Effect of Trehalose on Alkaline Transition of Cytochrome-c

Adalberto Bonincontro,*,† Emanuele Bultrini,† Vania Calandrini,† Stefania Cinelli,‡ and Giuseppe Onori‡

INFM-Dipartimento di Fisica, Università " La Sapienza ", Piazzale A. Moro 2, 00185 Roma, Italy, and INFM-Dipartimento di Fisica, Università di Perugia, Via A. Pascoli, I-06123 Perugia, Italy

Received: November 8, 1999; In Final Form: March 30, 2000

This work deals with a study of alkaline transition of ferricytochrome-c in water and in water−trehalose solutions using two different techniques: spectrophotometry in the region of the heme absorption spectrum and dielectric spectroscopy at radio frequencies. The first is a local probe and provides qualitative information on the identity of axial ligands to the heme iron. The second is able to reveal small variations in conformation and/or hydration state of the protein by two parameters: electric dipole moment and effective hydrodynamic radius. Optical absorption measurements of cytochrome-c in water as a function of pH show, in the basic range, a transition of the protein toward a partially denatured state, characterized by a displacement of methionine-S−Fe linkage in the heme. Dielectric measurements show that a simultaneous increase of the hydrodynamic radius and a decrease of the electric dipole moment of the protein accompany this alkaline transition. Thus, in water, conformational changes of cytochrome at basic pH result both in local structural changes in the heme pocket and in more global changes of the protein structure. In the water−trehalose solutions, optical measurements reveal a transition similar to that in water. On the contrary, dielectric parameters keep constant in the same pH range. Despite the displacement of the methionine-S−Fe linkage in the heme, the presence of trehalose stabilizes the global conformation of protein. This is coherent with the role of trehalose as bioprotector.

Introduction

The characterization of conformational transitions and folding intermediates in proteins is central for the study of protein folding and principles of structure stabilization.[1] Horse cytochrome-c is a well-characterized globular protein both in the crystalline state and in solution, and it was a very useful model for numerous folding studies.[2] At neutral pH, cytochrome-c has two strong field protein ligands, an imidazole nitrogen of histidine-18 and a sulfur of methionine-80, coordinated in the axial position of the heme iron. Axial ligation to the heme iron is sensitive to changes in pH, temperature, ionic strength, and solvent composition. In particular, the nature of alkaline transition, observed on raising the pH to alkaline values, has been the process of considerable interest in the last years, especially in connection with the role of conformational change in electron transfer.[3−6] The bulk of spectroscopic evidence suggests that the "alkaline" form of the protein possesses a six-coordinate, low-spin heme iron that retains the native imidazole ligand of His 18, while the sulfur atom of Met 80 is replaced by a strong-field ligand, most likely a lysine amino group. The conversion of the protein from the "neutral" to the "alkaline" form does not appear to involve any gross structural reorganization.[7]

This work deals with a study of alkaline transition of ferricytochrome-c in water and in water−trehalose solutions using two different techniques: spectrophotometry in the region of the heme absorption spectrum and dielectric spectroscopy at radio frequencies. The first is a local probe and provides qualitative information on the identity of axial ligands to the heme iron. The second is able to reveal small variations in conformation and/or hydration state of the protein by two parameters: electric dipole moment and effective hydrodynamic radius. Therefore, our approach allows obtaining information on the relationship between local structure and overall conformation.

Trehalose is a diglucose sugar that confers to certain plant and animal cells the ability to survive to dehydration.[8,9] Other sugars, such as maltose, sucrose, and glucose, have shown similar properties to protect against dehydration, but trehalose is significantly more effective.[10]

Although the role of trehalose as a bioprotector is well established and widely reported in the literature, the molecular processes involved remain unclear. Some of the evidence seems consistent with the hypothesis that the above effect is due to replacement of the main hydration shell of the polar groups in, for example, proteins and phospholipids, by OH groups of trehalose.[11,12] Some researchers, however, have suggested that the ability of the sugar to form a glass is essential for the macromolecule stabilization.[13−15]

Recently we used the dielectric technique to study lysozyme in solution under different conditions of pH, temperature, and solvent composition.[16−18] As a development of our research line on the role of solvent in protein conformation and dynamics we decided to investigate the cytochrome-c in water and in water−trehalose solutions at the fixed temperature of 25 °C, varying pH and sugar concentration. We focus on how the constraints imposed by the trehalose matrix influence the alkaline transition of cytochrome-c, looking both at local structural changes in the heme pocket and at more global changes of protein conformation.

* Corresponding author FAX NUMBER: 39 06 4463158 E-Mail: BONINCONTRO@AXRMA.UNIROMA1.IT

† Università "La Sapienza".

‡ Università di Perugia.

10.1021/jp993948s CCC: $19.00
Published on Web 06/30/2000

Materials and Methods

Cytochrome-c (from horse heart) was obtained from Sigma and it was used without ulterior purification. This could give some problems for the presence of impurities, usually of the order of few % w/w. We think, however, that these do not influence significantly the dielectric relaxation exhibited by the protein. A confirmation of this is the good agreement of the values estimated for hydrodynamic radius and dipole moment with literature data (see Results section). The presence of a small fraction of proteins in reduced form could be another ulterior problem. It has to be considered, however, that treatments with oxidizing agents, such as ferricyanide, for example, are strongly discouraged because it is very difficult to remove completely the reagent with an extensive dialysis.[19] Trehalose was a generous gift by Prof. L. Cordone of Palermo University. The solutions were prepared by weighing. Two concentrations of cytochrome, 5 and 10 mg/mL, were employed. The ionic strength of the samples was equivalent to a 2−4 mM NaCl solution, as deduced from conductivity measurements.

The pH was measured by a Crison micropH 2000. Viscosity of water−trehalose solutions was measured by a Rheolab MC1 with measuring system Z1 DIN.

The variations of pH were obtained by adding negligible volumes of concentrated acid or basic solutions. Therefore, the protein concentration was substantially unaltered.

In dielectric experiments permittivity ϵ' and dielectric loss ϵ'' were measured by means of a computer controlled Hewlett-Packard impedance analyzer, model 4194A, in the frequency range 10^5-10^8 Hz. The measuring cell, previously described,[20] is a section of a cylindrical waveguide, which can be partially filled with the sample solution. The system behaves as a waveguide excited far beyond its cutoff frequency mode, and therefore only the stray-field of the coaxial line−waveguide transition is used in the measurement. Cell constants were determined by measurements with electrolyte solutions of known conductivity, following well-defined procedures of literature.[21,22] The errors on $\epsilon'$and ϵ'' are within 1%.

A JASCO V-750 spectrophotometer with cells of 1 mm light path was used for the optical measurements. Both dielectric and optical experiments were performed at 25.0 ± 0.1 °C.

Results and Discussion

Spectrophotometric Measurements. A pH titration was carried out on a water−protein solution from pH = 4−7 (native state) to pH = 12 (denatured state). The protein concentration was 10 mg/mL. A more dilute solution (protein concentration 1 mg/mL in 1 cm length cuvette) was also measured (data not shown). No difference was evident.

In Figure 1 the absorption spectra for the native (ϵ_n) and denatured (ϵ_d) state of the protein in water in the range 450−750 nm are reported. At neutral pH (native state) the visible absorption spectrum of ferricytochrome-c has a maximum at 530 and 695 nm. In the denatured state (pH = 12) the absorption at 530 nm is notably lowered and the band at 695 nm completely disappears. The 695-nm absorbance band has been associated with the methionine-80−sulfur-heme iron bond of cytochrome-c.[23] This absorbance band is not present in the spectrum of cytochrome-c if the methionine-80 is lost as the sixth ligand.

All of the spectra $\epsilon(\lambda, \text{pH})$ in the pH range from 4 to 12 present isosbestic points at λ = 501, 515, and 600 nm. The presence of isosbestic points is a strong argument in favor of the existence of equilibrium between two optically absorbing species with band shapes and extinction coefficients insensitive to pH. This is in agreement with the known models of globular protein unfolding. In fact the protein denaturation is currently considered as a two-state conformational transition, where only native and unfolded states are significantly populated.[24] According to this, we verified that the $\epsilon(\lambda, \text{pH})$ spectrum can be accurately expressed, for any value of pH, as a linear combination

$$\epsilon = f_n\epsilon_n + f_d\epsilon_d \qquad (1)$$

where ϵ_n and ϵ_d are the spectra, f_n and f_d are the molar fractions ($f_n + f_d = 1$) of molecules in native and denatured state, respectively. The values of the fraction (f_d) of molecules in the denatured state, estimated for any pH value of solution are reported in Figure 2. These data were fitted to the equation $f_d = (1+10^{\,n(pK-pH)})^{-1}$ based on an equilibrium with a constant K between two states of the protein which differ by n protons. The continuous line, superimposed on calculated values of f_d, is the result of this best-fit procedure, which gives $n \cong 1$ and $pK \cong 9$. This result is in a good agreement with literature data, obtained by the same optical probe[7] or by other spectroscopic probe.[2]

An identical experiment was performed in the presence of trehalose (35% w/w). The optical spectrum of native state is not modified as respect to that in water. Moreover, the behavior

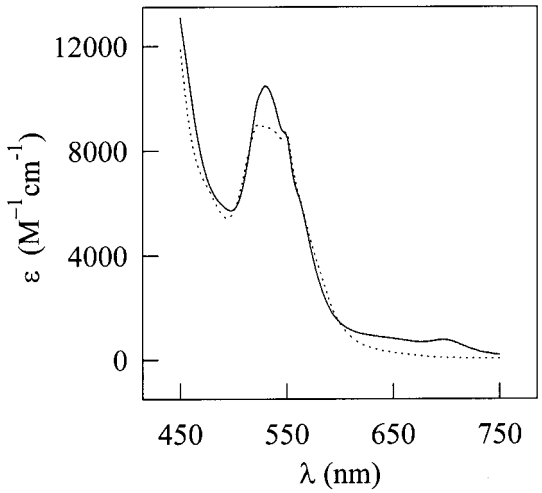

Figure 1. Molar extinction coefficient, ϵ, of cytochrome-c in water, in native (—) and denatured (...) state. Concentration 10 mg/mL. Temperature 25 °C.

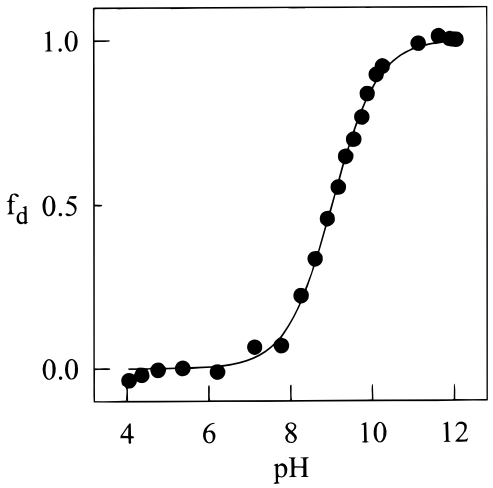

Figure 2. Molar fraction of denatured cytochrome-c, f_d, obtained by optical absorption spectra in aqueous solution, as function of pH. The solid line is a best fit based on a two-state model.

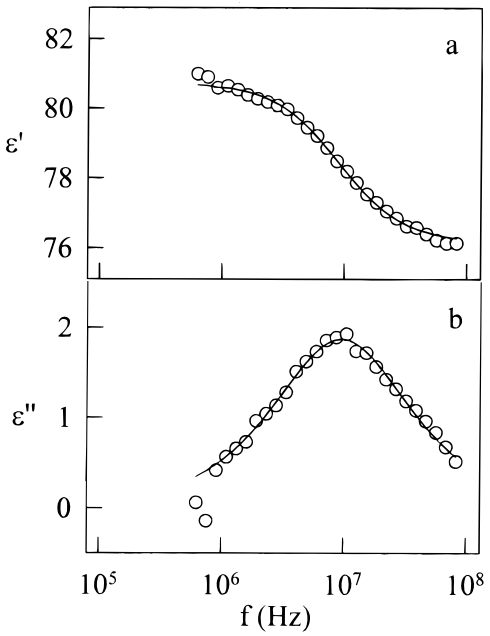

Figure 3. (a) Permittivity ϵ' and (b) dielectric loss ϵ'' of cytochrome-c aqueous solution (10 mg/mL) as function of frequency. Temperature 25 °C. The solid line is a Cole−Cole fit.

for higher pH values was the same, within the errors, of that obtained in absence of the disaccharide. Therefore, the presence of trehalose does not seem to alter the native state of the protein and its alkaline transition, observed as modifications of heme group.

The alkaline transition of cytochrome, which seems to follow a simple two-state model, is really more complicated. In fact, NMR studies[4] reveal that the alkaline form is not single species. However, this is not important for our objective consisting in a comparison between data monitored by a local and an overall conformation probe.

Dielectric Measurements in Water−Protein Solution. We measured permittivity ϵ' and dielectric loss ϵ'' of cytochrome-c dissolved in water in the frequency range 10^5-10^8 Hz, varying the pH of the solution. The variation of pH was within the interval 4−10. Values of pH higher than 10 produced a high electric conductivity that did not allow a good estimation of relaxation curve. The concentration of the protein (5 and 10 mg/mL in two repeated experiments) was low, but sufficient to ensure a good signal-to-noise ratio. The coincidence, within the errors, of the results for the samples with two different protein concentrations demonstrates that the solute−solute interactions produce negligible effects at these concentrations. At the frequencies used in the experiment, the cytochrome solution showed a well-defined dielectric dispersion due to the orientation polarization of the dipole moment of the protein (see Figure 3). The experimental data were fitted with the real part and imaginary part of the Cole−Cole equation[25]

$$\epsilon = \epsilon_\infty + \frac{\Delta\epsilon}{1 + i\left(\frac{f}{f^*}\right)^{(1-\alpha)}} \tag{2}$$

where ϵ is the complex dielectric constant, f is the measuring frequency, f^* is the relaxation frequency, i is the imaginary unit, $\Delta\epsilon$ is the dielectric increment, ϵ_∞ is the permittivity extrapolated at high frequency, and α is an empirical parameter taking into account a spread of relaxation times. The electric dipole moment μ of the cytochrome molecule was estimated from the dielectric

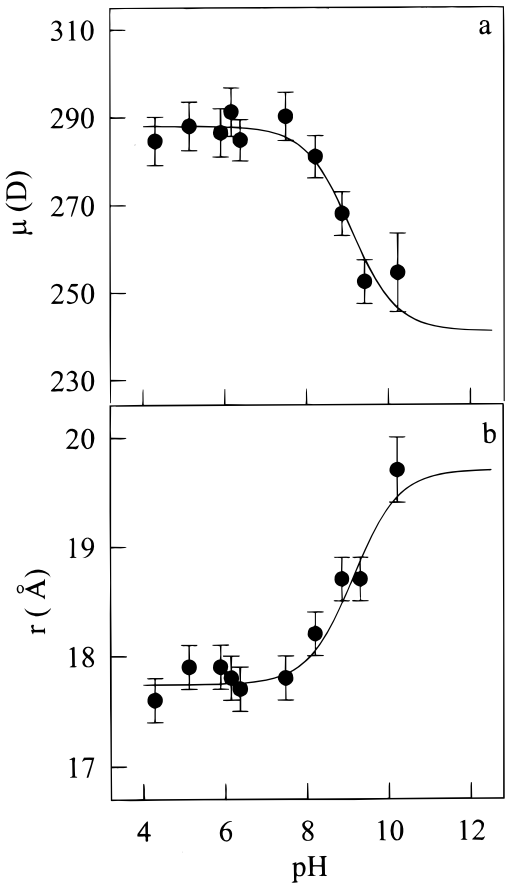

Figure 4. (a) Electric dipole moment μ and (b) effective hydrodynamic radius r of cytochrome-c in aqueous solution, as function of pH. The solid lines represent the best fit to eq 6 and 7 of text, respectively. The error bars are the result of a statistical analysis connected to the best fit procedure employed to analyze the experimental data.

increment $\Delta\epsilon$ using the Oncley formula[26]

$$\mu^2 = \frac{2\epsilon_0 MKT\Delta\epsilon}{Ncg} \tag{3}$$

where M is the protein molecular mass expressed in kilodalton, T is the temperature expressed in Kelvin, K is the Boltzmann constant, ϵ_0 is the vacuum dielectric constant, N is the Avogadro number, c is the protein concentration expressed in kg/m^3, and g is the molecular correlation parameter generally assumed as 1 in dilute protein solutions.[27]

The effective hydrodynamic radius r of the globular protein, assumed as a sphere, was calculated from the relaxation frequency f^* using the equation

$$f^* = \frac{KT}{8\pi^2\eta r^3} \tag{4}$$

where η is the viscosity of the solvent.[28]

The values of μ and r are shown as functions of solution pH in Figure 4. As it can be seen, μ and r are stable in a pH range approximately up to 7, but for basic pH we observe a decrease of dipole moment and an increase of effective hydrodynamic radius, while the optical absorption measurements evidence a clear two-state transition of the macromolecule. For such a two-state system, the dielectric response is the overlap of two distinctive, but very close relaxation processes, due to native and denatured molecules. In the case of noninteracting particles, the electrical polarization may be expressed in terms of the

single polarizations

$$\alpha = \sum_i f_i \alpha_i \tag{5}$$

where f_i are the molar fractions of the different species. The polarization is proportional to 2 power of electric dipole moment. Therefore, the evaluated μ at any pH may be related to the dipole moments of native and denatured molecule by the following formula:

$$\mu = [(1 - f_d)\mu_n^2 + f_d\mu_d^2]^{1/2} \tag{6}$$

where μ_n and μ_d are the dipole moments relative to the native and denatured states, respectively.

We fitted the μ experimental values to eq 6 using for f_d the values estimated by optics experiments. The continuous line in Figure 4a represents the results of the fitting procedure, which gives for the dipole moments associated to the native and denatured species the values $\mu_n = 288$ D and $\mu_d = 241$ D, respectively. Good agreement is observed, within the errors, of the experimental data with the fit curve.

The effective hydrodynamic radius as function of solvent pH was calculated by eq 4 using the experimental relaxation frequency f^*. On the basis of the two-state model, f^* is related to the relaxation frequencies of native and denatured proteins present in the sample. These frequencies seem be very close as it appears by comparing the relaxation frequencies estimated at the extreme pH values, where one species is predominant (the highest difference is about 30%). In this condition, we suppose a linear combination of the relaxation frequencies. The same approach is used in measurements of translational diffusion coefficient of proteins in solution by dynamic light scattering technique.[29,30] With this hypothesis, the following equation for the hydrodynamic radius is obtained

$$r = \frac{r_n r_d}{(r_d^3 + f_d(r_n^3 - r_d^3))^{1/3}} \tag{7}$$

where r_n and r_d are the hydrodynamic radii of the native and denatured species, respectively.

The values of r as functions of pH were fitted to eq 7, using for f_d the values estimated by optics experiments. The continuous line superimposed in Figure 4b to the experimental values represents the results of the fit, which give for the hydrodynamic radius of the protein in native and denatured states the values $r_n = 17.7$ Å and $r_d = 19.7$ Å, respectively. Also, here the experimental data are in good agreement, within the errors, with the fit curve. The radius increase is small compared with those observed in denaturation processes induced by temperature or denaturing agents.[29] This means that our effect caused by basic pH is expressive of a partially unfolded process related to displacement of the methionine-S−iron linkage.

Dielectric Measurements in Water−Trehalose−Protein Solution. Dielectric measurements were carried out on the protein dissolved in water−trehalose solution at different concentrations of the sugar. The pH of the solution was fixed at 6.5 (native state of the protein in water). The presence of the trehalose increases the viscosity of the solution and consequently the relaxation frequency of protein polarization migrates toward lower values. The greatest concentration of sugar realized was 35% w/w. In fact, at higher concentrations the dielectric relaxation curve is partially out of our frequency window. For any trehalose concentration the solvent viscosity was measured (the results are reported in Table 1). The dipole moment and hydrodynamic radius were therefore evaluated. The results are shown in Figure 5. An effect consisting of an increase of r and simultaneously decrease of μ is evident. On the other hand, optics experiments showed that the local structure of native protein (pH = 6.5) is not altered by the presence of sugar. We can therefore suppose that the effect on overall conformation of the protein, not expressive of an internal structural change, consists of a variation of protein hydration. For example, we can hypothesize that interactions between water and protein are replaced by interactions between sugar and protein. This might explain the increase of hydrodynamic radius with the rise of sugar concentration in solution; these new interactions would also probably cause a local redistribution of charge and consequently a variation of dipole moment, compatible with the one we observed. It is certainly curious that the presence of trehalose produces approximately the same quantitative effect on dipole moment than the increase of pH in the basic region. Dielectric data alone do not allow giving a convincing interpretation.

Next, we performed dielectric measurements on water−trehalose−protein solution (35% w/w of sugar), varying the pH

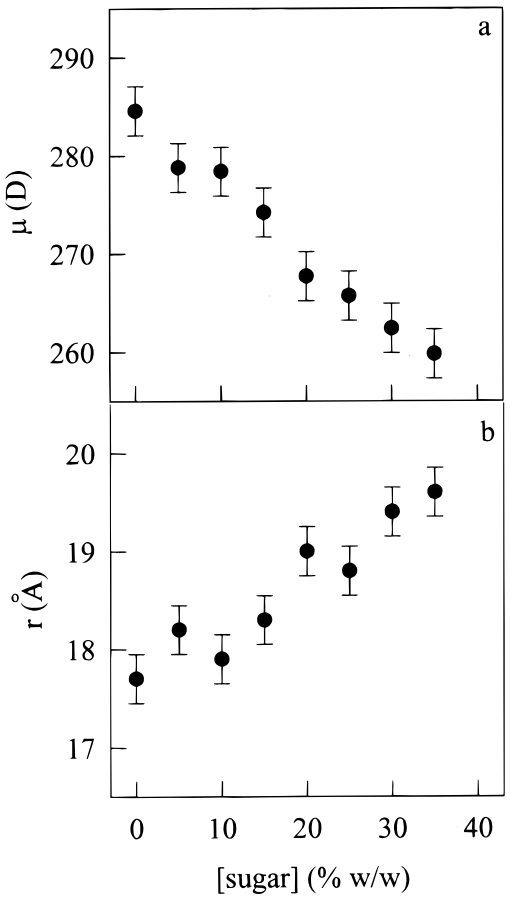

Figure 5. (a) Electric dipole moment μ and (b) effective hydrodynamic radius r of cytochrome-c in water−trehalose solution as function of sugar concentration. Temperature 25 °C, pH = 6.5. The error bars are the result of a statistical analysis connected to the best fit procedure employed to analyze the experimental data.

TABLE 1: Viscosity of Trehalose−Water Solutions at 25 °C

trehalose (% w/w)	viscosity (cp ± 0.01)	trehalose (% w/w)	viscosity (cp ± 0.01)
0	0.89	20	1.80
5	1.01	25	2.29
10	1.26	30	2.84
15	1.41	35	3.5

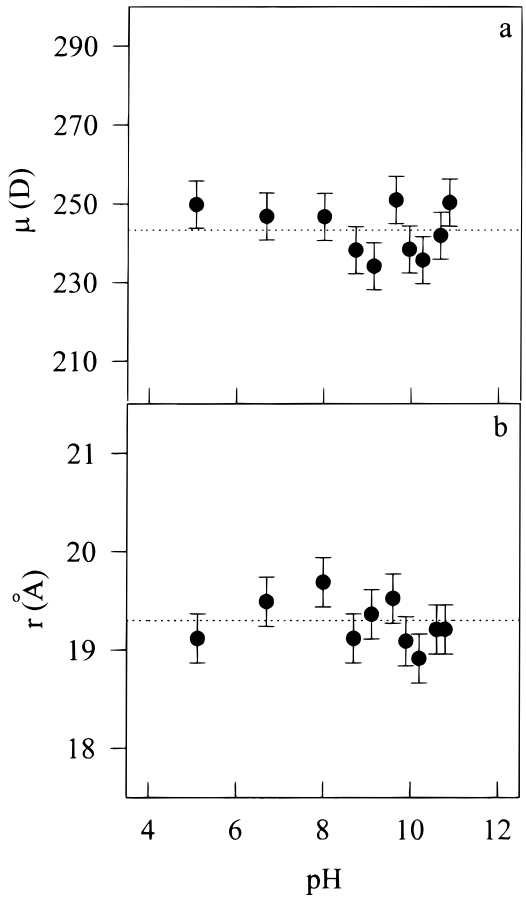

Figure 6. (a) Electric dipole moment μ and (b) effective hydrodynamic radius r of cytochrome-c in water−trehalose solution as function of pH. Sugar concentration 35% w/w. Temperature 25 °C. The error bars are the result of a statistical analysis connected to the best fit procedure employed to analyze the experimental data. The dotted lines are reported only for demonstration.

from 5 to 11. It was possible to carry out dielectric measurements up to pH 11 because in water−trehalose solution ion mobility and consequently electrode polarization are reduced. The dielectric parameters μ and r, obtained as before, are constant within the errors (see Figure 6), while optical measurements in the presence of trehalose show a perturbation in local structure characterized by the displacement of methionine-S−iron linkage in the heme group. This implies that the global conformation of protein, stabilized by interaction with the sugar, is insensitive to this effect in the heme group. All this seems coherent with the peculiar properties of trehalose in protecting living subjects against extreme dehydration conditions.

Conclusions

The main results obtained in this research can be summarized as follows.

In water: Spectrophotometry and dielectric spectroscopy probe the same transition in cytochrome. Conformational changes of cytochrome at basic pH result both in local structural changes in the heme pocket and in more global changes of the protein structure. The heme group with its axial ligands seems essential for stabilization of overall structure and conformation of cytochrome-c in water.

In water−trehalose: Basic pH conditions influence cytochrome only in local structural changes in the heme pocket. In fact, optical measurements revealed a transition similar to that in water, but dielectric parameters keep constant in the same pH range. Despite the displacement of the methionine-S−Fe linkage in the heme, the presence of trehalose stabilizes the global conformation of protein. This is coherent with the role of trehalose as bioprotector.

Acknowledgment. This work is part of a project co-financed by the European Community (European Fonds for Regional Development).

References and Notes

(1) Kim, P. S.; Baldwin, R. L.; *Annu. Rev. Biochem.* **1990**, *59*, 631.
(2) Scott, R. A.; Mauk, A. G., Eds. *Cytochrome-c: A Multidisciplinary Approach*; University Science Books: Sausalito, CA, 1996.
(3) Wilson, M. T.; Greenwood, C. In *Cytochrome-c: A Multidisciplinary Approach*; Scott, R. A., Mauk, A. G., Eds.; University Science Books: Sausalito, CA, 1996; p 611.
(4) Döpner, S.; Hildebrandt, P.; Rosell, F. I.; Mauk, A. G. *J. Am. Chem. Soc.* **1998**, *120*, 11246.
(5) Rosell, F. I.; Ferrer, J. C.; Mauk, A. G. *J. Am. Chem. Soc.* **1998**, *120*, 11234.
(6) Weber, C.; Michel, B.; Bosshard, H. R. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 6687.
(7) Greenwood, C.; Wilson, M. T. *Eur. J. Biochem.* **1971**, *22*, 5.
(8) Womersley, C.; *Comput. Biochem. Physiol.* **1981**, *70B*, 669.
(9) Crowe, J. H.; Crowe, L. M.; Carpenter, J. F.; Wistrom, C. A. *Biochem. J.* **1987**, *242*, 1.
(10) Crowe, J. H.; Crowe, L. M. *Biol. Membr.* **1984**, *5*, 57.
(11) Crowe, L. M.; Mouradian, R.; Crowe, J. H.; Jackson, S. A.; Womersley, C. *Biochim. Biophys. Acta* **1984**, *769*, 141.
(12) Carpenter, J. F.; Crowe, J. H. *Biochemistry* **1989**, *28*, 3916.
(13) Green, J. L.; Angell, C. A. *J. Phys. Chem.* **1989**, *93*, 2880.
(14) Levine, H.; Slade, L. *BioPharm.* **1992**, *5*, 36.
(15) Aldous, B. J.; Auffret, A. D.; Franks, F. *Cryo-Lett.* **1995**, *16*, 181.
(16) Bonincontro, A.; De Francesco, A.; Matzeu, M.; Onori, G.; Santucci, A. *Colloids Surf. B* **1997**, *10*, 105.
(17) Bonincontro, A.; De Francesco, A.; Onori, G. *Colloids Surf. B* **1998**, *12*, 1.
(18) Bonincontro, A.; De Francesco, A.; Onori, G. *Chem. Phys. Lett.* **1999**, *301*, 189.
(19) Goto, Y.; Takahashi, N.; Fink, A. L. *Biochemistry* **1990**, *29*, 3480.
(20) Bonincontro, A.; Briganti, G.; Giansanti, A.; Pedone, F.; Risuleo, G. *Colloids Surf. B* **1996**, *6*, 219.
(21) Bottomley, P. A. *J. Phys. E: Sci. Instrum.* **1978**, *11*, 413.
(22) Athey, T. W.; Stuckly, M. A.; Stuckly, S. S. *IEEE Trans. MTT* **1982**, *30*, 82.
(23) Shechter, E.; Saludjian, P. *Biopolymers* **1967**, *5*, 788.
(24) Privalov, P. L. *Adv. Protein Chem.* **1979**, *33*, 167.
(25) Hasted, J. B. *Aqueous Dielectrics*; Chapman & Hall, London, 1973. Originally reported by Cole, K. S.; Cole, R. H. *J. Chem. Phys.* **1949**, *9*, 341.
(26) Pethig, R. *Dielectric and Electronic Properties of Biological Materials*; Wiley: New York, 1979. Originally reported by Oncley, J. L. *Chem. Rev.* **1942**, *30*, 433.
(27) Pethig, R.; Kell, D. B. *Phys. Med. Biol.* **1987**, *32*, 933.
(28) Grant, E. H.; Sheppard, R. J.; South, G. P. *Dielectric behaviour of biological molecules in solution*; Clarendon: Oxford, 1978. Original model in Debye, P., Ed., Polar Molecules, Chemical Catalog Co., New York, 1929.
(29) Dubin, S. B.; Feher, G.; Benedek, G. B.; *Biochemistry* **1973**, *12*, 714.
(30) Nicoli, D. F.; Benedek, G. B. *Biopolymers* **1976**, *15*, 2421.