Effect of Polyanion on the Acidic Conformational Transition of Native and Denatured Ferricytochrome *C*. Circular Dichroism Study

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Abstract. Interaction of polyanion poly(vinylsulfate) with oxidized cytochrome *c* (cyt *c*) significantly affects the protein main characteristics. One of them, pK_a value of acidic transition, was shifted from an apparent pK_a value 2.5 (typical for cyt *c* in low ionic strength solvent) to $\sim 5.20 \pm 0.15$ upon polyanion binding to the protein, pointing to a likely involvement of histidines 26 and/or 33 in the protein acidic transition in complex with the polyanion. The acidic transition followed at 6 different wavelengths all over circular dichroism spectrum, monitoring different parts of the protein structure, revealed basically two-state character process. Only ellipticity at 262 nm indicated a low-cooperative pH-induced conformational transition in heme region with an apparent pK_a $\sim$ 4.34 $\pm$ 0.25 in accordance with absorbance change at 620 nm. Polyanion also interacts with chemically-denatured (in the presence of 9 mol/l urea) state of the protein as it follows from stabilization of protein residual structure at acidic pH and its effect on pK_a value of acidic transition of chemically-denatured cyt *c*. Destabilization effect of polyanions on native and, on the other hand, stabilization influence on partially unfolded conformations of the protein are discussed with an implication for their chaperone-like properties *in vivo* and *in vitro*.

Key words: Cytochrome *C* — Conformational change — Molten globule — Polyanion binding — Circular dichroism

Abbreviations: cyt *c*, cytochrome *c*; PVS, poly(vinylsulfate); CD, circular dichroism

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Introduction

Direct study of intermediate conformational states of proteins occurring during translation and translocation processes is significantly limited in living cells. Therefore, more simple systems, which can model conditions inducing formation of the physiological intermediate conformational states of proteins (molten globule-like states), are investigated. Detailed structural and kinetic description of intermediate state(s) in different conditions was performed on cytochrome *c* (cyt *c*) because of its physico-chemical properties that enabled its characterization by utilizing variety of methods. Mitochondrial cyt *c* is a small (containing 104 amino acid residues) basic (p$I \sim 10.1$) heme-containing protein that mediates electron transfer in the respiratory chain between cytochrome bc_1 and cytochrome *c* oxidase complexes. It was shown that cyt *c* could be transformed *in vitro* into molten globule-like states at relatively harsh conditions, e.g. low pH and high ionic strength, in the presence of denaturants, at elevated temperature (Goto et al. 1990; see Ptitsyn 1995 for review), i.e. at conditions far from those occurring in living cells. Nevertheless, it was suggested that the folding of cyt *c* did not go through the specific molten globule, which was identified in high salt acidic solution (Yeh et al. 1997). Since in a number of cases originally native protein conformation "partially" unfolds upon interaction with negatively charged membrane surface (Pinheiro et al. 1997), conditions simulating such situations were modeled. Concerted actions of local decrease of pH and that of dielectric constant, i.e. conditions occurring close at the membrane surface, have been modeled by water-methanol mixture at acidic pH (Bychkova et al. 1996). In fact, induction of molten globule state of cyt *c* was observed. On the other hand, we have studied different model system of protein-negatively charged surface represented by cyt *c*-polyanion complex (Sedlák and Antalík 1999). It was found out that cyt *c* upon binding to poly(vinylsulfate) (PVS) was in molten globule-like state under slightly acidic pH and low ionic strength conditions. Negatively charged surface, represented by polyanion, shows interesting properties related to processes of protein folding: (i) it can accelerate protein refolding (Dabora et al. 1991; Rentzeperis et al. 1999), (ii) it stabilizes protein structure (Burke et al. 1993; Rentzeperis et al. 1999), (iii) it prevents protein aggregation (Fedunová and Antalík 1998; Meng et al. 2001). In contrast with the previous works (Burke et al. 1993; Rentzeperis et al. 1999), we have now observed destabilization effect of PVS-binding on cyt *c* structure what is, however, in accordance with effect of membrane surface on the protein (Muga et al. 1991; Pinheiro et al. 1997).

For better understanding the effect of polyanions on cyt *c* conformation and stability at slightly acidic pH, the acidic transitions of cyt *c* in presence and the absence of PVS were investigated by circular dichroism (CD). CD of horse heart cyt *c* is particularly rich in details over the entire spectral region reflecting the contributions from transitions arising from various chromophores. Since the origins of most of these transitions are well characterized, the investigations of alternations of their rotatory strength may aid in interpretations of conformational changes of cyt *c* in studied conditions.

Materials and Methods

Horse heart cytochrome c (cyt c), type VI, was obtained from Sigma Chemical Co. (Germany) and was used without further purification. Potassium ferricyanide was purchased from Fisher Scientific Co. (USA). Urea was purchased from Bio-Rad Laboratories (USA). Urea concentration was determined from refractive index. Double distilled water was used in all experiments. Poly(vinylsulfate) (PVS) was purchased from Aldrich (Germany) before 1999. At this point, it is necessary to mention, for those who would attempt to repeat our experiments, that we have experienced problems regarding solubility of recently purchased poly(vinylsulfate) from Sigma-Aldrich. While PVS – Aldrich (purchased before 1999) was a fine powder highly-soluble at room temperature, with main characteristics and effects on cyt c comparable with other 'hydrophilic' polyanions (as heparin, polyadenylate, polyglutamate – see Antalík et al. 1992; Garber and Margoliash 1994; Bágeľová et al. 1994, 2001; Sedlák and Antalík 1998, 1999), PVS – Sigma-Aldrich (based on experiences with 3 different batches of the chemicals purchased within 2 year period) was hardly soluble, creating gel-like liquid state, even containing insoluble particles. Based on a number of experiments with 'hydrophilic' polyanions, we believe that previously published results (Sedlák and Antalík 1998, 1999) and results presented in this paper obtained with PVS – Aldrich are correct. We don't have any explanation regarding the quality of recently distributed PVS by Sigma-Aldrich.

Circular dichroism (CD) measurements were obtained using an OLIS Cary-16 spectropolarimeter (USA). CD measurements were performed over the wide wavelength range, from 190 to 460 nm, what made possible to follow conformational changes of the protein: (i) in its secondary structure, 190–250 nm, (ii) in the tertiary structure in a region of aromatic side chains, 250–350 nm, and (iii) in close vicinity of the heme, 350–460 nm. CD measurements were performed with 16–19 μmol/l cyt c in 10 mmol/l phosphate buffer solution in the presence or absence of 9.0 mol/l urea. In all cases the concentration of PVS was 1.0 mg/ml. Oxidized cyt c was prepared by an addition of 5 μmol/l potassium ferricyanide into solution. Protein concentration was determined by using the extinction coefficient at 410 nm, $\varepsilon_{410\,\mathrm{nm}} = 106\ (\mathrm{mmol/l})^{-1}\cdot\mathrm{cm}^{-1}$. CD spectra of ferricyt c were expressed as averages of 3–8 consecutive scans. 1 mm and 5 mm cuvettes were used for measurements in the regions 190–250 nm and 250–450 nm, respectively. Typically, a time constant of 1 s, band width of 1.0–1.5 nm and 200 points/100 nm were used. The ellipticity was reported as the mean residue ellipticity in the range 190–250 nm, and as the molar ellipticity in the range 250–460 nm, and calibrated with (+)-10-Camphorsulfonic acid.

The pH of solution was changed by an addition of suitably diluted HCl. The pH values were always measured before and after measurements with a Radiometer America Electrodes glass electrode using pH meter – EA 940 Expandable ion Analyzer Orion Research (USA), and were determined with uncertainty of ± 0.05 pH units. The normalized ellipticity values Θ were plotted against pH to obtain

curves that were fitted to the equation given below:

$$\Theta = \{\Theta_n + \Theta_u[10^{n(pKa-pH)}]\}/\{1 + 10^{n(pKa-pH)}\}$$

In this equation, Θ_n is the normalized ellipticity at neutral pH prior to transition, Θ_u is the normalized ellipticity in acidic pH after transition, pK_a is pH value corresponding to an inflextion point of the dependence and n value, corresponding to a slope at the inflextion point, determines the number of protons involved in the transition. Fitted values of Θ_n and Θ_u were in ranges 0–10 and 95–105, respectively. Corresponding pK_a and n values are listed in Table 1.

Table 1. The apparent pK_a and n (number of protons involved in the transition) values of cytochrome c in the presence and in the absence of polyanion – poly(vinylsulfate). The transition was followed at various wavelengths monitoring different part ("reporter group") of the protein, at temperature 20 °C. Uncertainty in pH determination was ±0.05 pH unit

		Cytochrome c			
		− polyanion		+ polyanion	
Wavelength (nm)	Reporter group	pK_a	n	pK_a	n
208	?			5.31 ± 0.07	3.24 ± 0.42
220	α-helix	2.36 ± 0.10	3.63 ± 0.38		
262	heme	2.91 ± 0.25	1.74 ± 0.80	4.34 ± 0.25	0.45 ± 0.25
282	Trp-59	2.46 ± 0.12	1.85 ± 0.47	5.12 ± 0.08	2.55 ± 0.33
288	Trp-59	2.58 ± 0.15	1.39 ± 0.43	5.12 ± 0.09	2.80 ± 0.66
404	heme	2.56 ± 0.11	2.56 ± 0.81	5.20 ± 0.14	2.71 ± 1.73
416	heme/Phe-82	2.43 ± 0.10	2.16 ± 0.46	5.10 ± 0.07	2.62 ± 0.30

CD spectra were measured at 20 °C. Temperature was kept constant by a water bath circulator.

Results

Absolute CD spectra of both free cyt c and cyt c in the presence of PVS in low ionic strength at different acidic pH values of solvent are shown in Figure 1.

The far-UV (190–250 nm) CD spectrum (Fig. 1A), sensitive probe for protein secondary structure, shows typical features of α-helical protein structure. While the 222 nm dichroic band is predominantly associated with α-helical $n \rightarrow \pi^*$ amide transitions (Myer 1968a), the dichroic band at 208 nm, corresponding to the $\pi \rightarrow \pi^*$

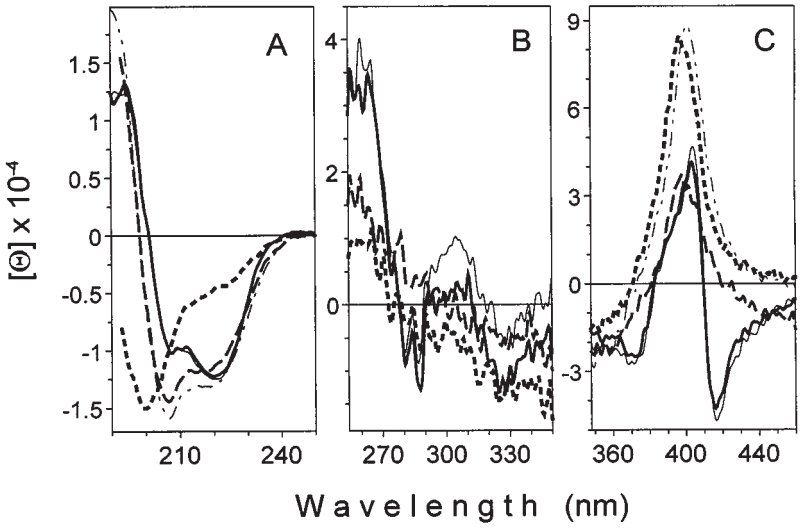

Figure 1. Far-UV (A), near-UV (B) and Soret (C) circular dichroism spectra of cyt *c* at: pH 7.0 (thick solid line), pH 1.9 (thick dotted line), and cyt *c* in the presence of PVS at: pH 7.0 (thin solid line), pH 3.7 (thin dashed line), pH 1.9 (thick dashed line) in low ionic strength (10 mmol/l phosphate buffer). (A) Far-UV, deg·cm^2/dmol of amide bonds; (B) and (C) near-UV and Soret, deg·cm^2/dmol of heme.

amide transitions may arise from changes in other secondary structure elements in the protein, or may be due to the presence of optically active heme transitions of the polypeptide chain. An addition of PVS to cyt *c* in low ionic strength in neutral pH did not affect CD spectrum of the protein in this region (Fig. 1A). However, acidification of solvent brought about different effects on free cyt *c* and cyt *c*-PVS complex. Dichroic minima of free cyt *c* at 208 and 222 nm were replaced by a minimum at ∼200 nm indicating a conformational transition to a random coil-like state of polypeptide chain at pH 2.0 (Myer and Saturno 1990). On the other hand, far-UV CD spectrum of cyt *c* in the complex with PVS was changed only slightly due to acidification of the solvent: the minimum at 222 nm was nearly unaffected and an increase in the rotatory strength at 208 nm was observed (Sedlák and Antalík 1999). Noteworthy, analogous changes in ellipticity at 208 nm were observed in transitions of cyt *c* into molten globule-like state at different conditions (Goto et al. 1990, Bychkova et al. 1996, Pinhero et al. 1997).

CD in near-UV (250–350 nm) (Fig. 1B) and Soret (350–450 nm) (Fig. 1C) regions reflect changes in protein tertiary structure that affect the environment of aromatic side chains and an environment close to heme, respectively. CD spectrum of cyt *c* in the near-UV region is characterized by two sharp minima at 282 and 288 nm assigned to the Trp-59 side chain (Davies et al. 1993; Myer 1968b). It may be pointed out that the rotatory strengths of these transitions are relatively insensitive

to presence of extrinsic ligands and pH variation over a wide pH range (3–11.6) (Myer 1968b). The band centered around 251 nm possibly reflects contribution from tyrosine side chains (Myer 1968b), and the broad positive CD transition around 262 nm has been attributed to porfyrin transitions with rotatory strength dependent on the immediate environment of the heme (Urry 1967). Similarly as in far-UV region, CD spectrum of cyt *c* in near-UV region is not affected upon binding to PVS at neutral pH (Fig. 1B). Acidification of solvent led in both free and complexed cyt *c* to simplification of the relatively complex dichroic pattern in this spectral region. Rotatory strength of all bands (at 251, 262, 282 and 288 nm) decreased what indicated a loosening of rigid environment around aromatic amino acids residues in the protein core.

Cyt *c* exhibits a characteristic CD doublet in the Soret region (Fig. 1C). The intensity of the negative band at 417 nm depends on heme-protein interactions (Myer 1968a) and reflects the direct interaction of the $\pi \rightarrow \pi^*$ transition of the aromatic ring of Phe-82 with $\pi \rightarrow \pi*$ transition of the heme group (Rafferty et al. 1990). Decrease in the intensity of the negative lobe in the Soret region sensitively reflects changes in the distance and/or orientation of the phenylalanine residue regarding to the heme in cyt *c* (Zheng et al. 2000). In fact, dissapearance of 417 nm negative Cotton effect was observed as a result of pH changes, replacement of 6^{th} heme-iron ligand Met-80 by extrinsic ligands as imidazole, cyanide, azide, perturbations induced by elevated temperature, by different denaturants such as urea, guanidine HCl, alcohols (Myer 1968b; Kaminsky et al. 1972a) and, as we have shown, also by complexation of the protein with 'hydrophobic' polyanions (Sedlák et al.1997; Sedlák and Antalík 1998). In accordance with that, upon binding of polyanion into cyt *c*, "invisible" in the other regions of CD spectrum, rotatory strengths at both positive and negative lobs slightly increased. Similar changes were observed in CD Soret spectrum of cyt *c* upon binding to negatively charged biomacromolecules such as cyt *c* peroxidase, polyglutamate, phosvitin (Garber and Margoliash 1994) and heparin (Sedlák 1997). Similar to the aromatic region, acidification of the solvent induced profound changes in the Soret CD expressed in disappearance of 417 nm negative Cotton effect and an increase of rotatory strength of positive peak at 404 nm. A difference between free and cyt *c* in the complex with PVS is in the pH range where observed change took place. While the rotatory strength of cyt *c*-PVS complex achieved maximum at pH ~ 4 in the Soret region, comparable CD spectrum of uncomplexed cyt *c* was observed at pH ~ 2. Further acidification (pH < 4) of cyt *c*-PVS complex led to a significant decrease in intensity of the positive peak pointing to a diminishing of asymmetry of heme environment (Fig. 1C).

More detailed analysis of pH dependences of the "reporter" bands – 208, 222, 262, 282, 288, 404, 417 nm – of cyt *c* and cyt *c*-PVS at low ionic strength conditions are shown in Figure 2. For sake of clarity, the dependences of ellipticities at the different wavelengths were normalized and fitted curves were not included into the figure. In Figure 2A, there are shown the curves with fitting parameters pK_a and n: 2.5, 1.0 (thin solid line); 2.5, 2.0 (thick solid line); 2.5, 3.0 (thick dotted line). Analogously, depicted curves in Figure 2B correspond to the fitting parameters:

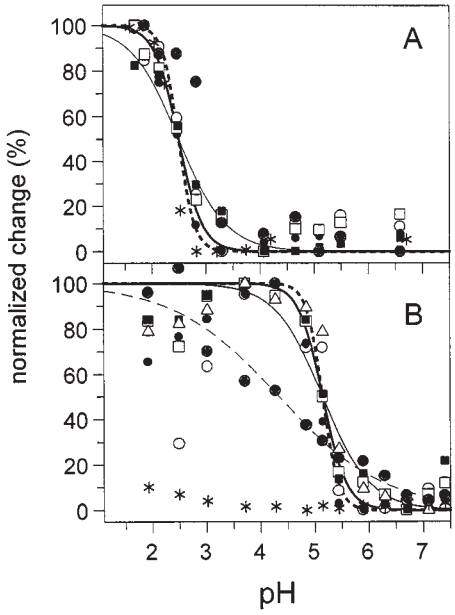

Figure 2. Acidic transition of cyt c in low ionic strength solvent (10 mmol/l phosphate buffer) followed at 208 nm (empty triangle), 220 nm (star), 262 nm (large solid circle), 282 nm (empty rectangle), 288 nm (solid rectangle), 404 nm (empty circle), 417 nm (small solid circle) of circular dichroism spectra in the absence (A) and the presence (B) of polyanion-PVS. Depicted curves are fitted according the equation in Materials and Methods with fitting parameters $\Theta_n = 0$ and $\Theta_u = 100$ for all curves and: (A) p$K_a = 2.5$, $n = 1$ (thin solid line), p$K_a = 2.5$, $n = 2$ (thick solid line), p$K_a = 2.5$, $n = 3$ (thick dotted line); (B) p$K_a = 5.2$, $n = 1$ (thin solid line), p$K_a = 5.2$, $n = 2$ (thick solid line), p$K_a = 5.2$, $n = 3$ (thick dotted line) and p$K_a = 4.34$, $n = 0.45$ (thin dashed line – the best fit into ellipticity dependence at 262 nm).

5.2, 1.0 (thin solid line); 5.2, 2.0 (thick solid line); 5.2, 3.0 (thick dotted line), and a curve fitted to pH-dependence of ellipticity at 262 nm with the parameters pK_a = 4.34 and $n = 0.45$ (thin dashed line). The best fitting parameters obtained from pH-dependences at different wavelengths are shown in Table 1.

From Figure 2A, it follows that free cyt c in low ionic strength proceeds the acidic transition in apparently two-state character transition with an apparent p$K_a = 2.5 \pm 0.15$, and $n = 2.5 \pm 1.0$. A slightly shifted pK_a value of the pH-induced transition at 262 nm to 2.91 ± 0.25 might reflect a mechanism of the acidic transition of cyt c in low ionic strength proposed Myer and Saturno (1990). The authors concluded that the neutral pH form, state III, changes to the acidic pH form, state I, through a three-step process: state III↔state III$_a$↔state II↔state I with pK_a's 3.6 ± 0.3, 2.7 ± 0.2, and 1.2 ± 0.2, depending on the monitoring probe, respectively. Thus 262 nm-band might be more sensitive probe for the transition state III$_a$↔state II which is connected with the change of spin-state of iron-heme, from low to high spin state. In accordance with the above proposed mechanism, transition from neutral pH into pH ∼ 3, state III↔state III$_a$, was accompanied by a slight increase in ellipticities in "reporter" bands as it is shown in Figure 2A.

Based on our previous and presented results, the acidic transition of cyt c in complex with PVS is shifted to higher pH with an apparent p$K_a = 5.20 \pm 0.15$ and $n = 2.8 \pm 0.3$ (Fig. 2B, Table 1), and expresses transition from native to molten globule-like states (Sedlák and Antalík 1999). Here, it should be pointed out that ellipticity at 208 nm apparently reflects changes in tertiary structure of the protein

as its pH-dependence overlaps observed transitions in aromatic and Soret regions. On the other hand, the best fit of two-state process of pH-dependent changes in ellipticity at 262 nm gave parameters $pK_a = 4.34$ and $n = 0.45$ supporting formerly obtained value of an apparent pK_a based on changes of extinction coefficient at 620 nm (Sedlák and Antalík 1999). More detailed analysis of pH dependences, especially at 404 nm, points to at least three state nature of the acidic transition – first transition with $pK_a \sim 5.2$ and the second process with $pK_a \sim 3$. This is demonstrated by the fact that after ellipticity at Soret region reached maximum at pH $\sim$ 4 a further acidification led to its significant decrease very likely connected with spin-state change of heme-iron. Thus, an intermediate apparent pK_a value and low value of n parameter of pH-dependence at 262 nm might be also a result of overlapping of these pH-transitions at this wavelength.

Effect of negatively charged surfaces, represented by polyanions or cardiolipin-containing micelles (Zardeneta and Horowitz 1992; Rentzeperis et al. 1999), on protein folding prompted us to investigate an effect of polyanion on unfolded state of cyt *c*.

In Figure 3, an effect of PVS on denatured state of cyt *c* (in the presence of 9 mol/l urea) in pH 7.0 and 3.0 is shown. Cyt *c* under such denaturing conditions has strongly perturbed both tertiary and secondary structures but still contained a residual structure (Myer et al. 1980; Takahashi et al. 1997; Russell et al. 2000). An

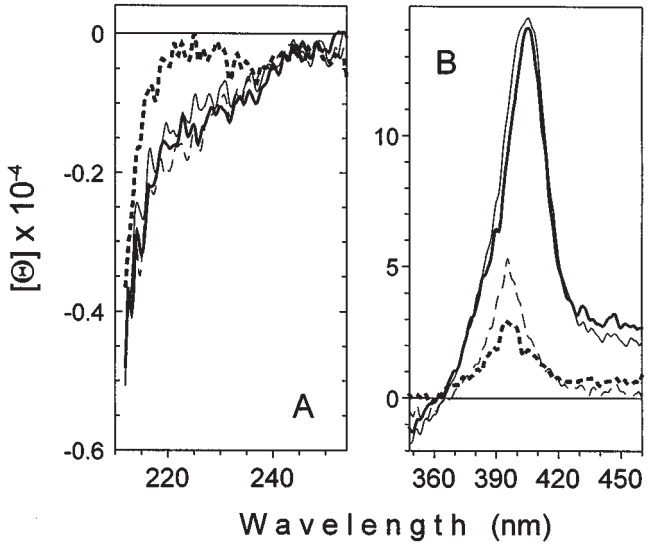

Figure 3. Far-UV (A) and Soret (B) circular dichroism spectra of cyt *c* at: pH 7.5 (thick solid line), pH 3.1 (thick dotted line), and cyt *c* in the presence of PVS at: pH 7.5 (thin solid line), pH 3.1 (thin dashed line) in 9 mol/l urea and low ionic strength (10 mmol/l phosphate buffer). (A) far-UV, deg·cm^2/dmol of amide bonds; (B) Soret, deg·cm^2/dmol of heme.

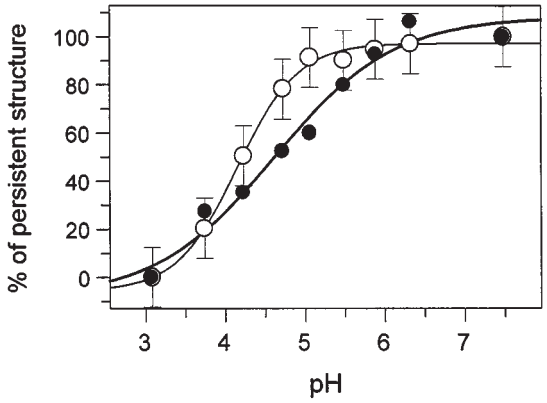

Figure 4. Acidic transition of cyt *c* in 9 mol/l urea in low ionic strength solvent (10 mmol/l phosphate buffer) followed at Soret region in the absence (empty circle) and in the presence of polyanion-PVS (solid circle). Depicted curves are fitted according the equation in Materials and Methods with fitting parameters: $pK_a = 4.15 \pm 0.20$, $n = 1.13 \pm 0.12$ (thin solid line), $pK_a = 4.62 \pm 0.20$, $n = 0.58 \pm 0.20$ (thick solid line). Estimated uncertainties in determination of ellipticity were $\pm$ 15% and are shown by error bars.

addition of PVS to cyt *c* did not affect denatured state of the protein as it follows from CD spectra in far-UV and Soret regions (Fig. 3). Interestingly, acidification of solvent from pH 7.0 to pH 3.0 (in 9 mol/l urea) led to a small but significant decrease of ellipticity in the case of free cyt *c* (Fig. 3A). Corresponding change in cyt *c*-PVS was not observed. Similarly, pH change from 7.0 to 3.0 led to a slightly more profound change in CD spectrum in Soret region of uncomplexed cyt *c* in comparison with the protein in complex with PVS. These observations indicate a stabilization effect of PVS on residual structure of denatured state of cyt *c*. pH-dependent acidic transitions of both denatured states monitored in Soret region are shown in Figure 4. The best fitting parameters for cyt *c* in 9 mol/l urea in absence and presence of PVS, based on an assumption of two-state transition, are $pK_a = 4.15 \pm 0.20$, $n = 1.13 \pm 0.12$ and $pK_a = 4.62 \pm 0.20$, $n = 0.58 \pm 0.20$, respectively. Less cooperative acidic transition of the denatured protein in the complex with polyanion with higher apparent pK_a value in comparison with free cyt *c* indicate stabilization tendency of polyanion on a "partially" unfolded state of cyt *c*. Although observed differences are relatively small, we believe that they are significant as it is not reasonable to expect large differences between stabilities of "partially" unfolded conformations of protein. Noteworthy, pK_a value 3.8 of transition of chemically unfolded cyt *c* (by 4.4 mol/l guanidine HCl) from 6-coordinated high-spin state to its 5-coordinated high-spin state has recently been determined by resonance Raman spectroscopy (Takahashi et al. 1997). Relatively close apparent pK_a value of the acidic transition of uncomplexed cyt *c*, in 9 mol/l urea, might indicate a sensitivity of Soret region of CD spectrum for this conformational transition.

Discussion

Uncomplexed cyt c in low ionic strength of solvent undergoes the acidic transition at an apparent p$K_a \sim 2.5$ as it was shown by absorbance spectroscopy 60 years ago by Theorell and Åkesson (1941). This view has been recently revisited by Myer and Saturno (1990) who showed that this transition consists of three-step process with pK_a's 3.6 ± 0.3, 2.7 ± 0.2, and 1.2 ± 0.2.

Presented results indicate that polyanion poly(vinylsulfate) affects both native and denatured states of cyt c as regards parameters characterising its acidic transition. An addition of PVS to native form of cyt c at neutral pH and low ionic strength solvent was registered only by tertiary structure of the protein reflected by slight changes in the aromatic and Soret regions of its CD spectrum (Fig. 1). Noteworthly, similar changes were observed in CD Soret spectrum of cyt c upon binding to negatively charged biomacromolecules such as cyt c peroxidase, polyglutamate, phosvitin (Garber and Margoliash 1994) and heparin (Sedlák 1997). However, the presence of polyanion significantly affects an apparent pK_a and a shift into slight acidic pH region is observed (Sedlák 1997; Sedlák and Antalík 1999). Previously determined pK_a's values of the transition, based on monitoring pH-dependence absorbance spectral changes in Soret region and at 620 nm (marker of high-spin state) 5.47 ± 0.15 and 4.55 ± 0.08, respectively, are in reasonable agreement with the obtained values presented in this work: 5.20 ± 0.15 in Soret region and 4.34 ± 0.25 at 262 nm of CD spectrum of complex cyt c with PVS. From the character of pH-dependences in Figure 2B, it is apparent that the acidic transition consists of at least 2 steps: (i) conformational transition at p$K_a \sim 5.2$ and (ii) final unfolding with p$K_a \sim 3$, the latter one reflected by the decrease of positive maximum in Soret region. The discussion below is confined only on the former of these transitions.

Besides significant distinction in pH region where the acidic transitions of free and complexed cyt c's occurred, the proteins also achieved different conformations in the corresponding acidic states, at pH ~ 2 and pH ~ 4, respectively. While free cyt c at pH ~ 2 is in "random coil" state with high-spin state of heme-iron, cyt c-PVS complex at pH ~ 4 is in molten globule (Sedlák and Antalík 1999) with likely mixed-spin state as it follows from relatively low absorbance at 620 nm (data not shown).

A question arises which amino acid residue was so efficient and shifted an apparent pK_a value of the acidic transition more than 2.5 pH unit towards neutral pH with significant effect on conformation of cyt c? At this point it is worth to mention results of several different groups that have determined pK_a value of the acidic transition of chemically-denatured cyt c at pH 5.1 ± 0.1 (Babul and Stellwagen 1971; Kaminsky et al. 1972b; Tsong 1975; Takahashi et al. 1997). This pK_a value was assigned to protonation of one of histidines 26 or 33 which were identified as predominant non-native ligands in unfolded cyt c (Muthukrishnan and Nall 1991; Colón et al. 1997). The nearly identical pK_a values of protonation of His-26 or -33 and the acidic transition of cyt c in complex with PVS led us to suggestion that both processes were interrelated. In addition, it was shown

that interaction of PVS with cyt *c* significantly perturbed thermal stability of the protein in slightly acidic pH (Sedlák and Antalík 1999). Important role of His-26 both for pH and for thermal stabilities of cyt *c* (Qin et al. 1995) indicate more specifically which histidine is likely involved in the acidic transition of cyt *c* in complex with PVS. In fact, the interaction of negatively charged sulfate group on polyanion with positively charged His-26 may perturb fine hydrogen bonding net around His-26 and affect both native and denatured states of the protein with severe consequences on its stability. This 'specific' interaction might explain such deleterious effect of polyanion on cyt *c* in slightly acidic pH as this observation has been in contrast with previously observed stabilization effect of polyanions on some protein structures (Burke et al. 1993; Rentzeperis et al. 1999). On the other hand, such strong electrostatic interaction can prevent complete unfolding of cyt *c* and stabilize a non-native conformation of the protein as it is demonstrated by the effect of PVS on residual structure of chemically-denatured cyt *c* in acidic pH and its influence on the pK_a of the transition (Fig. 3, 4). Stabilization of partially unfolded state of cyt *c* in acidic pH at elevated temperature upon binding of PVS observed in thermal-induced unfolding measurements performed by CD in far – UV region (Sedlák E., Petrenčáková D., Antalík M., unpublished results) further supports above mentioned results.

Unspecific interactions of polyanion that stabilize partially unfolded protein states may have important biological implication regarding an effect of negatively charged surfaces on protein folding. Recently, it has been shown that polyanion represented by PVS, heparin, polyglutamate, DNA and RNA accelerates refolding of certain proteins and prevents protein aggregation *in vitro* (Fedunová and Antalík 1998; Rentzeperis et al. 1999; Meng et al. 2001). On the other hand, one can speculate that at stress conditions in cells, accompanied by intensive transcription/translation processes, intrinsic chaperons might not be capable to fulfill their roles in time. Thus, the unspecific interaction with negatively charged surfaces (membranes, DNA, RNA) may stabilize proteins in partially unfolded state(s) (molten globule state?) and keep them available for actions of chaperons and preventing their aggregation. In addition, reduction dimensionality by binding on polyanions (Grasberger et al. 1986) enhances interaction between partially unfolded proteins, which may induce structure and stability of proteins (Uversky et al. 1999; Van den Berg et al. 1999) or may be part of a protein folding pathway (Segel et al. 1999). This can be the other effective way of polyanions how to accelerate protein refolding. All the mentioned qualities of polyanions (accelerating of refolding, preventing of aggregation) expressed through their capability to interact with both native and denatured states, may find interesting their utilization not only in bio/technology but also, due to their biocompatibility, in profylaction and treatment some of "protein misfolding and aggregation" diseases (Brimacombe et al. 1999).

Acknowledgements. This work was supported by Slovak Grant Agency (grants No. 7023 and 8047).

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Final version accepted: December 10, 2001