# Molten Globule-Like State of Cytochrome *c* under Conditions Simulating Those Near the Membrane Surface†

Valentina E. Bychkova,*,‡ Alexandra E. Dujsekina,‡ Stanislav I. Klenin,§ Elisaveta I. Tiktopulo,‡ Vladimir N. Uversky,‡ and Oleg B. Ptitsyn‡,||

*Institute of Protein Research, Russian Academy of Sciences, 142292 Pushchino, Moscow Region, Russia, Institute of High Molecular Compounds, Russian Academy of Sciences, 199004 St. Petersburg, Russia, and Laboratory of Mathematical Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892-5677*

*Received September 19, 1995; Revised Manuscript Received February 21, 1996*⊗

ABSTRACT: Methanol-induced conformational transitions in cytochrome *c* have been studied by near- and far-UV circular dichroism, Trp fluorescence, microcalorimetry, and diffusion measurements. The existence of at least two cooperative stages of transition has been shown. At the first stage, the native protein is transformed into an intermediate which has only traces of tertiary structure, but has a native-like secondary structure content and is relatively compact; i.e., it has properties of the molten globule state. On the second stage, the alcohol-induced molten globule is transformed into a more helical state, typical of proteins at high alcohol concentrations. The conditions at which the alcohol-induced molten globule exists (moderately low pH and moderately low dielectric constant) could be similar to those existing near negatively charged membrane surfaces. Consequently, these results might explain how the molten globule state can be achieved under physiological conditions.

In 1988, we predicted that the molten globule state, as well as other non-native states of proteins, can exist in a living cell and can be involved in a number of physiological processes (Bychkova et al., 1988). During the last 5 years, this prediction was confirmed by experiments which have shown that molten globules and/or other non-native states of protein molecules are involved in protein recognition by chaperones (Martin et al., 1991; van der Vies et al., 1992), protein penetration into membranes (van der Goot et al., 1991, 1992), release of protein ligands (Bychkova et al., 1992), and in some other processes [see Bychkova and Ptitsyn (1993) and Ptitsyn (1995) for reviews].

However, proteins can be transformed *in vitro* into the molten globule state at low pH, moderate concentrations of urea or guanidinium chloride (GdmCl), high temperature, etc. [see Ptitsyn (1992) for a review], far from usual physiological conditions. The question arises, how can proteins achieve their molten globule or other non-native states in a living cell. Of course, they can be trapped into these states by chaperones just after their biosynthesis (Bochkareva et al., 1988). It is also possible that some mutant proteins are arrested in the molten globule state, since these mutations prevent proteins from complete folding (Bychkova & Ptitsyn, 1995). However, in a number of cases (e.g., at penetration of toxins into membranes and, especially, at the release of ligands to the target cells), proteins almost certainly are originally native and denature only near membrane surfaces.

In fact, it was shown that the membrane surface can lead to a "partial denaturation" of proteins, transforming them into the non-native (though not completely unfolded) state (Eilers et al., 1988; de Jongh et al., 1992).

It was also shown that this effect is due to negative charges of a membrane (Endo & Schatz, 1988). The reason is that the negative electrostatic potential of a membrane surface attracts protons from a bulk solution leading to a local decrease of pH and may provoke acid denaturation of proteins (Eisenberg et al., 1979; van der Goot et al., 1991). However, even in salt-free solutions, this local decrease of pH does not exceed ∼2 pH units at 5−15 Å from the membrane surface (Prats et al., 1986), which is usually insufficient for acid denaturation.

Therefore, we have proposed an additional denaturing action of the membrane surface—a local decrease of the dielectric constant near the membrane surface (Bychkova & Ptitsyn, 1993; Ptitsyn et al., 1995). The classical electrodynamics [see, e.g., Landau and Lifshits (1982)] show that the effective dielectric constant of water near the organic surface should be twice smaller than its "bulk" dielectric constant. The decrease of the dielectric constant near the membrane surface can enhance the electrostatic repulsion of negatively charged groups at moderately low pH, thus "helping" to denature proteins.

The question arises, how can this "concerted" action of a local decrease of pH and that of the dielectric constant be modeled in more simple systems. Of course, the local decrease of pH near membranes can be simulated by moderately low "bulk" pH [see, e.g., van der Goot et al. (1991)]. More unexpected is that the local decrease of the dielectric constant may be modeled by water−alcohol mixtures since it was shown that the denaturing action of different alcohols depends only on the average dielectric constant of solution rather than on the specific properties of different alcohols (Wilkinson & Mayer, 1986; Dufour et al., 1993). This opens the possibility to use a "concerted" action

† This study was supported in part by the Human Science Frontier Program Organization (Grant RG-331/93 M).

* To whom correspondence should be addressed at LMMB, Molecular Structure Section, NIH, Building 12B, Room B116, MSC 5677, 12 South Dr., Bethesda, MD 20892-5677.

‡ Institute of Protein Research, Russian Academy of Sciences.

§ Institute of High Molecular Compounds, Russian Academy of Sciences.

|| National Cancer Institute.

⊗ Abstract published in *Advance ACS Abstracts,* April 1, 1996.

S0006-2960(95)02246-X CCC: $12.00 

of moderately low pH and moderately low dielectric constant in water−alcohol mixtures to model the denaturing action of membranes on a protein structure (Ptitsyn et al., 1995).

We have chosen cytochrome *c* for this modeling since this protein is located and functions near the surface of the inner mitochondrial membrane, but may not be directly associated with it under physiological conditions (Gupte & Hackenbrock, 1988). In addition, it was shown (de Jongh et al., 1992) that phospholipid vesicles transform this protein into a much more flexible state which is virtually denatured.

## MATERIALS AND METHODS

*Materials*. Pure horse cytochrome *c* was obtained from Serva (Germany). The protein concentrations were determined by absorbance at 280 and 409 nm on a Perkin-Elmer spectrophotometer using $\epsilon^{1mg/mL}_{1cm,280} = 1.79$ and $\epsilon^{1mg/mL}_{1cm,409} = 8.52$ (S. Yu. Venyaminov, private communication). The buffer components were analytical or extra pure grade and were used without additional purification. Desirable pH values were adjusted by the addition of 1.0 N HCl. Methanol (Reakhim, Russia) was extra pure grade. All solutions were prepared on bidistilled water.

*Circular Dichroism*. CD measurements were made on JASCO-41A and JASCO-600 spectropolarimeters (Japan) equipped with a temperature-controlled holder. The cell path length varied from 0.15 to 0.5 mm for far-UV CD and was 10.0 mm for near-UV CD. The molar ellipticity was calculated as

$$[\theta]_\lambda = \theta_\lambda \mathrm{MRW}/lc \qquad (1)$$

where $\theta_\gamma$ is the measured ellipticity (in millidegrees) at a wavelength $\lambda$, MRW is the mean residue molecular weight calculated from the protein sequence (for cytochrome *c*, MRW = 119), *l* is the cell path length (in millimeters), and *c* is the protein concentration (in milligrams per milliliter). The concentration *c* was varied from 0.3 to 1.5 mg/mL. The temperature was 24 °C.

*Fluorescence*. Fluorescence spectra were obtained for a 0.03 mg/mL protein concentration at 24 °C; tryptophan fluorescence was excited at 290 nm.

The protein solutions for CD and fluorescence measurements were prepared from stock solutions by dilution with a corresponding buffer. All solutions were incubated overnight before measurements.

*Microcalorimetric measurements* were made on a DASM-4A capillary scanning microcalorimeter, Russia (Privalov & Plotnikov, 1989), equipped with gold 0.5 mL cells. The rate of heating was 1 K/min. The protein concentration was 0.5−1.2 mg/mL. To calculate the protein heat capacity, the value of 0.72 for the partial specific volume was used. All the solutions were equilibrated overnight against a corresponding buffer by dialysis.

*Diffusion*. Measurements of diffusion coefficients (*D*) were performed at 24 °C by the method of macroscopic diffusion (Tsvetkov, 1951). This method provides quantitative information on individual components in complicated systems as interferometric optics allow observation of the Gaussian distribution of the concentration gradient (d*c*/d*x*) in a diffusion cell at different moments of the experiment. These Gaussian curves can be used for calculations of the diffusion coefficients of individual components (e.g., Tsvetkov & Klenin, 1953). This method has been successfully applied to the determination of the dimensions of protein molecules in different conformational states (Bychkova et al., 1990). The value of the Stokes radius ($R_s$) is determined as

$$R_s = kT/6\pi\eta_0 D \qquad (2)$$

where *k* is the Boltzmann constant, $\eta_0$ is the viscosity of a solvent, and *T* is a temperature.

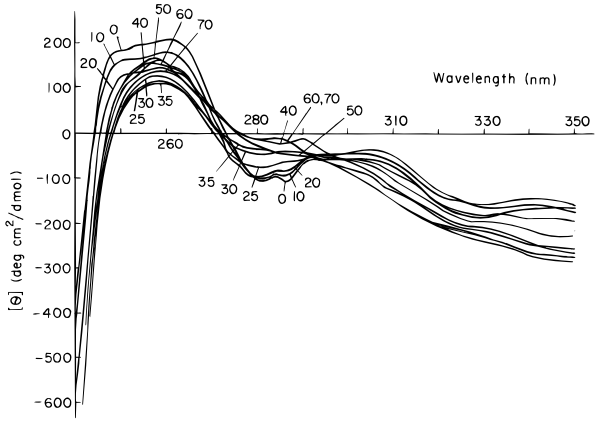


FIGURE 1: Near-UV CD spectra of cytochrome *c* (pH 4.0 in 0.5 M NaCl, 24 °C) at methanol concentrations shown near the curves.

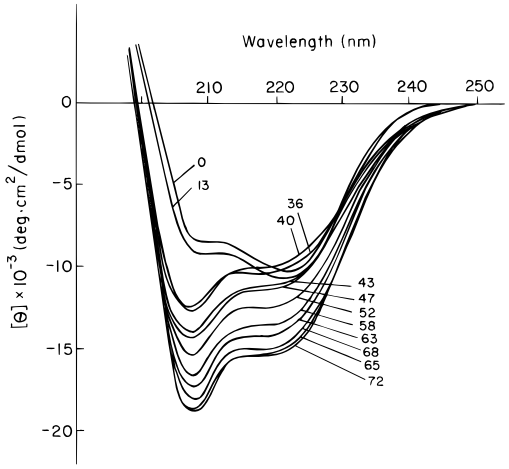


FIGURE 2: Far-UV CD spectra of cytochrome *c* (pH 4.0 in 0.5 M NaCl, 24 °C) at methanol concentrations shown near the curves.

## RESULTS

*Two Stages of Methanol-Induced Transitions*. Near- and far-UV CD spectra of horse cytochrome *c* have been obtained in water−methanol mixtures with different concentrations of methanol (from 0 to a 70% volume) at pH 4.0 and in three different salt concentrations: 0.5 M NaCl; 0.15 M NaCl; and 6 mM sodium acetate. Figures 1 and 2 present the results in 0.5 M NaCl as an example. Figure 1 shows that native cytochrome *c* has a broad positive band at 250−270 nm and two negative bands between 280 and 290 nm. The positive band has been attributed to transitions in a heme (Urry, 1967; Blauer et al., 1993). The negative bands have been long ago attributed to electronic transitions in a side chain of the single Trp59 which has been confirmed by their vanishing in a mutant where Trp59 is replaced by Phe (Davies et al., 1993).

In water−methanol mixtures, both negative bands decrease and practically vanish at methanol content >40%. The

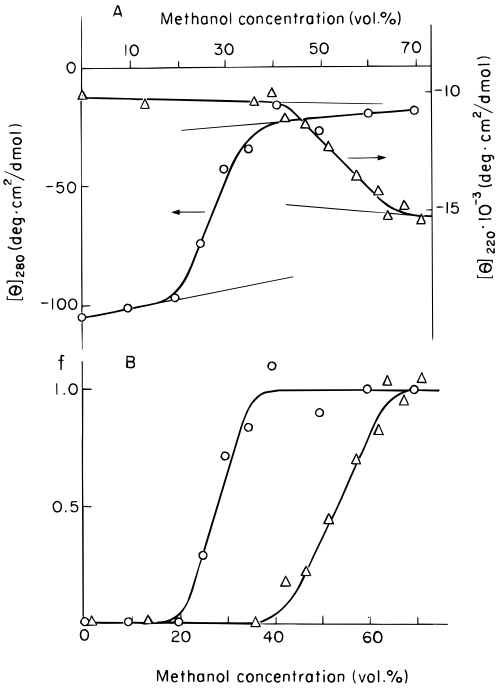


FIGURE 3: (A) Dependence of the molar ellipticities $[\theta]_{280}$ and $[\theta]_{220}$ (pH 4.0 in 0.5 M NaCl, 24 °C) on the methanol concentration. Thin lines represent base lines extrapolated from small and large methanol concentrations. (B) The same data shown in relative units.

positive band changes its shape, becoming more narrow when the methanol content increases to 25%. The intensity of this band (in its maximum around 260 nm) decreases when the methanol content is increased to 35% and then increases again. At high methanol concentrations, this band is less intensive than in the native protein but substantially more intensive than that in 7 M GdmCl or at pH 2 in water without salt (see below, Figure 5), i.e., under conditions where cytochrome *c* is practically unfolded (Babul & Stellwagen, 1972; Ikai et al., 1973). These data suggest that the rigid tertiary structure of cytochrome *c* near Trp59 is practically destroyed at methanol concentrations about 40%. On the other hand, some traces of tertiary structure may remain in the neighborhood of the heme giving rise to its near-UV CD spectrum.

Figure 2 presents far-UV CD spectra of cytochrome *c* under the same conditions. The figure demonstrates the increase of the far-UV CD spectrum with the increase of methanol concentration, which is the usual feature for all alcohol-denatured proteins studied so far (Tanford, 1968; Timasheff, 1970; Wilkinson & Mayer, 1986; Dufour et al., 1993; Alexandrescu et al., 1994; Ptitsyn et al., 1995; Shiraki et al., 1995) and is believed to reflect an increase of their helicity. Similar behavior of cytochrome *c* far-UV CD spectra was observed by Kaminsky et al. (1972) upon increase of 1-propanol content.

Figure 3A compares the methanol dependence of two molar ellipticities: at 280 nm (near-UV) and at 220 nm (far-UV) in 0.5 M NaCl. The figure shows that, while $[\theta]_{280}$ changes mainly in the interval from 20 to 40% methanol, the main changes of $[\theta]_{220}$ occur at methanol concentrations >40%, i.e., when the change of $[\theta]_{280}$ is almost over. To illustrate this result, Figure 3B shows the changes of both molar ellipticities in relative units:

$$f = (X - X_N)/(X_A - X_N) \qquad (3)$$

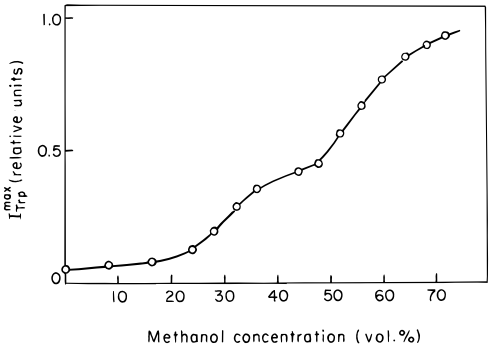


FIGURE 4: Dependence of the intensity of the Trp fluorescence on the methanol concentration at pH 4.0 in 0.5 M NaCl, 24 °C.

where $X$ is the measured parameter, while $X_N$ and $X_A$ are their values in the native and the alcohol-denatured states, respectively, obtained from base lines in Figure 3A.

The existence of two stages of alcohol-induced transition was also confirmed by the fluorescence of the single Trp59. Figure 4 illustrates the methanol dependence of fluorescence intensity at the maximum of the fluorescence spectrum ($\lambda_{max}$). The figure shows that the increase of fluorescence consists of two stages. The first stage almost coincides with the loss of protein tertiary structure monitored by $[\theta]_{280}$ (see Figures 1 and 3). The second stage occurs at methanol concentrations similar to those corresponding to the increase of the protein secondary structure (see Figures 2 and 3). It should be mentioned that the first stage is accompanied by a negligible red shift of $\lambda_{max}$ as compared with the native value (339 nm), while the second stage is coupled with a small blue shift of $\lambda_{max}$ to 334 nm (at 80% methanol).

It is well-known that the fluorescence of Trp59 in cytochrome *c* is a very sensitive test for protein unfolding [see, e.g., Elöve et al. (1992)] because in the folded state this fluorescence is very strongly quenched by a heme (Tsong, 1974, 1976; Roder et al., 1988). Upon complete or partial unfolding of a protein, the distance between Trp59 and heme increases, and removing tryptophan from the heme leads to reduction of the heme quenching of Trp59 fluorescence, i.e., to the enhancement of fluorescence intensity. Two stages of this enhancement suggest that both stages of protein unfolding monitored by CD spectra contribute to this process.

*Intermediate State*. Two stages of the methanol-induced transition in cytochrome *c* documented by Figures 3 and 4 are well resolved, and this suggests the presence of almost a 100% population of an intermediate state. This 100% population corresponds to 40% methanol at pH 4.0 in 0.5 M and 0.15 M NaCl and shifts to 50% methanol at low ionic strength (0.006 M sodium acetate).

The physical properties of this intermediate state can be described as follows:

*Tertiary Structure*. Figure 5 presents a comparison of the near-UV CD spectra for the intermediate state in 40% methanol with spectra for native and unfolded proteins as well as for the pH-induced molten globule state of cytochrome *c* in water solution, described by Ohgushi and Wada (1983). One can see that the near-UV CD spectrum of the alcohol-induced intermediate state is similar to that of the pH-induced molten globule in water. Both have no "native" negative bands between 280 and 290 nm, and both have a relatively narrow positive band around 260 nm instead of a broad band at 250−270 nm in the native protein. This

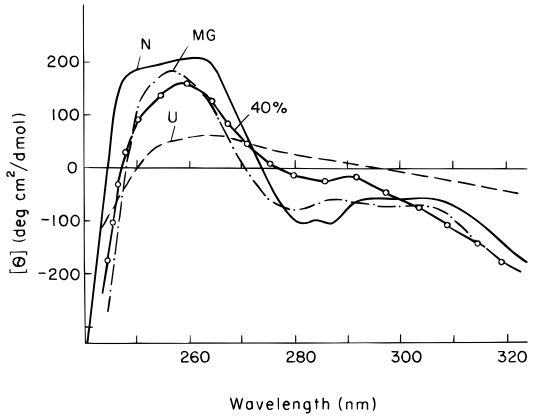


FIGURE 5: Near-UV CD spectrum of the alcohol-induced intermediate state of cytochrome *c* (pH 4.0 in 0.5 M NaCl at 40% methanol) as compared with spectra in the native (N) state (pH 4.0 in 0.5 M NaCl), the unfolded (U) state (in 7 M GdmCl), and in the pH-induced molten globule (MG) state (pH 2.0 in 0.5 M NaCl). The near-UV CD spectrum at pH 2.0 without NaCl (not shown) is very close to that in 7 M GdmCl. All spectra were recorded at 24 °C.

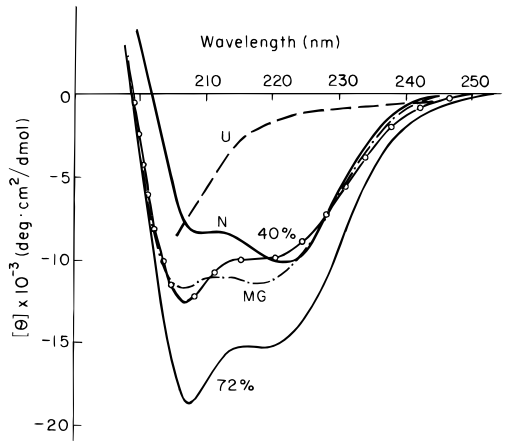


FIGURE 6: Far-UV CD spectra of cytochrome *c* in 40% and 72% methanol as compared with those of the native (N) state, the unfolded (U) state, and in the pH-induced molten globule (MG) state. Experimental conditions are the same as in Figure 5.

suggests that both these states may share similar traces of the native tertiary structure, which completely disappear only in the unfolded state. Near-UV CD spectra of the pH-induced molten globule in cytochrome *c* have been observed by Goto and Nishikiori (1991) and by Kuroda et al. (1992) at pH 1.8 in 0.5 M KCl and at pH 2.0 in 0.5 M KCl, respectively. These spectra are not identical to our spectrum observed at pH 2.0 in 0.5 M NaCl, but all these spectra share common features—the existence of a positive band at 260 nm and the absence of fine structure at 280−290 nm.

*Secondary Structure*. The far-UV CD spectrum of cytochrome *c* in the methanol-induced intermediate state is compared in Figure 6 with spectra of this protein in the native and unfolded states as well as in the pH-induced molten globule state in aqueous solution. The figure shows that the far-UV CD spectra of cytochrome *c* in 40% methanol and in the pH-induced molten globule state are similar to each other and different both from the native and from the high methanol content states.

*Compactness*. Hydrodynamic dimensions of cytochrome *c* in the methanol-induced intermediate state have been measured by translational diffusion. The results are presented in Table 1. In this method, the diffusion coefficient $D$ is measured as the slope of the time dependence of dispersion, $\sigma_i^2 = 2D_i t$, for each component (Tsvetkov & Klenin, 1953). It should be noted that for cytochrome *c* in 40% methanol this curve has a remarkable downward curvature showing the presence of slowly moving associates. The presence of associates can influence even the apparent initial slope of this curve, which may present the data averaged over a monomeric protein and its associates. Therefore, the initial slope in this case can only give the minimal value of the diffusion coefficient, i.e., the maximal value of the Stokes radius (see eq 2). Thus, we conclude that the Stokes radius of the methanol-induced intermediate cannot be larger than 21 Å. This is close to the value for the molten globule state in aqueous solution (19 Å) and lies between the values for the native (15 Å) and unfolded (28 Å) states.

Table 1: Molecular Dimensions of Cytochrome *c* in Different Conformational States

| state | conditions | diffusion coefficient, $D_{20,w} \times 10^7$ ($cm^2/s$) | Stokes radius, $R_s$ (Å) | $D^N_{20,w}/D_{20,w}$ |
|---|---|---|---|---|
| native | pH 4; 0.15 M NaCl | 14.0 | 15 | (1.00) |
| molten globule | pH 2; 0.5 M NaCl | 11.5 | 19 | 1.22 |
| methanol-induced intermediate | pH 4; 0.15 M NaCl, 40% methanol | >10.2 | <21 | <1.37 |
| unfolded | pH 2; $H_2O$−HCl | 7.7 | 28 | 1.82 |

*Temperature Melting*. Denatured proteins usually have no cooperative temperature melting. However, cytochrome *c* is an exception—its pH-induced molten globule state shows cooperative temperature melting with the temperature of the transition $T_m = 50$ °C and the melting enthalpy $\Delta H \sim 35$ kcal/mol (Potechin & Pfeil, 1989; Hagihara et al., 1994; Hamada et al., 1994). Temperature melting of cytochrome *c* in this state was also monitored by CD spectra. In this case, there is a significant decrease of intensity for both near- and far-UV CD spectra (data not shown). Similar results were obtained by Kuroda et al. (1992).

We have studied the temperature melting of cytochrome *c* at different concentrations of methanol. Figure 7 presents these data. First of all, the temperature dependence of the excess heat capacity, $C_p(T)$ (see Figure 7A), shows that both $T_m$ and $\Delta H$ decrease with increasing methanol concentration (Figure 7B,C), emphasizing that methanol destabilizes the native structure of cytochrome *c*. Comparison of Figure 7A with the dependency of the near-UV CD on methanol content (see Figures 1 and 3) shows that cytochrome *c* remains native at 24 °C if the methanol content does not exceed 20%. Thus, $C_p(T)$ curves at 0, 10, and 20% methanol reflect the melting of the native protein.

At higher methanol concentrations, cytochrome *c* is denatured by ~75% at 30% methanol and practically by 100% at 35 and 40% methanol at 24 °C. Therefore, $C_p(T)$ curves at 35 and 40% reflect the melting of "denatured" cytochrome *c*, i.e., of cytochrome *c* without a rigid tertiary structure around Trp59, though probably with some traces of tertiary structure in the neighborhood of the heme. Surprisingly enough, our measurements of the temperature dependence of the near-UV CD spectrum at 40% methanol showed that this spectrum practically does not change upon the increase of temperature from 10 to 40 °C, i.e., almost in the whole interval of "temperature melting". It means that

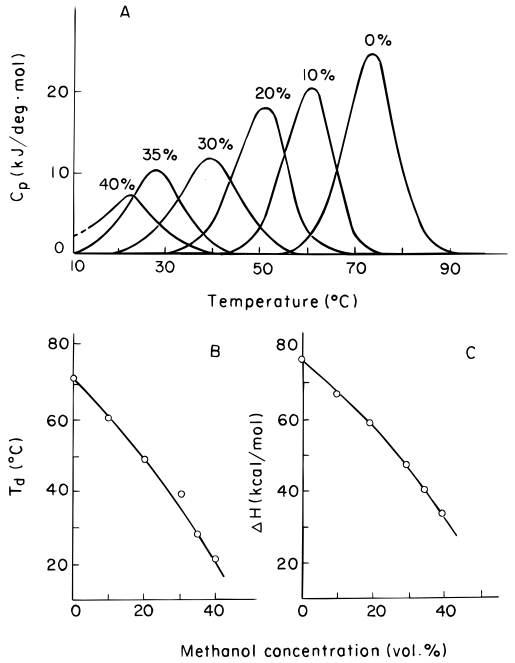


FIGURE 7: (A) Excess heat capacity curves *versus* temperature for cytochrome *c* at pH 4.0 in 0.15 M NaCl at different methanol concentrations (shown at corresponding curves). (B) Dependence of the melting temperature ($T_m$) on the methanol concentration. (C) Dependence of the calorimetric melting enthalpy ($\Delta H^{cal}$) on the methanol concentration.

the influence of tertiary structure on the CD of Trp59 is already lost at 10 °C, while its influence on the CD of the heme around 260 nm is still present at 40 °C. The far-UV CD spectrum also does not change strongly in this temperature interval.

These results may help one to understand the structural nature of the exceptional temperature melting of the denatured cytochrome *c*, though many more experiments are needed to clarify this point. Nevertheless, the alcohol-induced intermediate of cytochrome *c*, likely its pH-induced molten globule state, is shown to have temperature melting. It should also be mentioned that for the temperature melting of both intermediates the calorimetric $\Delta H$ is equal to the van't Hoff $\Delta H$ values within experimental error; i.e., this melting is an "all-or-none" process (Privalov, 1979).

## DISCUSSION

We have shown that there are two different alcohol-induced denatured forms of cytochrome *c*: the "final" form stable at high alcohol concentrations and the intermediate form, which is stable at moderate alcohol concentrations.

Proteins denatured by high alcohol concentrations have been studied by many authors (Weber & Tanford, 1959; Tanford et al., 1960; Tanford, 1968; Timasheff, 1970; Wilkinson & Mayer, 1986; Buck et al., 1993; Dufour et al., 1993; Fan et al., 1993; Alexandrescu et al., 1994; Ptitsyn et al., 1995; Shiraki et al., 1995), and the general result is that all of them have a more pronounced far-UV CD as compared with the native proteins (or the molten globule state), suggesting a substantial increase of their helicity. This conclusion has been supported by NMR data which show that some $\beta$-structural or irregular chain regions of the native protein become helical upon their alcohol-induced denaturation (Stockman et al., 1993; Buck et al., 1993; Alexandrescu et al., 1994).

There are also some observations of intermediate states of proteins upon their alcohol denaturation. Tanford and his collaborators (Weber & Tanford, 1959; Tanford et al., 1960) have demonstrated the existence of an extremum of optical properties of some proteins at moderate alcohol concentrations. This means the existence of at least two stages of alcohol-induced denaturation. The first stage has been described as "an unfolding or partial unfolding of the native structure", while the second one as "a folding to the new conformation" (Tanford, 1968).

The existence of an intermediate in alcohol-induced protein denaturation was supported by Timasheff (1970). He showed that the near-UV CD spectra of lysozyme and some other proteins change at smaller alcohol concentrations than their far-UV CD spectra, which suggests that small conformational changes precede the large ones.

Wilkinson and Mayer (1986) have observed two comparable stages of the increase of the far-UV CD spectrum for ubiquitin and have explained this by the separate denaturation of two protein domains, accompanied with the increase of their helicity.

Two stages of a methanol-induced transition in cytochrome *c* observed in this paper hardly can be explained by domain melting since circular dichroism shows only one methanol-induced transition in far-UV region (as well as in the near-UV region).

The alcohol-induced intermediate state of cytochrome *c* revealed in this paper met all the requirements of the molten globule state [see, e.g., Ptitsyn (1992)]—it has a strongly reduced tertiary structure but has a pronounced secondary structure and is compact. Moreover, both CD spectra and hydrodynamic dimensions of the methanol-induced intermediate are similar to those of the pH-induced molten globule state.

There is a large difference between "final" denatured states of cytochrome *c* in the presence of strong denaturants and in mixtures of water with organic solvents: unfolded chain in GdmCl or urea and highly helical state in alcohols. However, the *intermediate states* in both these types of denaturation are rather similar and have the main properties of the molten globule state.

The transformation of the native state of cytochrome *c* into the molten globule-like state at moderately low pH and intermediate concentrations of methanol can be compared with the transition of the same protein from the native to a much more flexible state in the presence of phospholipid vesicles (de Jongh et al., 1992). This state has the far-UV CD spectra even more pronounced than the native state, but its deuterium exchange is much faster than that in the native state and its stability against urea and cooperativity of urea-induced unfolding are substantially less than those of native molecules. Analysis of these experimental data has led to the conclusion that surfaces of phospholipid vesicles transform cytochrome *c* into the molten globule state (Bychkova & Ptitsyn, 1993). An interesting but not solved problem is whether the molten globule state of cytochrome *c* is relevant to its functioning in the respiratory chains of mitochondria.

Thus, we have shown that the state of cytochrome *c* near membrane surfaces can be mimicked in simple artificial systems—in water−alcohol mixtures with moderately low pH. These systems simulate both denaturing actions, suggested for membrane surfaces: local decrease of pH and local decrease of the dielectric constant.

We consider the results described above as the first evidence that proteins can achieve their molten globule state under conditions which may mimic those near membrane surfaces.

## ACKNOWLEDGMENT

We thank K. S. Vassilenko for help in CD measurements and H. Roder for valuable advice.

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BI9522460