Encapsulation of Ferricytochrome *c* into the Nanoparticle Made from a Natural Polysaccharide: Schizophyllan

Mika Kasuga,[1] Naohiko Shimada,[1] Yoichi Takeda,[1] Seiji Shinkai,[2,3] and Kazuo Sakurai*[1]
[1]*Department of Chemical Process & Environments, Faculty of Environmental Engineering, The University of Kitakyushu, 1-1 Hibikino, Wakamatu-ku, Kitakyushu, Fukuoka 808-0135*
[2]*Department of Chemistry & Biochemistry, Graduate School of Engineering, Kyushu University, Motooka 744, Nishi-ku, Fukuoka 819-0395*
[3]*Center for Future Chemistry, Kyushu University, Motooka 744, Nishi-ku, Fukuoka 819-0395*

(Received July 12, 2006; CL-060792; E-mail: sakurai@env.kitakyu-u.ac.jp)

A novel protein/polysaccharide complexation was found when unfolded ferricytochrome *c* (Cyt-*c*) was mixed with denatured schizophyllan (SPG) and the renaturation of both protein and polysaccharide was carried out simultaneously. Upon the complexation, the Q band was split, indicating that ferric Cyt-*c* was converted into the ferrous state. The changes in circular dichroism and fluorescence from tryptophan showed that the entire protein conformation was drastically deformed.

Polymeric nanoparticles have been demonstrated to be able to deliver drugs to enhance therapeutic efficacy.[1] Akiyoshi et al.[2] are the first to demonstrate that cholesterol-bearing pullulan can form a nanosize gel and the gel particle spontaneously encapsulates proteins. They explained this encapsulation by the distinct hydrophobic domain that is provided by the combination of the hydrophilic polysaccharide and the hydrophobic cholesterol, and thus can move dynamically to engulf hydrophobic materials. Meanwhile, Sakurai, Shinkai, and co-wokers found[3–5] that a natural polysaccharide called schizophyllan (SPG, Figure 1A) forms a complex with polynucleotides, carbon nanotubes, and some hydrophobic polymers. SPG is composed of β-(1→3)-D-glucan main chain and one β-(1→6)-D-glycosyl side chain links to the main chain at every three glucose residues.[6] In nature, SPG takes a triple helix, and it can denature to a random coil by dissolving with DMSO or alkaline solutions. They showed that the major driving force of the complexation of SPG is the combination of hydrophobic and hydrogen-bonding interactions.[3] Recently, Hasegawa et al. showed that SPG can encapsulate porphyrin molecules and that the complexation induces them to assemble in a fibrous manner.[7] Their work intrigued us to investigate whether heme proteins such as Cytochrome *c* (Cyt-*c*) can interact with SPG.

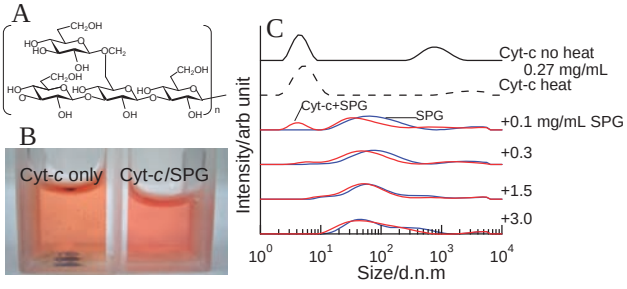

Figure 1. Repeating unit of SPG (A), comparison of the solution colors between Cyt-*c*/SPG and Cyt-*c* only (B) and the mixing ratio dependence of the particle size distribution determined by dynamic light scattering (DLS).

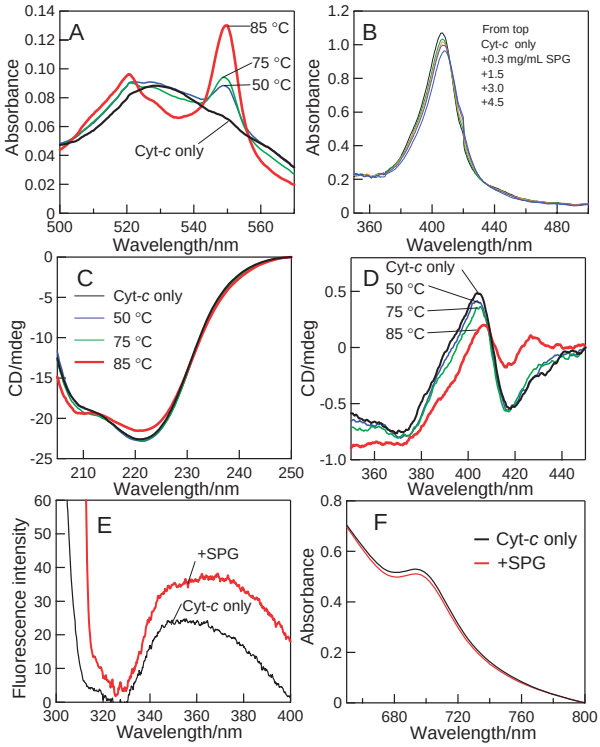

Figure 2. Spectral changes induced by complexation between Cyt-*c* and SPG. A: absorption spectra in Q band after SPG was added at different temperatures, B: absorption spectra in Soret band after SPG added at different compositions, C: CD spectra for the amide region, D: CD spectra in Soret band, E: fluorescence spectra from Trp59, λ_{ex}: 295 nm. [SPG] = 3 mg/mL, [Cyt-*c*] = 0.27 mg/mL, F: charge-transfer band from the sulfur of Met80 to heme iron(III), [SPG] = 5 mg/mL, [Cyt-*c*] = 12.5 mg/mL, All samples were measured at 20 °C after addition of SPG. Cyt-*c* only indicates spectra of Cyt-*c* only after heating at 85 °C.

When Cyt-*c* (12,327 Da)[8] was heated above 85 °C in 100 mM Tris-HCl (pH = 7.3), the complete unfolding of the original three-dimensional structure was observed with the circular dichroism (CD) for the Soret and amide-bond regions. To this unfolded Cyt-*c* solution, a SPG/NaOH (0.1 M, pH = 13) solution was added. Mitsui Sugar Co. kindly supplied a SPG sample($M_w = 1.5 \times 10^5$).[3,9] Upon mixing, the Tris buffer adjusted pH to be around 7–8, and the mixed solution was cooled

on standing to room temperature (denoted by Cyt-*c*/SPG). SPG takes the random coil conformation in 0.1 M NaOH solution and the triple helix at pH = 7–8, and the renaturation (i.e., random coil to triple helix) due to pH change takes about a few hours.[7] Therefore, during the above-mentioned cooling process, the renaturation of both Cyt-*c* and SPG occurs concurrently.

Figure 1B compares the solution colors between Cyt-*c*/SPG and Cyt-*c* only, showing that the addition of SPG changed the color from orange to faint red. The color change became less distinct when the mixing was carried out at lower than 50 °C. These features suggest that SPG interacts with Cyt-*c* during the renaturation process of SPG and Cyt-*c*. Figure 1C shows the particle size distributions when the different amounts of SPG were added. Native and renatured Cyt-*c* show the particle size of about 5 nm and renatured SPG appears around 30 nm. With increasing the amount of SPG, the Cyt-*c* peak became smaller and disappeared when the SPG concentration was more than 1.5 mg/mL. This change suggested that Cyt-*c* was encapsulated into the SPG particle.

The visible absorption spectrum of Cyt-*c* consists of two main bands: the Q and Soret bands. The Q band is related to electronic transitions for the porphyrin ring, and it becomes one broad peak at the ferric (oxidized) state and splits into two relative sharp peaks at the ferrous (reduced) state. The Soret band has a large molar absorption coefficient and has been known to be sensitive for the conformational changes of the protein. Generally, upon the reduction of the heme iron, the Soret band shows the red shift and increases the intensity.[10]

Figure 2A shows the Q bands when SPG was added to the ferric Cyt-*c* solutions at different temperatures. As mentioned above, mixing at 85 °C provides the most distinct change. Upon the addition, the broad Q band around 530 nm splits into two bands, indicating that some amount of Cyt-*c* was converted into the ferrous form. From the reported extinction coefficients of 28.0 and 8.4 (E^{mM}) for the reduced and oxidized forms,[11] approximately 40 mol % of Cyt-*c* was converted to the ferrous state. The intensity of the Q band increased proportionally to the amount of added SPG (data not shown). Figure 2B compares the Soret band when different amounts of SPG were added, showing that the addition of SPG induced the red shift and decreased the intensity of the band. The red shift is consistent with the reduction of Cyt-*c*, however, the decreased intensity is not. The decreased intensity may be related to deformation of the entire protein conformation upon mixing. Other polymers such as dextran, pulluran, and PEG were examined if they induce the similar spectral changes when they are added to Cyt-*c* in the same way as SPG. We found that the changes are considerably small compared with that of SPG.

Figure 2C shows the CD spectral changes in the amide region upon mixing with SPG. The 222-nm band decreased and the 208-nm band increased. These changes were small, but significant, considering that only 40 mol % of Cyt-*c* was interacting with SPG. The CD spectral changes suggest that the amount of α-helix was decreased and thus the entire protein's conformation was altered upon the complexation. Figure 2D shows the CD spectra for the Soret band. The intensities of both negative and positive bands were decreased upon the mixing, compared with the original ferric state. The ferrous state shows a reverse Cotton effect of the ferric one, and the observed spectra should consist of both states. Therefore, the decreased bands in Figure 2D are consistent with the other spectral changes. Figure 2E shows the fluorescent spectra comparing between before and after addition of SPG. The fluorescence from tryptophan was increased upon mixing. It is known that the Trp59 of Cyt-*c* is located close to the heme, and thus the fluorescence is reduced owing to energy transfer. The increased fluorescence confirms that protein's conformational changes were induced upon the SPG mixing.

DLS suggested that most Cyt-*c* were ingested in the SPG particle when they were renatured in the same solution. The spectral observation indicated that SPG complexed about 40 mol % of Cyt-*c*, and the complexed Cyt-*c* had the ferrous iron and the protein conformation was deformed. The complexation was only observed when we mixed base-renatured SPG with unfolded Cyt-*c*. Referring to Hasegawa's work,[7] we can presume that SPG is bound to the heme-porphyrin during the renaturation processes. The base-renatured SPG at pH = 13 should have some alkoxide anions, and these anions may reduce the iron of the heme. If this speculation is correct, Met80 (axial ligand) should be replaced by the oxygen atom of SPG ($M_w = 6000$). We checked whether decrease was observed in the charge-transfer band from the sulfur of Met80 to heme iron(III) at 695 nm. A faint decrease of the peak was observed (Figure 2F), although the peak change was very small.

To sum up our finding, SPG can encapsulate unfolded Cyt-*c* during the renaturation process. The complexed Cyt-*c* has the ferrous iron, probably coordinated by SPG oxygen atom, and the protein conformation is deformed. This finding should provide a new approach to study protein–polysaccharide interactions that become an important issue recently. Furthermore, the Cyt-*c*/SPG particle may be applicable to chemotherapy such as an apoptosis inducer.

This work is finically supported by JST SORST program and Grant-in-Aid for Scientific Research (Nos. 16350068 & 16655048).

References and Notes

1 K. Emoto, M. Iijima, Y. Nagasaki, K. Kataoka, *J. Am. Chem. Soc.* **2000**, *122*, 2653.
2 K. Akiyoshi, S. Kobayashi, S. Shichibe, D. Mix, M. Baudys, S. W. Kim, J. Sunamoto, *J. Controlled Release* **1998**, *54*, 313.
3 K. Sakurai, S. Shinkai, *J. Am. Chem. Soc.* **2000**, *122*, 4520.
4 K. Sakurai, M. Mizu, S. Shinkai, *Biomacromol.* **2001**, *2*, 641.
5 K. Sakurai, K. Uezu, M. Numata, T. Hasegawa, C. Li, K. Kaneko, S. Shinkai, *Chem. Commun.* **2005**, 4383.
6 K. Tabata, W. Ito, T. Kojima, S. Kawabata, A. Misaki, *Carbohydr. Res.* **1981**, *89*, 121.
7 T. Hasegawa, F. Tomohisa, M. Numata, C. Li, A.-H. Bae, S. Haraguchi, K. Sakurai, S. Shinkai, *Chem. Lett.* **2005**, *34*, 1118.
8 a) *Handbook of Biochemistry*, CRC Press, **1968**. b) SIGMA, Product information. Cytochrome *c* from bovine heart (C 2037).
9 T. Itou, A. Teramoto, M. Matuo, H. Suga, *Carbohydr. Res.* **1987**, *160*, 243.
10 I. Hamachi, A. Fujita, T. Kunitake, *J. Am. Chem. Soc.* **1997**, *119*, 9096.
11 B. F. Van Gelder, E. C. Slater, *Biochim. Biophys. Acta* **1962**, *58*, 593.

Published on the web (Advance View) September 9, 2006; doi:10.1246/cl.2006.1120