Structural Transformation of Cytochrome c and Apo Cytochrome c Induced by Sulfonated Polystyrene

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The structural transformation of cytochrome c (cyt c) and its heme-free precursor, apo cyt c, induced by negatively charged sulfonated polystyrene (SPS) with different charge density (degree of sulfonation) and chain length was studied to understand the factors that influence the folding and unfolding of the protein. SPS forms stable transparent nanoparticles in aqueous solution. The hydrophobic association of the backbone chain and phenyl groups is balanced by the electrostatic repulsion of the sulfonate groups on the particle surface. The binding of cyt c to negatively charged SPS particles causes an extensive disruption of the native compact structure of cyt c: the cleavage of Fe-Met80 ligand, about 40% loss of the helical structure, and the disruption of the asymmetry environment of Trp59. On the other hand, SPS particle-bound apo cyt c undergoes a conformational change from the random coil to α-helical structure. The folding of apo cyt c in SPS particles was influenced by pH and ionic strength of the solution, SPS concentration, and the degree of sulfonation and chain length of SPS. The folding can reach more than 90% of the α-helix content of native cyt c in solution. Poly(sodium 4-styrenesulfonate) (PSS), which is 100% sulfonated polystyrene and cannot form hydrophobic cores in the solution, induces only two-thirds of the α-helix content compared with SPS. It appears that the electrostatic interaction between PSS/SPS and apo cyt c induces an early partially folded state of apo cyt c. The hydrophobic interaction between nonpolar residues in apo cyt c and the hydrophobic cores in SPS particles extends the α-helical structure of apo cyt c.

Introduction

The biophysical basis of how a polypeptide chain self-assembles into a stable, native protein within a biologically relevant time scale has been a problem fascinating theoreticians and experimentalists for decades. The occurrence of diseases that resulted from the structural transformation and mis-folding of a peptide has made the study of structural diseases that resulted from the structural transformation and mis-folding of a peptide has made the study of structural transformation one of the most attractive research fields.1–4 The structural transformation can take place when the environment of a peptide is changed. For example, the protein folding and unfolding that happens on the surface of lipid membranes are essential biological processes in protein translocation across membranes, protein assembly, and protein functions.5–9

Cytochrome c (cyt c) plays an electron-transfer role in biological systems and also plays a role in programmed cell death.10,11 Because of its small size and stability, as well as the increasing evidence for its physiological roles in both soluble and membrane-bound forms,7,12 cyt c has been a subject of extensive studies in the general areas of protein chemistry and protein folding.8,13–17 The interactions of cyt c or its heme-free precursor, apo cyt c, with lipid membranes have been well studied. Bound to negatively charged lipid membranes, cyt c undergoes a partial unfolding,8,18–23 whereas apo cyt c undergoes a conformational transition from the random coil to α-helical structure.24–28 Cyt c also interacts with other anionic surface, such as heteropolytungstates, SDS, and sulfopropyl surfaces.29–32 It has been reported that the structural changes of the protein on negatively charged surface are the result of protonation of the surface residues, caused by a local acidic environment.33 The negatively charged surface-bound proteins are characterized by a highly helical conformation without stable tertiary structure.5,30

Synthetic polymers such as polyelectrolytes provide good models to explore the roles of electrostatic and hydrophobic interactions of the protein structures because polyelectrolytes are available in various charged forms, with varying degrees of hydrophobicity and different architecture.34,35 Proteins interact strongly with polyelectrolytes, and the interactions can be modulated by pH, ionic strength, and hydrophobicity of the solvent.36–38 It is widely recognized that the structure of a protein is a result of a delicate balance between the interactions within and outside the protein. In our present work, we have studied the structural transformation of cyt c and its heme-free precursor, apo cyt c, induced by negatively charged sulfonated polystyrene (SPS) with different charge density (degree of sulfonation) and chain length to understand the factors that influence the folding and unfolding of the
**Experimental Section**

**Reagents.** Styrene was washed with 5% sodium hydroxide and water, followed by vacuum distillation after drying with MgSO₄. Benzyl bromide was vacuum distilled. 2,2′-Bipyridine was re-crystallized in acetone. 1,2-Dichloroethane was distilled after drying with P₂O₅. Other chemicals were used as received without purification. Poly(sodium 4-styrenesulfonate) (PSS) with an average Mw of 7 × 10⁴ was from Aldrich.

**Synthesis of Polystyrene.** PS was synthesized by atom transfer radical polymerization. The molecular weight and molecular weight distribution were determined using size exclusion chromatography Agilent 1100 series with THF as the mobile phase at a flow rate of 1.0 mL/min.

**Sulfonation of PS.** The preparation of sulfonated PS based on the method described by Lantman et al. was carried out in 1,2-dichloroethane using acetyl sulfate as the sulfonating agent. The SPS was characterized by FTIR (Nicolet Magna 550 spectrophotometer). The degree of sulfonation of SPS was calculated based on sulfur analysis.

**Protein Purification.** Horse heart cyt c (type VI) from Sigma was dissolved in 10 mM phosphate buffer, pH 7.0. After oxidation of ferro cyt c with K₃[Fe(CN)₆], the protein was loaded on Sephadex G-25 column and eluted with the same buffer. Apo cyt c was prepared according to the method of Fisher et al. The concentrations of cyt c and apo cyt c were determined spectrophotometrically using the values of ε₁₆₀ = 106 100 M⁻¹ cm⁻¹ and ε₂₇₇ = 10 580 M⁻¹ cm⁻¹, respectively.

**Preparation of Solutions.** Stock Solution. A transparent stable colloidal dispersion of SPS in water was prepared according to the method of Jiang and co-workers. A total of 70 mg of SPS sample was initially dissolved in 10 mL of 50% THF in water and then further diluted 10 fold in water, by dropwise addition under stirring. The pH of the solution was adjusted with HCl or NaOH.

**Samples for Spectrophotometric Determination.** The samples were prepared by diluting 0.7 mg/mL SPS stock solution and 100 μM of cyt c or apo cyt c stock solution under stirring with solution of desired pH and ionic strength.

**Spectra Measurements.** CD spectra were recorded with Jasco J-715 spectropolarimeter equipped with Naslab temperature controller. The cell lengths are 0.1 and 1 cm for 190–250 and 250–500 nm regions, respectively. The ellipticity was recorded at a speed of 100 nm/min, 0.2 nm of resolution, 5–16 of accumulations, 1.0 nm of bandwidth, and 27 °C. UV spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer. Background (buffer with or without SPS/PSS) was recorded and subtracted from the original spectra.

**Results**

**Characterization of SPS.** The molecular weight and its distribution of two PS samples determined by size exclusion chromatography are shown in Table 1. The results indicate that the two samples possess narrow molecular weight distribution.

The SPS samples characterized by FTIR show the bands at 1010 and 1127 cm⁻¹ due to the in-plane bending vibration and stretching vibration of the sulfonated phenyl, respectively (data not shown). The degree of sulfonation (DS) of SPS was measured from sulfur analysis (Table 2) and calculated based on the average number of repeating units of the polymer chain. Therefore, 1 mol % of DS refers to an average of one out of every 100 repeating units styrene being sulfonated.

The introduction of sulfonic groups to PS shows a noticeable effect on the solubility behavior because of the big difference in polarity between the ionic group and hydrocarbon main chain and phenyl group. THF as a solvent of weak polarity was found to be able to dissolve all SPS samples except 55S-PS2, which has the highest DS. Ethanol and water can only dissolve the SPS samples with high DS. Both of the mixed solvents, ethanol/THF (1/1) and water/THF (1/1), can dissolve all of the SPS samples.

In this report, two series of SPS were used. (1) xS-PS1 series, the low Mw PS of 6100 Da with average repeating units of 60 and a broad DS (x) from 19 to 38 mol %. (2) xS-PS2 series, the much higher molecular weight of PS, (28 500 Da) with average repeating units of 280 and x from...
14 to 55 mol%. Additionally, 100% sulfonated polystyrene, PSS, with 340 repeating units, which are close to that of xS-PS2 series (280), and the monomer, SSS were also used to compare the effects with SPS on the structural transformation of cyt c and apo cyt c.

**Unfolding of Cyt c Induced by PSS and SPS. Soret Absorbance of Cyt c.** Horse heart cyt c is a well-characterized small protein (Table 3). Cyt c contains 24 basic residues and is a positively charged protein at neutral pH. A single heme moiety is covalently attached to a polypeptide chain by two thioether bridges. The heme iron is coordinated with two strong-field ligands, His18 and Met80. A Soret absorption at 410 nm indicates a strong-field low-spin state of the heme iron, whereas a Soret absorption at 394 nm and a new band at 620 nm indicate a high-spin state of the heme iron; that is, the ligands of the heme iron are replaced by two weak-field ligands from solvent, such as water. The absorption at 695 nm is the feature of Met80-iron ligands.\(^{44-46}\)

*Figure 1.* Absorbance spectra of mixtures between cyt c and different concentrations of 36S-PS2. Ten \(\mu\)M aliquots of cyt c were mixed with 0, 0.035, 0.07, 0.14, and 0.35 mg/mL of 36S-PS2, in 10 mM phosphate, pH 7.0. Inset: The spectra between 600 and 800 nm of (a) 40 \(\mu\)M cyt c alone and (b) 40 \(\mu\)M cyt c + 0.35 mg/mL 36S-PS2.

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Circular Dichroism of Cyt c. Figure 2 shows far-UV, near-UV, and Soret region CD spectra of the mixtures of cyt c with various concentrations of PSS in 10 mM phosphate buffer, pH 7.0. The CD spectra of cyt c in aqueous solution are identical to those previously reported.\(^8\) The far-UV CD spectrum of cyt c (Figure 2A) shows a typical \(\alpha\)-helical structure. There was a decrease in the negative peak at 222 nm when PSS was added to the protein solution, suggesting a decrease in \(\alpha\)-helical content in cyt c.

*Figure 2.* (A) Far-UV, (B) near-UV, and (C) Soret regions of CD spectra of mixtures between cyt c and different concentrations of PSS. Ten \(\mu\)M aliquots of cyt c were mixed with 0, 0.015, 0.035, 0.07, 0.14, and 0.35 mg/mL PSS, in 10 mM phosphate buffer, pH 7.0.

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The near-UV CD spectra of cyt c at 282 and 288 nm have been assigned to the tertiary structural packing of Trp59.\(^{50}\) With the increasing concentration of PSS, these two peaks gradually decrease and finally disappear (Figure 2B), which is similar to the structural change of cyt c in 6.5 M GdnHCl.
solution (data not shown). 38S-PS1 shows the same effect on the changes of near-UV CD spectra of cyt c (data not shown). These results indicate that both PSS and 38S-PS1 disrupt the asymmetry environment of Trp59.

The Soret CD spectrum of cyt c in aqueous solution shows a strong negative band at 418 nm and a positive band at 405 nm due to coupling of heme $\pi-\pi^*$ electric dipole transition moments with those of nearby aromatic residues of the polypeptide. After each addition of PSS, there was a decrease in intensity of the negative band with concomitant increase of the positive band, with a small red-shift (Figure 2C). These are consistent with the changes observed on the GdnHCl-denatured cyt c (data not shown) and the SDS-denatured cyt c, a characteristic of the breakage of Met80-iron ligand.

Figure 3A shows the changes of molar ellipticity of cyt c at 222 nm with the increase of PSS or 38S-PS1 concentration. The molar ellipticity changed linearly when PSS concentration increased from 0 to 0.07 mg/mL and then did not change significantly as the concentration increases further. The maximum change of molar ellipticity of cyt c here is about 50% of the change of cyt c denatured by GdnHCl (data not shown). This result means that a significant amount of helical structure of cyt c remains. Compared with PSS, 38S-PS1 shows less effect on the changes of molar ellipticity. It is interesting to note that the small monomer, SSS, shows no significant effect on the molar ellipticity of cyt c.

Figure 3B shows the changes of molar ellipticity of cyt c at 405 nm with increasing concentration of PSS or 38S-PS1. The molar ellipticity increased sharply when the concentration of PSS increased from 0 to 0.07 mg/mL and then leveled off at higher concentration of PSS. Compared with PSS, the same range of concentration of 38S-PS1 caused similar change on the ellipticity but with smaller maximum effect. In contrast, SSS, the monomer, did not cause any significant CD changes of cyt c over the whole concentration range studied.

**Folding of Apo Cyt c Induced by PSS and SPS.** The far-UV CD spectrum of apo cyt c in aqueous solution, pH 2.1, indicates that apo cyt c mainly possesses a random coil conformation (Figure 4). Upon addition of 38S-PS1 to apo cyt c, there was a gradual decrease in intensity of the band around 200 nm with concomitant appearance of the bands at 208 and 222 nm. These results indicate that the interaction of apo cyt c with 38S-PS1 can induce a helical structure.

**Effects of Ionic Strength.** Electrostatic interaction plays an important role in the binding of apo cyt c to PSS. Figure 5 shows the effect of NaCl concentration on the molar ellipticity of apo cyt c at 222 nm induced by 0.035 mg/mL PSS and indicated concentrations of NaCl, pH 2.0. At pH 2.0, an apo cyt c molecule carries...
24 positive charges. Obviously, low concentration of NaCl can partially screen the electrostatic repulsion caused by positive charges and helps the folding of apo cyt c. In contrast, higher concentration of NaCl can disrupt the electrostatic attraction between apo cyt c and PSS and then decreases the α-helix level. In addition, turbidity appeared in the apo cyt c-PSS solutions with high concentration of NaCl, which may decrease the CD signal, as well.

Effects of pH. Figure 6 shows the influence of pH on the folding of apo cyt c induced by 29S-PS1. The α-helix content of apo cyt c induced by 29S-PS1 reached its maximum at pH 3.5. It is known that apo cyt c carries 24 positive charges at pH 2.0, and generally, the positive charges decrease when pH increases. Perhaps, fewer positive charges at pH 3.5, as compared to pH 2.0, suppress the electrostatic repulsion within apo cyt c so that 29S-PS1 can induce the α-helix lever to about 90% of the native structure. When the pH was increased from 3.5 up to 10.5, the helical structure induced by SPS decreased sharply because of the reduction of positive charges in apo cyt c. At pH 10.5, apo cyt c carries negative charges, but the signal at 222 nm of the mixture was larger than that of apo cyt c alone. There may be local positive charges in apo cyt c around pH 10.5 that can interact with SPS and resulted in increase in helical structure.

Effects of Degree of Sulfonation of SPS. Figure 7 shows the changes of molar ellipticity of apo cyt c at 222 nm induced by four SPS samples with different DS, at pH 2.0. The chain lengths of the four SPS samples are the same (repeating units 280). The solutions of the four samples were prepared to keep the sulfonate group content identical at 200 μM. This means that the weight concentration is higher for the SPS sample with lower DS. The result in Figure 7 shows the higher the DS, the more effective on the induction of structural transformation of apo cyt c. However, the SPS with lower DS can still induce a substantial content of helical structure, provided the SPS concentration is high enough (similar results can be seen in Figure 4).

Effects of Hydrophobicity. Figure 8 compares the changes of molar ellipticity of apo cyt c at 222 nm induced by PSS and 36S-PS2 at pH 2.0. At the concentration of 0.035 mg/mL, PSS shows a stronger effect on inducing the helical structure than 36S-PS2. However, further addition of PSS cannot significantly change the α-helix extent of apo cyt c.

On the other hand, the helical structure induced by 36S-PS2 continues a gradual increase over the whole concentration range especially at the concentration larger than 0.14 mg/mL. Overall, 36S-PS2 induced more helical structure than PSS did. As the chain lengths of 36S-PS2 and PSS are similar, the effect of molecule weight can be excluded. Considering that 36S-PS2 contains 64% of un-sulfonated styrene units, the fact that 36S-PS2 can induce one-third more helical structure than PSS at high polymer concentration indicates that the un-sulfonated phenyl groups in 36S-PS2 also contribute the structural transition of apo cyt c.

Effects of Chain Length of SPS. Figure 9 shows the molar ellipticity of apo cyt c at 222 nm with the addition of PSS or 36S-PS2. The apo cyt c concentration is 10 μM, and the pH is 2.0.

Discussion

SPS with a low degree of sulfonation is insoluble in water. Jiang et al. developed a “microphase inversion” method to produce surfactant-free nanoparticles, composed of lightly
sulfonated polystyrene, by mixing SPS-THF solution with an excess amount of water.\textsuperscript{39,40} Such nanoparticle dispersion is transparent and stable. Fluorescence and dynamic laser light scattering studies demonstrate that the ionic groups on the particle surface stabilize the hydrophobic core made of the polystyrene chains. Such nanoparticles have a relatively narrow size distribution. The particle size is determined by the degree of sulfonation, the initial concentration of the SPS-THF solution, as well as the mixing order. Obviously, the SPS chains would be more extended with higher degree of sulfonation, which makes SPS carry more ionic groups. The results from the studies of carboxylated polystyrene particles, with degree of carboxylation similar to our degree of sulfonation, show that the particle size increases and the particle density decreases with increasing degree of carboxylation.\textsuperscript{52} For the carboxylated polystyrene with 49.2\% (mol \%) degree of carboxylation, the hydrodynamic radius of the particles is less than 100 nm.

THF is a weak polar solvent. The final concentration of THF in the nanoparticle solution is usually less than 2.5 v\%. It was confirmed that removal of THF by dialysis did not show a substratal effect on the stability and size distribution of the particles.\textsuperscript{40,52} On the other hand, THF has no effect on the structural transformation of cyt c and apo cyt c at the concentration used in the preparation (data not shown). Therefore, we did not remove THF in the preparation procedure for convenience.

The intrinsic fluorescence of Trp59 is a sensitive probe for cyt c unfolding. In the native structure, Trp59 fluorescence is largely quenched by the adjacent heme group because of fluorescence resonant energy transfer (FRET). In the unfolded structure, Trp59 fluorescence intensity increases because of the decrease of FRET, which is caused by the increase of the distance between Trp59 and heme.\textsuperscript{45} Fluorescence emission spectra were measured with an excitation wavelength of 295 nm and a spectral bandwidth of 5 nm for excitation and emission (Cary Eclipse fluorescence spectrophotometer). The spectrum of cyt c shows a maximum at 340 nm, the fluorescence intensity of apo cyt c is stronger than that of cyt c, and the peak shifts to 356 nm, meaning that Trp59 of apo cyt c is in a more hydrophilic environment compared to cyt c Trp59. On the other hand, SPS shows very strong fluorescence intensity at 374 nm. After mixing SPS with apo cyt c, the spectrum exhibits a strong fluorescence with the maximum at 356 nm. Interestingly, for the mixture of SPS and cyt c, the fluorescence peak appears at 348 nm with much weaker intensity compared to that of SPS-apo cyt c. The most noticeable difference between the fluorescence spectra of SPS-cyt c and SPS-apo cyt c seems to be the quenching effect of the heme of cyt c on the fluorescence of SPS. This speculation was confirmed by comparison with the fluorescence spectra of SPS and hemin chloride (data not shown). Because of the complexity of the fluorescence spectra, we could not obtain quantitative information of the unfolding of cyt c by SPS particles. However, we may draw a conclusion from the fluorescence quench and the red shift of SPS-cyt c that the environment of Trp59 and heme of cyt c is changed, which is consistent with the results of CD and UV-vis studies.

SPS and PSS are negatively charged polyelectrolytes. Cyt c carries 7 positive charges at pH 7. Therefore, the electrostatic interaction may result in complexes of SPS-cyt c or PSS-cyt c. The binding of cyt c to negative charges of SPS or PSS causes an extensive disruption of the native compact structure of cyt c. Both SPS-bound and PSS-bound cyt c lack many features of the native structure: the cleavage of Fe-Met80 ligand, a significant loss of the helical structure, and the disruption of the asymmetry environment of Trp59. As discussed above, the disruption of the native structure of cyt c by SPS is not as extensive as that by PSS. This is understandable when the structural difference between SPS and PSS in solution is taken into account. SPS chains aggregate into particles; thus, only the negative charges on the particle surface could interact with the positive charges in cyt c. In contrast, PSS is 100\% sulfonated polystyrene, and PSS chains dissolve in the solution molecularly; thus, all of the negative charges have the opportunity to interact with cyt c. SSS does not show any effect on the conformational transition of cyt c although it is expected to interact with cyt c. This clearly shows the importance of the chainlike structure of polyelectrolytes.

It has been known that cyt c binding on a negatively charged lipid membrane remains a highly helical structure but has no tightly packed native tertiary structure including the cleavage of Met80 heme ligation.\textsuperscript{8,18–23} Spectral and CD analysis (Figures 1 and 2) show that the interactions between SPS/PSS and cyt c are similar to the results of the negatively charged lipid L-PG micelle-bound cyt c.\textsuperscript{8} Lipid forms micelles or vesicles, whereas SPS forms particles in aqueous solution. The cores of the SPS particles are more hydrophobic than the surface. SPS-bound cyt c has 60\% and PSS-bound cyt c has 50\% of helical structure, and both are less than the lipid-bound cyt c.

It was reported that anions could induce the conformational transition of unfolded proteins, including cyt c.\textsuperscript{53–55} The effectiveness of the anions on inducing the conformational transition of acid-denatured cyt c is in the order of ferricyanide > ferrocyanide > sulfate > trichloroacetate > thiocyanate > perchlorate > iodide > nitrate > trifluoroacetate > bromide > chloride. To investigate whether the structural transition of apo cyt c mixed with SPS results from anions or not, the monomer, SSS was used instead of SPS. At the
concentration similar to SPS used, SSS had no effect on the conformational transformation (data not shown). In the experiments, the pH of the solutions was adjusted with HCl because the induction ability of chloride is not as effective as other anions. We confirmed that sodium chloride alone had no significant induction on the structural transformation of apo cyt c at the concentration of 100 mM.

Apo cyt c undergoes a conformational transition from a random coil to α-helical structure on binding to negatively charged lipid membranes. Kinetic investigation showed that the folding proceeded via a collapsed intermediate state and the formation of the α-helical structure preceded membrane insertion. In this study, we demonstrated that SPS particles could induce structural transformation of apo cyt c. The helical structure content of apo cyt c induced by SPS particles depends on pH and the ionic strength of the solution, as well as the concentration, degree of sulfonation, and chain length of SPS. The α-helix content induced by SPS particles can reach more than 90% of native cyt c in the aqueous solution, which is close to that induced by negatively charged lipid micelles. Therefore, both of the third-thirds of the α-helix content compared with SPS. It appears that the electrostatic interaction of PSS/SPS and apo cyt c induces an early partially folded state of apo cyt c in lipid micelles and vesicles. Therefore, both of electrostatic and hydrophobic interactions contribute to the folding of apo cyt c in SPS particles.

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Abbreviations

cyt c, cytochrome c
DS, degree of sulfonation
GdnHCl, guanidine hydrochloride
PS, polystyrene
PS1, polystyrene with 60 repeating units
PS2, polystyrene with 280 repeating units
SPS, randomly sulfonated polystyrene
PSS, poly(sodium 4-styrenesulfonate) with 340 repeating units
SDS, sodium dodecyl sulfate
THF, tetrahydrofuran
SO3H, sulfonated styrene sodium

References and Notes

(1) Gorman, P. M.; Chakrabarty, A. *Biomacromolecules* 2001, 60, 381–94.

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