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Influence of NaCl and sorbitol on the stability of conformations of cytochrome *c*

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ABSTRACT

Influence of ionic (NaCl) and non-ionic (sorbitol) additives on structural transitions of cytochrome *c* was investigated by circular dichroism, optical and EPR spectroscopy. Transformations of cytochrome *c*, induced by the acidification of solution and temperature perturbation, were monitored in the heme pocket together with changes in the secondary structure. NaCl and sorbitol exhibited antagonistic effect on the acid-induced transition of the protein. Sorbitol enhanced the stability of native conformation while NaCl destabilized this state. The midpoints of acid-induced transitions in the axial coordination of heme as well as in the secondary structure occurred nearly at the same pH values. However, temperature-induced transitions in the unfolding of the secondary structure were almost coincidental with the cleavage of Met80–Fe bond only in the sorbitol solutions. In the salt solution the Met80–Fe bond was markedly more labile than the secondary structure.

1. Introduction

Cytochrome *c* (cyt *c*) is one of the most studied proteins with respect to its conformational intermediates [1,2]. In the native state (N) the heme is covalently bound by two thioester bridges to two cysteine residues. In this conformation the heme is axially ligated with His18 and Met80 resulting in the low spin state of ferric iron [3]. Acidification of a salt-free solution of cyt *c* to pH 2.0 by HCl leads to the unfolding of the protein associated with a conversion of the low to the high-spin state [4]. The structure of unfolded protein (U) is an extended coil having a dimension greater than that of a random coil owing to electrostatic repulsions among the positively charged lysine and arginine residues [5,6].

Upon addition of salts to the acid unfolded cytochrome *c* the protein cooperatively folds to a compact structure with a molten globule (MG) character [7–10]. Molten globular conformation of cyt *c* has the secondary structure content similar to that of the native conformation, but less packed tertiary structure. Conformational transition from the unfolded to the molten globule is also promoted by variety of uncharged molecules and polyanions [11–13] and several studies were concerned with the role of the axial ligand Met80 for stabilizing the MG state [14–15].

To understand the principles of cyt *c* stability it is necessary to elucidate the stabilizing mechanisms for the native as well as MG states. The temperature- and pH-induced protein conformational transitions under selected conditions serve as a useful tool for revealing the role and importance of various interactions. In this work the effect of NaCl and sorbitol, as representatives of ionic and non-ionic additives, on native and acid-denatured cyt *c*, was studied by several spectroscopic techniques. Conformational transitions of native, molten globule and unfolded state of cyt *c* were induced by variation of the pH of solution, temperature and by the nature of cosolvent. This investigation determined selectively the stability of the Met–Fe bond and its relation to that of the secondary structure.

2. Experimental

2.1. Materials

Horse heart cytochrome *c* (type VI) and sorbitol were purchased from Sigma-Aldrich. Cytochrome *c* and reagents of analytical grade were used without purification. All samples of cyt *c* are in water solutions containing no buffer. The homogeneity of cytochrome *c* was verified by SDS PAGE electrophoresis. Concentration of cyt *c* was determined from the optical absorption at 410 nm using the extinction coefficient of $\varepsilon_{410}^{ox} = 106.1\ mM^{-1}\ cm^{-1}$ [16].

2.2. Optical absorbance measurements

Optical spectra were collected in Specord S 100 spectrophotometer (Analytic Jena). Temperature of the optical cell was controlled by a thermostat water bath. The heating rate was approximately 1 °C/min.

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2.3. EPR spectroscopy

EPR spectra were recorded with Brucker EMX spectrometer. The conditions for EPR measurements were: frequency, 9.6 GHz; power, 3 mW; modulation amplitude, 10 G; the modulation frequency, 100 kHz; temperature, 10 K. The high-spin signal at g=6 was quantified by double integration with the lower integration limit taken below the low-field end of the spectrum, and the upper limit at a field corresponding to a g value of 4.67. This integrated intensity was compared with the signal of a standard high-spin complex of metmyoglobin at pH 7.5.

2.4. Circular dichroism (CD) measurements

CD spectra were monitored in JASCO 810 spectropolarimeter equipped with a temperature-controlled cell holder. Quartz cell with 1 mm optical pathlength was used and during measurements the cuvette was continuously purged with nitrogen. CD spectra of cytochrome c are averages of three to five scans. Cyt c concentration was 25 μM and the heating rate 1 °C/min.

2.5. pH titration

The acid titration was carried out by direct addition of concentrated HCl solution at 25±0.2 °C. The pH of samples was measured in the cuvette using a HI 9017 pH-meter (Hanna Instruments Srl. Italy) coupled to a SG901C pH electrode (Sensorex, USA).

2.6. Data analysis

The thermal transitions of cyt c were fitted to the two-state equation:

$$y_{obs} = \frac{y_I + m_I \cdot T + (y_F + m_F \cdot T)\exp\left\{\frac{\Delta H_{vH}}{R}\left(\frac{1}{T} - \frac{1}{T_{trs}}\right)\right\}}{1 + \exp\left\{\frac{\Delta H_{vH}}{R}\left(\frac{1}{T} - \frac{1}{T_{trs}}\right)\right\}} \quad (1)$$

where y_{obs} is the observed spectral parameter, y_I and y_F are spectral parameters of the initial (with slope m_I) and final form (with slope m_F), T is temperature (in K), R is the gas constant 8.314 J/K/mol, ΔH_{vH} is the apparent van't Hoff enthalpy, and T_{trs} is the apparent midpoint temperature of transition. The data were fitted using a simple weighted nonlinear regression analysis.

3. Results

3.1. Stability of the heme iron–Met 80 bond: Optical and EPR spectroscopy data

It is known that in optical spectrum of native cyt c the charge transfer band at 695 nm is linked to the presence of Met80–heme iron bond (Met80–Fe). By lowering the pH to the value of 2.0 Met80 is replaced with H_2O and this structure is characterized by the new peak at 620 nm [17,18]. Since the spectrum of cyt c at pH 3.2 and low ionic strength (Fig. 1) corresponds to the native cyt c we use the term native cyt c (N) for this conformation. However, under these conditions the protein is on the edge of acid transition and is more sensitive to various small perturbations like the pH, temperature or additives.

Addition of 1 M NaCl to native cyt c reduces the 695 nm band to ~70% of intensity of the native cyt c as well as an increase of the absorption at 620 nm (Fig. 1a). Contrary, 3 M sorbitol does not change the spectrum and all absorption bands are almost the same as those for cyt c without cosolvent (Fig. 1b). Adding either 1 M NaCl or 3 M sorbitol to acid unfolded cyt c at pH 2.0 results in the decrease of 620 nm band and the 695 nm band is partially restored (Fig. 1a, b). The recovery of 695 nm band is ~40% in the presence of 1 M NaCl and ~60% with 3 M sorbitol in solution.

The effects of salt and sorbitol on the heme pocket of native and unfolded cyt c was extended by more detailed investigation at various pH. Insets in Fig. 1 show the influence of NaCl and sorbitol concentrations on the pH-induced transitions monitored at 695 nm. In the absence of cosolvents the transition of cyt c produced by HCl occurs in a very narrow pH range with the midpoint (pK) of 2.5±0.1 and number of exchanged H^+=3. An increase of NaCl concentration results in the shift of the apparent pK to higher values. The pK of 3.3±0.1 is established in solution with 1 M NaCl (Fig. 1a, inset). In this case the end of the transition is also reached at pH 2.0 but it is less cooperative (number of exchanged H^+=1.6) than that observed for cyt c without

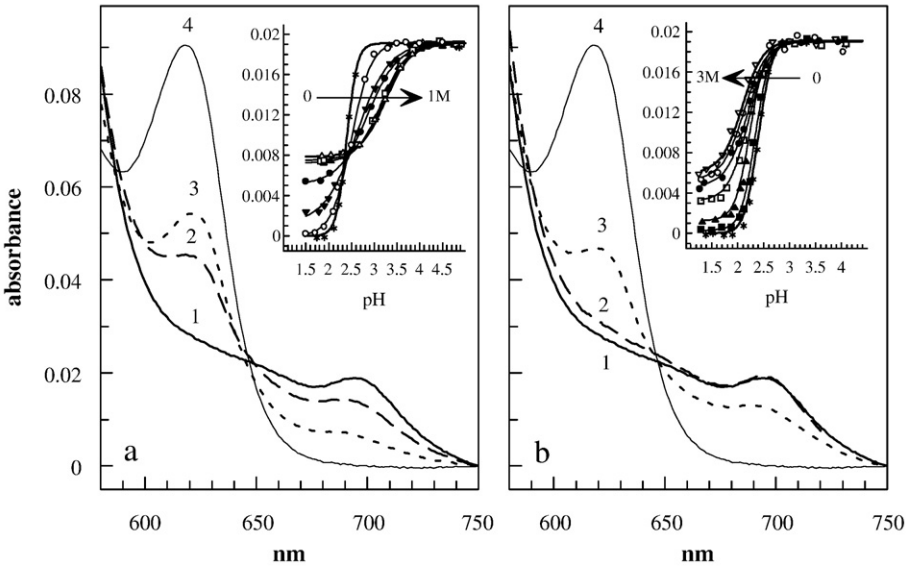

Fig. 1. Absorption spectra of cytochrome c in the presence of 1 M NaCl (a) and 3 M sorbitol (b). Cyt c concentration, 27 μM. Temperature, 25 °C. a: (1) cyt c, pH 3.2; (2) cyt c, pH 3.2+NaCl; (3) cyt c, pH 2+NaCl; (4) cyt c, pH 2. Inset: The pH dependencies of absorption of cytochrome c at 695 nm on concentration of NaCl (0.025, 0.05, 0.1, 0.3, 0.5 and 1 M). b: (1) cyt c, pH 3.2; (2) cyt c, pH 3.2+sorbitol; (3) cyt c, pH 2+sorbitol; (4) cyt c, pH 2. Inset: The pH dependencies of absorption band at 695 nm at various concentrations of sorbitol (0.5, 1, 1.5, 2, 2.5 and 3 M).

additives. In contrast, an increase of sorbitol concentration enhances the stability of Fe–Met80 bond. The disruption of this bond starts at lower pH values comparing to free cyt *c* (Fig. 1b, inset). From the dependence of 695 band on pH the pK of 2.2±0.1 is determined and number of exchanged H^+=2.3 for cyt *c* in 3 M sorbitol. Interestingly, in the presence of either 1 M NaCl or 3 M sorbitol there is still a fraction of cyt *c* having the Fe–Met80 ligation indicated by the residual absorption at 695 nm (Fig. 1, insets).

Conclusions from optical spectroscopy about the heme iron axial ligation are also corroborated by the EPR data (Fig. 2). At pH 3.2 with no additives the EPR spectrum of cyt *c* (Fig. 2, pH 3.2) is almost identical to that collected on the sample at pH 8.0 (not shown). The rhombic low spin signal is characterized by g_z=3.00 and g_y=2.28 (g_x position is out of the scale) and only a small fraction (~1%) is in the high-spin state visualised as the signal at g=5.94. The whole population of ferric iron is basically in the single low spin state (Table 1). The homogeneity and symmetry of iron center is not changed by the addition of 3 M sorbitol (Fig. 2, pH 3.2). In this case the ferric iron exhibits again the low spin rhombic signal (g_z=3.04 and g_y=2.25). However, the single low spin coordination at pH 3.2 is destabilized by addition of NaCl (Fig. 2, pH 3.2). In 1 M NaCl ~30% of heme iron is found in the high-spin state with nearly axial symmetry (g_x=g_y=6.00 and g_z=1.95). The dominant fraction of cyt *c* (~70%) exhibits two rhombic low spin signals: one is characterized by the g_z=2.98, g_y=2.29 and the other is described by g_z=2.76, g_y≈2.29. The former signal makes the major contribution to the spectrum (see bellow).

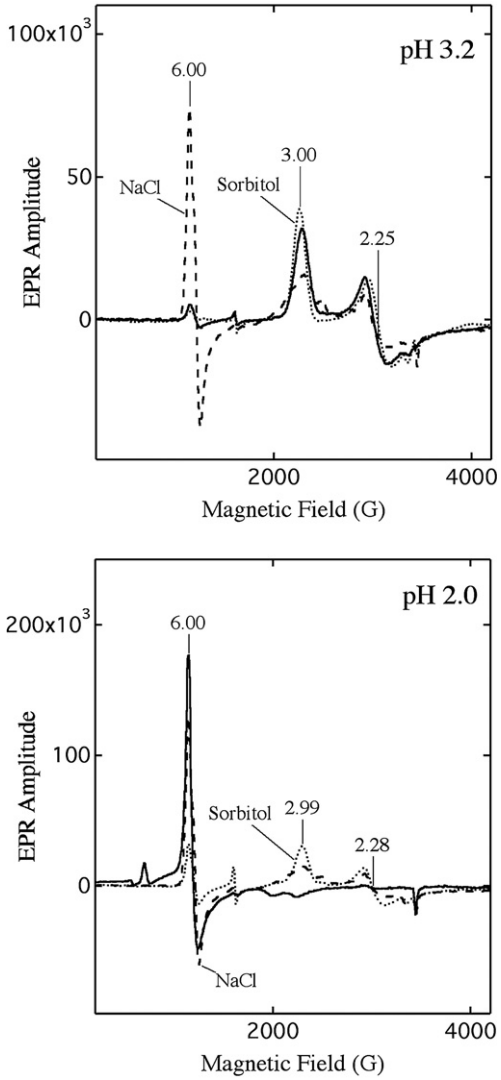

Fig. 2. Influence of NaCl and sorbitol on the EPR spectrum of cytochrome *c* at both pH 3.2 (upper box) and pH 2.0 (lower box). Full line spectra (——): no additives; Dashed line (- - - -): spectra in the presence of 1 M NaCl; Dotted line (……): cyt *c* in the presence of 3 M sorbitol. Numbers above the spectra indicate the *g* values. Each sample contained 100 μM cytochrome *c* and 1 μM ferricyanide.

Table 1
Dependence of the yield of the heme iron of cytochrome *c* in the high-spin state on the cosolvents

Cosolvent	Yield of high-spin signal (%)	
	pH 3.2	pH 2.0
None	1	100
3 M Sorbitol	1	13
1 M NaCl	30	48

Taking the *g* values of low spin signals the crystal field parameters were calculated [19,20]. These parameters were used to assign the heme axial ligands from the diagram of Blumberg and Peisach [19,20]. From this diagram His–Fe–Met axial coordination is found for ~99% of cyt *c* in solutions with either no additives or 3 M sorbitol. However, in the presence of 1 M NaCl ~60% of ferric iron is having His–Fe–Met ligation, ~10% is in His–Fe–His state and the residual ~30% contributes to the high-spin signal. Comparison of the high-spin signal of cyt *c* with both the EPR and optical spectra of metmyoglobins [21] suggests that this ~30% of the heme iron has His–Fe–H_2O structure.

The same EPR measurements together with the assignment of the axial ligands were performed on the samples at pH 2.0. Without cosolvents the decrease of pH from 3.2 to 2.0 value brings about the transition in the coordination structure of iron manifested by the change of low to the high-spin state (Fig. 2, pH 2.0). Two high-spin signals are distinguished in the spectrum: main is the axial signal (g_{xy}=6.0 and g_z=1.99; yield ~96%) and a small fraction (~4%) displays the signal at g=9.85. However, this complete change of the spin state is not observed in the presence of either 1 M NaCl or 3 M sorbitol (Fig. 2, pH 2.0 and Table 1). Addition of the sorbitol stabilizes ~87% of iron in the rhombic low spin state (g_z=2.99, g_y=2.28) and only ~13% shows the axial high-spin component (g_{xy}=5.98). In 1 M NaCl the yield of the axial high-spin component (g_{xy}=6.0 and g_z=1.99) is ~48% and ~52% of cytochrome *c* exhibits two rhombic low spin signals (g_z=2.98, g_y=2.28 and g_z=2.75, g_y≈2.28). The two signals are essentially the same as those determined at pH 3.2. Nearly identical the *g* values and the symmetry of EPR signals at both pH 3.2 and 2.0 suggests the same axial ligation of Fe at these pH values. However, a distribution of three structures (His–Fe–H_2O, His–Fe–Met and His–Fe–His) is changed by the pH decrease. At pH 2.0 and no additives nearly all cytochrome *c* has His–Fe–H_2O coordination. With 3 M sorbitol ~13% of iron has His–Fe–H_2O structure and ~87% is in His–Fe–Met state. In solution with 1 M NaCl the estimated ~48% belongs to His–Fe–H_2O fraction, ~44% is in His–Fe–Met state and the residual ~8% has His–Fe–His coordination.

To further characterize the stability of Fe–Met80 bond the effect of temperature on this ligation was studied at both pH 3.2 and 2.0 in the presence of cosolvents (Fig. 3a). For native cyt *c* the bond is interrupted at 58.6 °C with an enthalpy of transition ΔH_{vH}=229 kJ/mol. In the presence of 1 M NaCl the transition temperature is 40.8 °C and ΔH_{vH}=65 kJ/mol. In spite the fact that adding of 3 M sorbitol to cyt *c* does not change the spectrum at pH 3.2 and 25 °C, the rupture of Met 80–heme iron bond is shifted to the higher T_m of 73.5 °C with ΔH_{vH}=330 kJ/mol.

At pH 2.0 and in the presence of 1 M NaCl the Met80–heme iron bond cleavage occurs at 34.2 °C with ΔH_{vH}=92 kJ/mol. In 3 M sorbitol the process is more cooperative and described with T_m=39 °C and

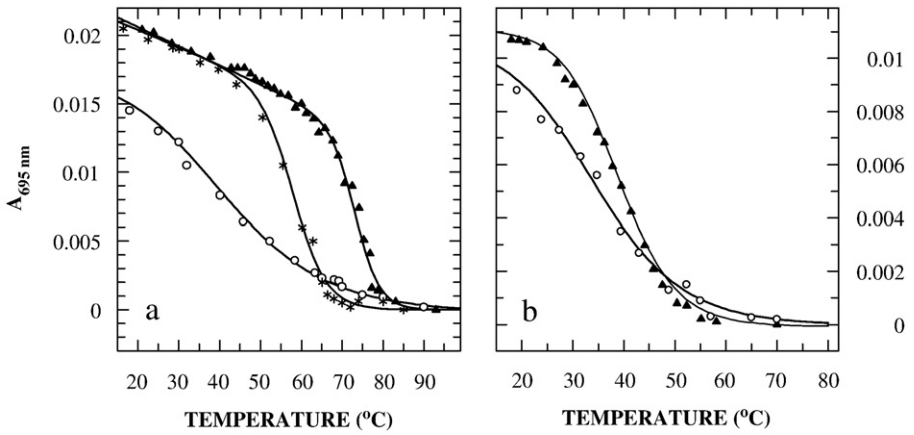

Fig. 3. Influence of temperature on the absorption band at 695 nm. Transitions were measured at both pH 3.2 (a) and pH 2.0 (b). Cyt *c* (*); cyt *c* plus 1 M NaCl (○); cyt *c* plus 3 M sorbitol (▲).

ΔH_{vH} = 145 kJ/mol (Fig. 3b). Altogether data show that 3 M sorbitol stabilizes and 1 M NaCl destabilizes the heme region of cyt *c* towards the temperature-induced transitions at both pH values.

3.2. Transitions in the secondary structure: Circular dichroism data

Circular dichroism (CD) spectroscopy was used to investigate the changes in protein secondary structure (Figs. 4 and 5). The far-UV CD spectra of native and unfolded cyt *c* (Fig. 4a) are consistent with data published in the previous studies [8,22,23]. Moreover, at pH 3.2 the spectra of native cyt *c* and cyt *c* in the presence of 3 M sorbitol are almost indistinguishable (Fig. 4a). However, the spectrum of cyt *c* is influenced by 1 M NaCl and indicates a formation of the state possessing a structural similarity with the molten globule state (MG).

Significant changes in the spectrum of unfolded protein (pH 2.0) are observed after addition of either1 M NaCl or 3 M sorbitol. Both spectra reflect the increased α-helical content in these states relative to that of the unfolded protein (Fig. 4a). It is evident that these additives promote a formation of MG state from unfolded cyt *c* as noticed previously [12].

Since there are minimal differences in the ellipticity of native and MG states at 220 nm (Fig. 4a) the ellipticity at 208 nm was chosen to monitor the pH-induced changes in the structure [24,25]. From the pH-dependencies obtained in the presence of cosolvents (Fig. 4b) the pK values of the transition from native to MG state were determined. In presence of 1 M NaCl the pK is 3.3 ± 0.2 and number of exchanged H^+ = 1.9. In 3 M sorbitol the apparent pK of 2.2 ± 0.1 was established and number of exchanged H^+ = 2.0. For free cyt *c* insignificant changes in ellipticity occur at 208 nm within a range of the pH titration (not shown).

The thermal transitions from native (N) or molten globule (MG) to unfolded state (U) are shown in Fig. 5. The denaturation temperature (T_m) of cyt *c* without additives is 59.8 °C and ΔH_{vH} = 247 kJ/mol. An addition of 3 M sorbitol to native cyt *c* increases T_m for N ↔ U transition to 76 °C as well as enthalpy to 300 kJ/mol. On the other hand, in the presence of 1 M NaCl T_m for MG ↔ U transition is 75 °C and ΔH_{vH} = 190 kJ/mol. Fig. 5b shows the thermal denaturation profiles of the 1 M NaCl and 3 M sorbitol-induced MG states of cyt *c* at pH 2.0. The MG state, relatively stable at low temperature (<30 °C), shows a loss of ellipticity with T_m of ~39.5 °C. At temperatures above 55 °C the CD spectrum indicates a typical random coil structure. In the presence

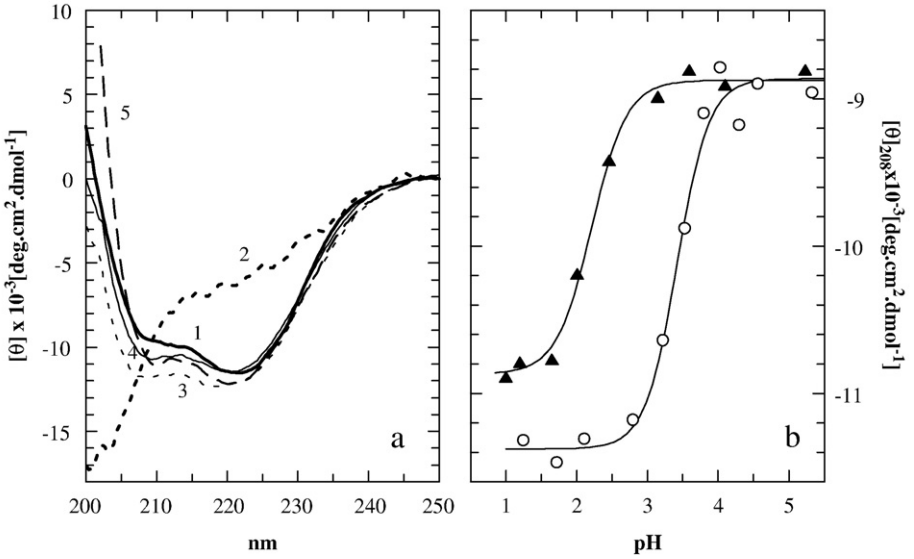

Fig. 4. Variation of circular dichroism spectra of cytochrome *c* with pH and additives. a: CD spectra in far-UV region after addition of 1 M NaCl and 3 M sorbitol to the native (pH 3.2) and unfolded cyt *c* (pH 2). (1) cyt *c*, pH 3.2; (2) cyt *c*, pH 2.0; (3) cyt *c*, pH 2.0 plus 1 M NaCl; (4) cyt *c*, pH 3.2 plus 1 M NaCl; (5) cyt *c*, pH 2.0 plus 3 M sorbitol. b: pH-induced conformational transition of cyt *c* in both 1 M NaCl (○) and 3 M sorbitol (▲) measured by the changes in the ellipticity at 208 nm. Temperature, 25 °C.

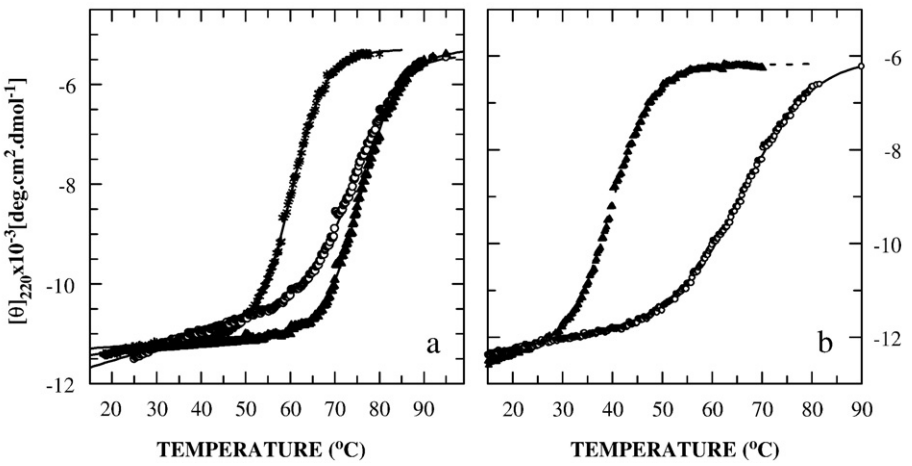

Fig. 5. Temperature-dependent changes in the secondary structure of cytochrome *c* determined at pH 3.2 (a) and pH 2.0 (b). All transitions were monitored by the changes in ellipticity at 220 nm. Cyt *c* (*); cyt *c* plus 1 M NaCl (○); cyt *c* plus 3 M sorbitol (▲).

of 3 M sorbitol the transition of MG↔U occurs at 39.5 °C with ΔH_{vH}= 191 kJ/mol (Fig. 5b) and in 1 M NaCl T_m=66.8 °C and ΔH_{vH}=142 kJ/mol were determined.

4. Discussion

Protein stability is dependent upon a fine balance between favourable and unfavourable interactions of native and denatured protein states with cosolvent molecules [26]. Thus the stabilizing effect results from the nature of the protein as well as cosolvents. The structure and mechanisms of stabilization of the solute-induced MG states generated from both the unfolded [10,11,22,27–29] and native cyt *c* have been already investigated [29–31].

While the assignment of the optical spectra of native cytochrome *c* to the heme ligands is possible due to the known X-ray structure, this is yet not the case for the multiple conformations induced by the variation of pH, temperature, ionic strength or denaturants. In the acid pH region (low ionic strength) the existence of high-spin state of cyt *c* or cytochrome *c*′ was identified by several spectral methods [32–34] and is also confirmed by our EPR data (Fig. 2). The nature of axial ligands of heme iron in this high-spin state is indicated by the striking spectral resemblance of the acid unfolded cyt *c* to that of metmyoglobin [21] and to the site-directed mutant of cyt *c* [35]. Both optical and EPR spectra of metmyoglobin at neutral pH, when the heme iron is ligated by His and H_2O [36], are very similar to cyt *c* at pH 2.0. This similarity extends to the mutant of cyt *c* in which Met 80 was substituted by Ala [35]. These observations strongly suggest that in the unfolded cyt *c* at pH 2.0 one axial ligand of the ferric iron is His and the other is water (His–Fe–H_2O).

Addition of either NaCl or sorbitol to the unfolded cyt *c* results in the partial recovery of Met80–heme iron bond and formation of MG state (Figs. 1 and 2). The main driving force of salt-induced MG state at pH 2.0 is a reduction of the electrostatic repulsion between the charged groups of protein molecule [37]. In case of sorbitol-induced MG is enhancement of hydrophobic interaction or the increased steric repulsion between the protein and the sugar solution [28] overcoming the electrostatic repulsion between charged residues [11].

The opposite effect of NaCl and sorbitol on the native conformation of cyt *c* (pH 3.2) was observed. While NaCl destabilizes Met80–Fe bond (Figs. 1 and 2) and produces the MG state, 3 M sorbitol maintains and enhances the stability of the native cyt *c* structure (Figs. 1 and 2). We assume that salt ions in solution disrupt the coupled charge pairs in the protein resulting in a destabilization and a release of Met80 from heme iron. At the same time the formation of MG state (Fig. 4a) is probably driven by the shielding of the free (not involved in ionic pairs) positive protein charges by the salt.

Antagonistic influence of NaCl and sorbitol on native cyt *c* is also demonstrated by the acid-induced transitions (Figs. 1 and 4b). In the presence of additives the decrease of pH from 3.2 to 2.0 leads to the formation of MG state with the almost simultaneous release of the Met80 from the heme iron (Figs. 1 and 4a). However, sorbitol stabilizes the native structure of cyt *c* whereas NaCl decreases the stability of the protein.

In contrast, a contribution of salt and sorbitol to the structural stability of cyt *c*, determined from the acid-induced denaturation, was not established in the studies of the temperature-induced unfolding of N and MG states. The salt increased the stability of the secondary structure and the transition temperatures of MG↔U were higher than those observed for a free cyt *c* (Fig. 5). However, transitions of the Met80–heme iron bond were strikingly below the temperatures at which the unfolding of α helices was observed (Fig. 3). Quite unexpected and puzzling is a destabilization of the secondary structure by sorbitol during the conversion of MG↔U at pH 2.0 (Fig. 5). Yet, only in the presence of sorbitol no significant differences were identified between transition temperatures of the secondary structure and a cleavage of the Met80–Fe bond.

5. Conclusions

Using both the pH and temperature perturbation of the solutions of cyt *c* containing either salt (NaCl) or sugar (sorbitol) the influence of the electrostatic interactions and the steric repulsion between a protein and solutes (crowding effect) were investigated. Generally, both NaCl and sorbitol promote a transition of U↔MG state and the stability of the secondary structure is increased relative to the stability of Met80–Fe bond. Transitions in the secondary structure and the axial ligation of heme iron are cooperative but the midpoints do not coincide under all investigated conditions. The largest differences in the stability between the heme pocket and the secondary structures were identified in the temperature-induced perturbations. In contrast the acid-induced transformations of the secondary structure as well as the heme coordination occurred nearly at the same pH values. Antagonistic influence of NaCl and sorbitol on the acid-induced transitions of cyt *c* was revealed. Sorbitol contributed positively to the stability while the salt destabilizes the native conformation of cytochrome *c*.

Acknowledgements

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