NMR studies on binding sites and aggregation–disassociation of fluorinated surfactant sodium perfluorooctanoate on protein ubiquitin

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A central goal of the studies on protein folding is to obtain information on how the binding of different ligands can affect protein structure and function and how the disordered polypeptide chain of a denatured protein folds into the native structure. Surfactants, a group of amphiphatic substance with hydrophilic groups at one end and hydrophobic groups at the other, are important protein ligands. They can induce the unfolding of proteins, and in some special cases, stabilize proteins at a very low concentration [1]. They are also solubilizing agents for membrane proteins [2]. Organisms contain a lot of amphiphatic substances, and many biological and medical products as well as food contain both proteins and surfactants [2]. There are some reports studying protein-surfactant interaction by such as surface-tension, viscosity, dialysis, and dynamic light scattering, and speculating the binding mechanism of surfactant to protein [2]. However, few works study how surfactant binding affects protein structure and function at the molecular level. Otzen studied the effect of sodium dodecyl sulfate micelle structure, ionic strength, pH, and temperature on the unfolding of protein S6 [3]. Sun et al. studied the interaction of BSA with cetylpyridinium bromide and found that cetylpyridinium bromide at low and high concentrations could induce the unfolding and refolding of BSA, respectively [4]. The existence of specific binding and cooperative binding in the binding process of surfactants to protein is now widely accepted [2], but detailed mechanisms at the molecular level, such as the surfactant binding sites and the binding process, remains elusive. Our approach to this problem makes use of NMR spectroscopy to observe the detailed structural changes of protein when interacting with surfactants.

The fluorinated surfactant sodium perfluorooctanoate (SPFO) could bind onto ubiquitin (UBQ) and induce the unfolding of UBQ. By using 15N-edited heteronuclear single-quantum coherence (HSQC) NMR and 19F NMR to monitor 15N-labeled UBQ and SPFO, respectively, the binding sites and the aggregation process of SPFO on UBQ at various SPFO concentrations were observed. A detailed process from specific binding to cooperative binding of SPFO on UBQ, and a detailed structure change of UBQ upon the increase of SPFO concentration were obtained. The refolding of UBQ in UBQ–SPFO complex was carried out by adding cationic surfactant. It was shown that added cationic surfactants formed mixed micelles with SPFO and resulted in the dissociation of the UBQ–SPFO complex, and consequently, most ubiquitin could be refolded to its native state.

1. Introduction

A central goal of the studies on protein folding is to obtain information on how the binding of different ligands can affect protein structure and function and how the disordered polypeptide chain of a denatured protein folds into the native structure. Surfactants, a group of amphiphatic substance with hydrophilic groups at one end and hydrophobic groups at the other, are important protein ligands. They can induce the unfolding of proteins, and in some special cases, stabilize proteins at a very low concentration [1]. They are also solubilizing agents for membrane proteins [2]. Organisms contain a lot of amphiphatic substances, and many biological and medical products as well as food contain both proteins and surfactants [2]. There are some reports studying protein-surfactant interaction by such as surface-tension, viscosity, dialysis, and dynamic light scattering, and speculating the binding mechanism of surfactant to protein [2]. However, few works study how surfactant binding affects protein structure and function at the molecular level. Otzen studied the effect of sodium dodecyl sulfate micelle structure, ionic strength, pH, and temperature on the unfolding of protein S6 [3]. Sun et al. studied the interaction of BSA with cetylpyridinium bromide and found that cetylpyridinium bromide at low and high concentrations could induce the unfolding and refolding of BSA, respectively [4]. The existence of specific binding and cooperative binding in the binding process of surfactants to protein is now widely accepted [2], but detailed mechanisms at the molecular level, such as the surfactant binding sites and the binding process, remains elusive. Our approach to this problem makes use of NMR spectroscopy to observe the detailed structural changes of protein when interacting with surfactants.

The fluorinated surfactant sodium perfluorooctanoate (SPFO) was a protein denaturant [5]. In our previous work [6], we found that fluorinated surfactants exhibited stronger interactions with proteins than hydrogenated ones with similar critical micelle concentrations (cmc). In order to study the detailed mechanisms of surfactant–protein interaction at the molecular level, especially the binding sites and the binding process of surfactants on proteins, in this study SPFO and 15N-labeled ubiquitin (UBQ) were used. As SPFO contains no H and N atoms, and UBQ contains no F atoms, we used 15N-edited heteronuclear single-quantum coherence (HSQC) NMR and 19F NMR to monitor 15N-labeled UBQ and SPFO, respectively. First of all, the binding sites and aggregating process of SPFO on UBQ were determined. Subsequently, we tried to refold UBQ by adding a cationic surfactant.

2. Materials and methods

Sodium perfluorooctanoate (C17H35COONa, SPFO) was prepared by the procedure described in our previous article [7]. Dodecyltrimethylammonium chloride (C12H25N(CH3)3Cl, DTAC) and cetyltrimethylammonium chloride (C16H33N(CH3)3Cl, CTAC) were from Alfa Aesar. All
Fig. 1. The HSQC spectra of ubiquitin (UBQ) in the presence of SPFO at 298 K.
surfactant solutions were prepared in 25 mM PBS (pH 5.7) buffer. The water was deionized, distilled and passed through a Millipore Q purification system (Millipore Corporation, Bedford, MA). All other chemicals were of analytical grade.

Ubiquitin sample was prepared by dissolving 2.2 mg uniformly $^{15}$N-labeled ubiquitin (provided by Beijing Nuclear Magnetic Resonance Center, BNMRC) in 500 μl buffer (25 mM PBS, pH=5.7, 90% H$_2$O and 10% D$_2$O) to the final concentration of 0.5 mM.

All the NMR experiments were carried out at 25 °C on the Bruker Avance 400-MHz spectrometer equipped with Z-axis pulsed field gradient BBO probe in BNMRC. For the two-dimensional $^{15}$N-edited heteronuclear single quantum coherence (HSQC) spectra, the spectral widths were set to 5592.8 Hz (14 ppm) and 1338.1 Hz (33 ppm) with the carrier frequencies at 1880.8 Hz (4.7 ppm) and 4744.0 Hz (117 ppm) for $^1$H and $^{15}$N, respectively. 1024 and 128 complex points were collected for $^1$H and $^{15}$N dimensions with 4–128 transients (depending on the peak strength) and 1.1 s recycle delay. The WATERGATE method was applied to suppress the water magnetization and the quadrature detection in the indirect dimension was achieved by the States-TPPI method. All the 2D HSQC spectra were processed using the program NMRPipe [8] and analyzed using the program NMRView [9]. All the 1D $^{19}$F spectra were collected with 16 k complex points, 128 transients and 2 s recycle delay. But for the $^{19}$F NMR experiments of LPFN, the spectral widths were set to 20325.2 Hz (54 ppm) with the carrier frequencies at $-39532.3$ Hz ($-105$ ppm). And for the $^{19}$F NMR experiments of SPFO, the spectral widths were set to 22522.5 Hz (60 ppm) and the carrier frequencies at $-39532.3$ Hz ($-105$ ppm).

The experiments were carried out by addition of surfactant into UBQ solution. The spectra of $^{19}$F NMR of surfactant were measured and the change of chemical shifts of $^{19}$F, $\Delta\delta$, was analyzed among different concentration of surfactant. The values of $^{19}$F chemical shift changes, $\Delta\delta$, of fluorinated surfactant were defined as $\Delta\delta(F)=\delta_{\text{mix}}-\delta_{\text{mono}}$, where $\delta_{\text{mix}}$ was the chemical shifts obtained in the mixtures of
3. Results and discussion

3.1. Binding sites of SPFO on UBQ

Fig. 1a shows the NMR spectra of UBQ. When SPFO is gradually added into 0.5 mM UBQ at pH 5.7, at SPFO concentration of 0.99 mM, the HSQC spectrum of UBQ does not show significant changes, indicating that SPFO does not bind to UBQ at low SPFO concentration (Fig. 1b). By increasing SPFO to 2.9 mM, three main segments in the UBQ molecule are destabilized, which is shown in the disappearance or shift of the peaks (Fig. 1c). The first segment contains residues V5, T7, L8, T9, K11, I13, T14, and E16, which are located in the loop connecting two strands of β-sheet. The second one contains K29, D32, K33, E34, and I36, which is in the α-helix. The third one includes Q41, R42, L43, I44, F45, A46, K48, and Q49, which are in the β-sheet [10,11]. These binding sites are consistent with those predicted by Irbäck et al. by Monte Carlo simulation [12]. Notably, these sites are positively charged and located on the protein surface (Fig. 2). In addition, they are also near the hydrophobic region of UBQ (Fig. 2), which suggests that hydrophobic interaction might also be involved. At this SPFO concentration which is far below cmc of SPFO (∼31.0 mM in water) [5], the molar ratio of SPFO to UBQ is about 6:1, so SPFO binds to UBQ as a monomer, which is called specific binding. The driving force is predominantly electrostatic interactions, as SPFO does not form aggregates on UBQ.

When the concentration of SPFO is increased to 4.7 mM, the affected segments are extended, most of which are in the residues 40–76 of the peptide sequence (Fig. 1d). These residues are mostly positively charged. With the increase of SPFO concentration, the positively charged binding sites of UBQ are gradually occupied until saturation is achieved. By further increasing SPFO concentration to 7.4 mM, most of the UBQ structure is modified, which is shown in Fig. 2.

Schematic representation of the structural changes of UBQ when binding SPFO. a, c and e are the electrostatic potential diagrams of UBQ, and b, d and f are the ribbon diagrams. In b and d, the residues labeled as yellow correspond to the changes of UBQ HSQC spectra at SPFO 2.99 mM. In f, the residues in the circle are those that keep intact in UBQ HSQC spectra at SPFO 7.4 mM.

Fig. 2. Schematic representation of the structural changes of UBQ when binding SPFO. a, c and e are the electrostatic potential diagrams of UBQ, and b, d and f are the ribbon diagrams. In b and d, the residues labeled as yellow correspond to the changes of UBQ HSQC spectra at SPFO 2.99 mM. In f, the residues in the circle are those that keep intact in UBQ HSQC spectra at SPFO 7.4 mM.

Fig. 3. The relative fluorine chemical shifts of α-CF₂ and α-CF₃ peaks in SPFO at 298 K. The UBQ concentration was 0.5 mM.
that most of the UBQ peaks disappear (Fig. 1e). In the HSQC spectrum, the peaks that are kept intact include G10, T12, Z18, D21, T22, I23, K27, D39, S57, D58, N60, and K63. These amino acid residues are mostly in the negatively charged region of UBQ, which are unfavorable for the binding of anionic SPFO due to electrostatic repulsion (Fig. 2).

In the presence of 14 mM SPFO, no dispersed peaks could be observed, which means that UBQ structure is almost modified by SPFO. Our recent work shows, by circular dichroism measurement, that SPFO induces extra helical secondary structure of UBQ [6]. SPFO starts to increase the helical secondary structure content of UBQ at the concentration of about 3 mM, which is consistent to NMR results. When SPFO concentration increases, UBQ helical structure content also increases and reaches a maximum platform at SPFO concentration above 10 mM. For some proteins with β-sheet secondary structure, surfactant binding would cause an augmentation of helical content at the loss of β-sheet [6].

3.2. Aggregation state of SPFO on UBQ

To see the state of SPFO when bound to UBQ, we measure the $^{19}$F chemical shift of SPFO (Fig. 3). At this SPFO concentration, the fluorine chemical shift of SPFO is similar to that of SPFO micelle as shown by $^{19}$F NMR, indicating that cooperative binding occurs in which SPFO forms aggregates on UBQ with hydrophobic interaction as the main driving force. In addition, it is also shown that the fluorine nuclei closer to the hydrophobic end has larger chemical shift than those near the headgroups. This result indicates that SPFO molecules bind to UBQ molecule possibly in the fashion that the SPFO headgroups bind to the positively charged sites of UBQ and the SPFO hydrophobic chain is buried in the hydrophobic region of UBQ (Fig. 4). This result is consistent with the above UBQ HSQC spectrum change when binding SPFO, and further confirms that SPFO binds to UBQ and forms aggregates around UBQ molecular chain.

3.3. Disassociation of UBQ–SPFO complex and refolding of UBQ

It is of great importance to study the refolding of protein when the protein has been unfolded by surfactants. Our previous work observed that cationic surfactants can induce the refolding of lysozyme unfolded by anionic surfactants [13]. So we try to disassociate the UBQ–SPFO complex by adding cationic surfactants.

We have used NMR to study the interaction of UBQ with cationic surfactants dodecyl/cetyltrimethylammonium chloride (DTAC/CTAC). Both DTAC and CTAC have very weak binding affinity to UBQ up to their cmc. It might be that the hydrophobic segments in the acidic residues of UBQ are short, and the acidic residues of UBQ form the salt bridges [14–16], so both the hydrophobic and electrostatic interactions between UBQ and cationic surfactants are weak.

Therefore, we try to refold UBQ by using DTAC. UBQ and SPFO concentrations are initially at 0.5 mM and 10 mM respectively, and different amounts of DTAC are added. When DTAC concentration is increased up to 5 mM, some peaks appear which do not overlap with those of native UBQ (Fig. 5a). It might mean that DTAC begins to weaken the binding tendency of SPFO to UBQ and decrease the effect of SPFO on modifying UBQ structure.

When DTAC concentration is close to that of SPFO, the solutions become turbid because SPFO and DTAC form precipitates at molar ratio around 1:1 [17]. By centrifugation the SPFO–DTAC precipitates could be separated, which is confirmed by $^{19}$F NMR because no $^{19}$F signal could be observed in the supernatant. The HSQC spectrum of UBQ in the supernatant contains all the N-H peaks of the native UBQ (Fig. 5b), and they well overlap with those of native UBQ, which indicates that some UBQ molecules fold to its native state.

When DTAC concentration added is very low, DTAC and SPFO form mixed aggregates in which SPFO is in excess. With the increase of DTAC concentration, they tend to form equimolarly mixed aggregates and the cmc becomes lower [5]. The lowering of cmc makes more SPFO molecules to be incorporated to the mixed aggregates and form precipitates. The concentration of SPFO monomers in aqueous

Fig. 4. Schematic representation of the interaction between UBQ and SPFO and DTAC.
solution is so low that no SPFO NMR signal can be detected, and UBQ molecules fold to its native structure without the effect of SPFO. However, in the above described supernatant, some new peaks in the HSQC spectra appear. By using HPLC and Bio-MS we also find that some UBQ molecules form aggregates, and most of the aggregates are UBQ dimmers (see Supplementary materials).

The refolding of SPFO-denatured UBQ can be achieved by DTAC shown by above result. It is due to the formation of mixed micelles of anionic–cationic surfactants is a more favorable process than that of protein–surfactant complexes [13]. Wand et al. found a new approach to study large proteins by the encapsulation of a protein in a reverse micelle [18]. This work also provides a good way to study the refolding mechanism of protein. Furthermore, an excellent new protein separation method using a pair of cationic and anionic surfactants can be developed. Our previous work observed the unfolding and refolding of lysozyme by anionic and cationic surfactants, in which the anionic and cationic surfactant aggregates are in the same system with refolded lysozyme [13]. In this work, when UBQ is refolded, anionic surfactant and cationic surfactant form precipitates and almost no surfactant molecule is in solution, which bring much convenience when separating proteins.

4. Conclusion

By using NMR technology, the binding sites and the aggregation process of SPFO on UBQ were observed. A detailed process from specific binding to cooperative binding of surfactants on a protein, and detailed structural changes of a protein induced by anionic surfactant were observed for the first time (Fig. 4). Furthermore, the disassociation of protein–anionic surfactant complex and the refolding of unfolded protein were achieved by adding cationic surfactant.

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Appendix A. Supplementary data


References