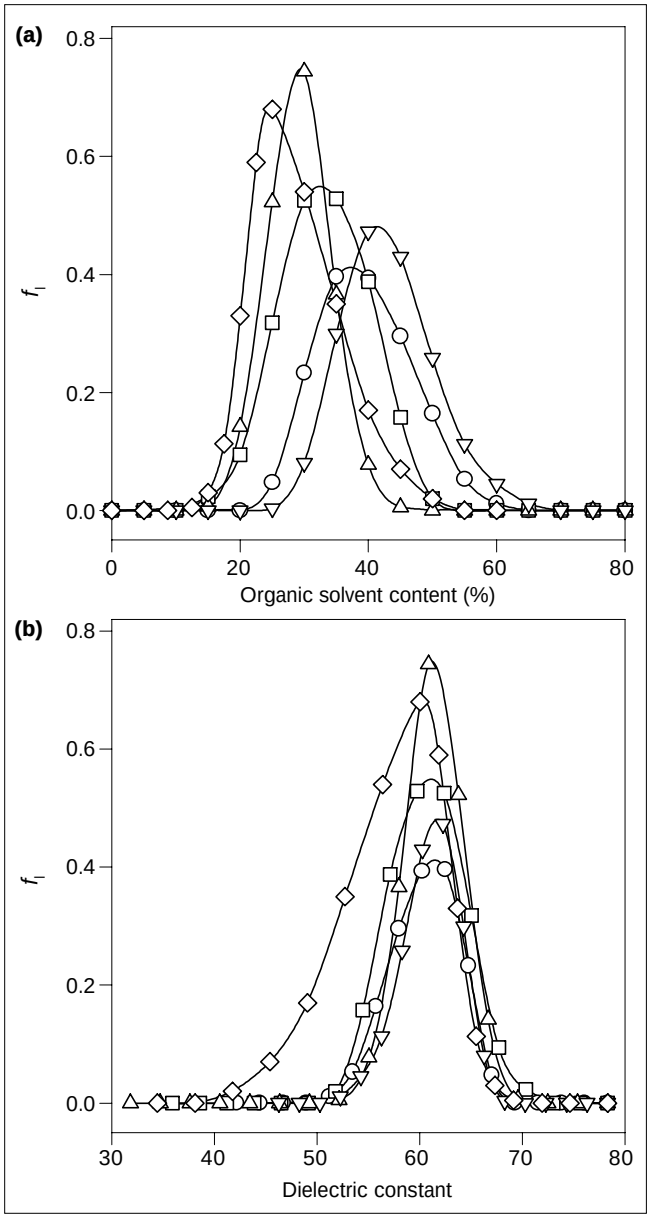


Dependence of the relative number of helical molecules (f_H) on (a) concentration of different organic solvents and (b) dielectric constant of solution. Organic solvents: methanol (circles), ethanol (triangles), isopropanol (reversed triangles), DMF (squares) and dioxane (diamonds).

figures show that the position of the dependence shifts toward the smaller content of the organic solvent on the increase in its hydrophobicity, i.e. with the decrease in the corresponding dielectric constant. The same observation was made earlier by Dufour *et al.* [26] for the effect of alcohols on the structural properties of this protein.

Figures 7b, 8b and 9b represent the recalculated data as the dependence of f_D, f_H or f_I, respectively, versus dielectric constant. Figure 7b shows that in such coordinates, the denaturation effect of different organic solvents is described by a 'master curve'. In other words, the denaturation of β-lactoglobulin in water/organic mixtures is provoked by the decrease in the dielectric constant of the solvent. Figure 8b shows that there is a master curve of the transition to the helical state induced by such solvents as methanol, ethanol, isopropanol and DMF, while the curve describing the effect of dioxane shares a common dependence. This observation may reflect the presence of

Figure 9



Dependence of the relative number of molten globule intermediate molecules (f_i) on (a) concentration of different organic solvents and (b) dielectric constant of solution. Organic solvents: methanol (circles), ethanol (triangles), isopropanol (reversed triangles), DMF (squares) and dioxane (diamonds).

some specific interaction between the solvent and protein molecules on the formation of the helical state. Finally, Figure 9b shows that the point of the maximal population of the intermediate state (molten globule like intermediate), being practically identical for all solvent systems studied, is observed at a dielectric constant $\epsilon \sim 62$. This means that a decrease in the dielectric constant of the solution can induce the molten globule like intermediate state in β-lactoglobulin.

Conclusions

The most intriguing finding of this work is that an intermediate state with molten globule properties can be induced in β-lactoglobulin by an increase in the solvent hydrophobicity. This observation is the first direct confirmation of the hypothesis on the denaturing action of dielectric constant suggested recently by Ptitsyn and coworkers [16,21] for the explanation of protein denaturation near the membrane surface.

Materials and methods

Materials

β-Lactoglobulin was purified from bovine milk by NV Kotova (Institute of Protein Research, Russian Academy of Sciences). Mg^{2+} salts of 8-anilinonaphthalene-1-sulfonate (8-ANS), dimethylformamide (DMF), dioxane and isopropanol were purchased from Sigma. Ethanol and methanol were from JT Baker BV (Deventer, Holland) and Carl Roth GmbH (Karlsruhe, Germany), respectively. Organic solvents concentrations are given as % v/v.

Equipment

Fluorescence lifetimes were measured by means of a SLM 48 000 MHF Fourier transform spectrofluorimeter (SLM Instruments, IL, USA) and streak camera C-4334 (Hamamatsu Inc, Japan). The first instrument was used for the investigations of methanol-induced changes in fluorescence decay parameters of 8-ANS–β-lactoglobulin complexes, while the second was applied for the studies of other solvent (ethanol, isopropanol and DMF) effects on protein structure. The 325 nm line of a helium cadmium laser (Plazma, Ryazan, Russia) was used for fluorescence excitation in a SLM 48 000 fluorimeter. This line is not very intense, allowing us to measure fluorescence decay characteristics only in solutions with relatively high fluorescence intensity. For the estimation of fluorescence decay times in the solutions with weak fluorescence intensity, we have used the time domain fluorescence lifetime method. In this case, we used for the fluorescence excitation the frequency-tripled output of a titanium sapphire laser Tsunami (Spectra Physics Ltd, Mountain View, USA). The laser was run in mode-locked operation at 82 MHz producing pulses of 250 fs pulse width with an averaged energy of 42 mW at 263 nm. The fluorescence decay was recorded by streak camera C-4334 (Hamamatsu Inc, Japan) in the 2 ns time window. The fluorescence emission was spectrally resolved by a polychromator in front of the streak camera.

Steady-state measurements of tryptophan fluorescence were performed using a SFM-25 spectrofluorimeter (Tegimenta, Switzerland). The tryptophan fluorescence was excited at 286 nm. Absorption spectra were recorded by Cary 4E UV-VIS spectrophotometer (Varian, Mulgrave, Australia).

Circular dichroism measurements were carried out by JASCO-700 spectropolarimeter (Japan). The cell path length was 0.1 mm for far UV CD and 10.0 mm for far UV CD. The protein concentrations were kept at 0.82 mg ml^{-1} throughout all the experiments.

Solution preparation

The protein was incubated in the desired solution for 24 h before measurements. The pH of solution was measured on a Radelkis laboratory digital pH-meter OP-211/1 (Hungary). The pH of samples in water/organic solvent mixtures was adjusted by HCl after the addition of the organic component. The pH values reported are direct meter readings uncorrected for organic solvent effect. The values of dielectric constant (ϵ) for pure solvents were taken from the reference books and were equal to 78.3, 38.3, 33.1, 25.3, 20.2 and 2.98 for water, DMF, methanol, ethanol, isopropanol and dioxane, respectively. In the cases where mixtures of two different solvent were investigated, the averaged dielectric constant, $\langle\epsilon\rangle$, was calculated as $\langle\epsilon\rangle = C_1\,\epsilon_1 + C_2\,\epsilon_2$, where C_i

is the relative concentration of components ($C_1 + C_2 = 1$), and ϵ_i is their dielectric constant.

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