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# Fluorescence studies on the microenvironments of proteins in $CO_2$-expanded reverse micellar solutions

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**Abstract**

The effect of compressed $CO_2$ on the microenvironment of the two proteins (cytochrome *c* and RNase A) in sodium bis(2-ethylhexyl)sulfosuccinate (AOT) reverse micelles was investigated using high-pressure steady-state fluorescence and time-resolved fluorescence. It is found that RNase A can shift from the water core to the interface of the reverse micellar cores as $CO_2$ is dissolved, while cytochrome *c* maintains at the interface of the micellar core. As the pressure reaches to a high enough value, the proteins can be precipitated from the reverse micelle, which has been detected by high-pressure UV–vis technique. The results show that cytochrome *c* was easier to be precipitated from the reverse micellar solution by $CO_2$ than RNase A. The possible reasons for these behaviors were discussed based on the changes of the microenvironment of the two proteins, which could be tuned by the dissolution of compressed $CO_2$.
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*Keywords:* Fluorescence studies; RNase A; Cytochrome *c*

## 1. Introduction

Many kinds of reverse micelles are able to solubilize fairly large amount of water and hydrophilic molecules, such as proteins. The interest in these protein-containing micellar systems lies in biotechnological applications, in the mimicking of biological structures, and in the contribution to basic problems of structural biochemistry in particular to that part of biochemistry concerned with the relationship between environment and conformation/activity of functional biopolymers [1–7]. Some properties of protein-containing reverse micelles have been studied by spectroscopic measurements, such as CD [8,9], Raman [10] and fluorescence [11].

The properties of biomolecules in reverse micelles can be studied by fluorescence because different fluorophores like indole, tryptophan and tyrosine residues are sensitive to the physicochemical properties of their environment [12–15]. Steady-state fluorescence can reflect the influence of microviscosity, micropolarity and rigidity within the water pool on biomolecules in reverse micelles [16]. Time-resolved fluorescence studies of proteins enable us to better understand how proteins behave in their native form or when sequestered within the water cores of reverse micelles [11,17–23].

It is known that compressed $CO_2$ can dissolve in many organic solvents, which results in considerable change in the solvent power of the solvents. Thus, changing the pressure can control the property of the liquid solvents, and separation of the gas and the liquid solvent can be achieved easily by depression. This principle has been used in extraction and fractionation, recrystallization of chemicals, and preparation of nanoparticles [24–28].

Dissolution of compressed $CO_2$ in the reverse micellar solution may adjust the properties of reverse micelles continuously. Our recent research demonstrated that the microenvironment of the water core in the reverse micelles, such as the polarity, ionic strength and pH value, could be tuned by the pressure of $CO_2$ [29], and the precipitation of inorganic nanoparticles in the reverse micelles were achieved at suitable pressure [30,31]. The application of compressed $CO_2$ into the protein-containing reverse micelles has also shown to be an interesting area. Zhang et al. have studied the recovery of proteins in reverse micelle by compressed $CO_2$, and pure and dry protein

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particles were obtained [32]. In our previous work, we have studied the effect of compressed $CO_2$ on microenvironment of protein (trypsin) in the reverse micelles using UV and FT-IR spectroscopy and precipitation of the protein [33]. It was found that dissolution of $CO_2$ in the micellar solution could cause the changes of the microenvironment of the proteins in the reverse micelles.

The fluorescence of proteins is a very sensitive indicator of the microenvironment of the tryptophan residues. In general, the wavelength of maximum emission should exhibit a blue shift as the polarity of the tryptophan environment decreases [16,34,35]. In present work, we further our studies on the $CO_2$-expanded protein-containing reverse micellar system using steady-state fluorescence and time-resolved fluorescence and UV. Two proteins, RNase A and cytochrome *c*, were selected. The fluorescence emission of RNase A arises from the six tyrosine residues [36,37]. And the fluorescence emission of cytochrome *c* is from one tryptophan residue [38]. The former is located in the water core of the reverse micelles and the latter is located at the interface of the micellar cores [39] in the absence of $CO_2$. RNase A contains 19 of the 20 natural amino acids, lacking only tryptophan. Three of the six tyrosines in RNase A are known to be buried in the native molecule whereas the other three are located on the protein surface. The enzyme is cross-linked by four disulfide bonds and the predominant elements of secondary structure are a long four-stranded antiparallel β-sheet and three short α-helixes. The overall shape of the enzyme resembles that of a kidney. The stability of RNase A is legendary. RNase A has no change of secondary structure in the high $w_0$ (water-to-surfactant molar ratio) reverse micelles [40,37]. Cytochrome is strongly associated with the inner mitochondrial membrane in vivo. This protein contains 19 lysine residues, which can be expected to make electrostatic interaction with anionic surfactants. Most of these residues are distributed on the two flanks of the protein molecule. However, this protein does not show a drastic change of secondary structure upon the incorporation into the reverse micelles and the addition of sodium dodecyl sulphate (SDS) to the aqueous protein solutions [41]. The effect of the compressed $CO_2$ on the microenvironment and micro-behavior of the proteins (cytochrome *c* and RNase A) in the reverse micelles were discussed. The investigations provide fundamental understanding for such systems and necessary information of the related potential applications.

## 2. Experimental

### 2.1. Materials

Cytochrome *c* (bovine, $M_w = 12{,}310$, PI = 10.0) was purchased from DongZhiKai Biotechnology Co. in Beijing and RNase A (bovine, $M_w = 13{,}690$, PI = 7.8) from Sigma. The two proteins were used directly without further purification. AOT was purchased from sigma and used after drying under vacuum for at least 2 h. *n*-Decane was purchased from Tianjin Wendaxigui Chemical Co. $CO_2$ (99.995%) was provided by Beijing Analytical Instrument Factory.

### 2.2. Preparation of reverse micellar solution

Concentrated stock solution of protein (150 mg/ml) was prepared in twice distilled water. A suitable amount of protein aqueous solution was injected into the AOT/decane solution, then appropriate amounts of water was subsequently added to achieve desired $w_0$ (water-to-surfactant molar ratio). The solution was shaken until it was transparent. The molar ratio of fluorophore to surfactant was carefully chosen to give an optimum signal-to-noise ratio with minimal perturbation to the micellar organization and negligible interprobe interactions. The final protein concentration in reverse micellar solution after expanded by $CO_2$ was 0.5 mg/ml, and the concentration of AOT was 100 mmol/l in all cases. The final molar ratio of protein to surfactant is 1:2462 for cytochrome *c* and 1:2738 for RNase A, respectively. At such a low fluorophore to surfactant molar ratio, the number of protein molecule in each reverse micelle would be no more than one on an average, which rules out molecule aggregation effects [42,43].

### 2.3. Phase behavior of reverse micellar solution in $CO_2$

The apparatus used to study the expansion curves and the cloud point pressure of the solution was the same as that used previously [28]. In the expansion experiment, suitable amount of reverse micellar solution was added into the view cell. The temperature of the water bath was controlled by a HAAKE D3 digital controller, and the accuracy of the temperature measurement was ±0.1 °C. After the thermal equilibrium had been reached, $CO_2$ was charged into the cell to a suitable pressure. A magnetic stirrer was used to enhance the mixing of $CO_2$ and reverse micellar solution. The pressure and the volume at equilibrium condition were recorded. More $CO_2$ was added and the volume of the liquid phase at another pressure was determined. The volume expansion coefficients were calculated on the basis of the liquid volumes before and after dissolution of $CO_2$.

### 2.4. UV measurements

A UV spectrophotometer (TU-1201, Beijing Purkin General Instrument Co., Ltd.) was used to examine the precipitation of the proteins from the reverse micelles at different pressures. The UV sample cell was the same as that used previously [44], and the maximal operating pressure and temperature of the cell is about 20 MPa and about 60 °C, respectively. The optical path length and the volume of the sample cell were 1.12 cm and 1.76 ml, respectively. In the experiments, suitable amount of protein-containing reverse micellar solution was loaded into the cell. The temperature of the cell was maintained at 298.2 K. After the thermal equilibrium was reached, $CO_2$ was charged into the sample cell until the cell was full. The UV spectrum of solution was obtained by repeated scans until it was unchanged. The concentration of the protein in the solution was known from the absorption maximum.

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