Forward and Backward Extractions of Hemoglobin in an AOT Reverse Micellar System

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ABSTRACT

Forward and backward extraction behavior of hemoglobin using a bis(2-ethylhexyl) sulfosuccinate sodium (AOT) reverse micellar system was investigated, and successful extractions of hemoglobin in this system were achieved. Forward extraction of hemoglobin from the feed solution to the reverse micellar organic phase was dependent on the feed conditions such as pH, salt concentration, and initial protein concentration. Optimal pH of the feed solution was found to be 6.0. The forward extraction of hemoglobin decreased with increasing salt concentration, increased with decreasing initial protein concentration. The forward extraction percentage of 3 µM hemoglobin increased with the AOT concentration in the reverse micellar organic phase and reached a plateau of ca. 100% at an AOT concentration of 100 mM. Almost complete backward extraction from the micellar organic phase was easily obtained by ingeniously controlling the salt concentration in the recovered aqueous phase. Higher order structure of the hemoglobin recovered from the reverse micellar organic phase was examined by circular dichroic measurement, which suggested that the extracted hemoglobin strongly interacted with AOT molecules.

KEYWORDS

reverse micelle, extraction, hemoglobin

INTRODUCTION

Reverse micelles are spontaneous aggregates of amphiphilic molecule in non-polar media, and capable to solubilize water and hydrophilic proteins. Protein extraction using reverse micellar solutions can provide a cost-effective approach for the separation of proteins, because liquid-liquid extraction can be performed, which is especially attractive for use in large-scale, continuous processing (Naoe at al., 2002). Reverse micellar systems formulated with amphiphilic molecule bis(2-ethylhexyl) sulfosuccinate sodium (AOT) are well known to be the most general systems for protein extraction. However, it was believed that the extraction of large-molecular-weight proteins using this system was difficult (Kinugasa et al., 1994; Shiomori et al., 2000), especially for hemoglobin (M.W. = 64750), which is an important protein in pharmaceuticals.

This paper reports the results of a careful investigation of the extraction behavior hemoglobin using an AOT reverse micellar system.

MATERIALS AND METHODS

Chemicals
Hemoglobin from bovine (M.W. = 64750, pI = 7.0) was purchased from Sigma Chemical Co. (H-2500). Bis(2-ethylhexyl) sulfosuccinate sodium (AOT) was used as an amphiphilic molecule, which (purity 99.8%) was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Organic solvents, isooctane (2,2,4-trimethylpentane), n-octane, and n-hexane were purchased from Wako Pure Chemical Industries (Osaka, Japan). All these chemicals were used without further purification. The buffer solutions used were phosphate buffers (pHs 6.0, 7.0, and 8.0), citrate buffers (pHs 3.5, 4.5, and 5.5), and tris-HCl buffers (pHs 8.5 and 9.5) at 0.1 M.

Methods

The forward extraction was carried out by mixing an equal volume (10 mL) of an AOT reverse micellar solution with the initial aqueous phase for 15 min with a magnetic stirrer, then reverse micellar organic phase containing hemoglobin was separated by centrifugation. In the backward extraction, the reverse micellar phase containing hemoglobin was mixed with a fresh buffer solution for 20 min with a magnetic stirrer. After centrifugation, the recovered aqueous phase could be obtained. The concentrations of hemoglobin in the aqueous and organic phases were determined by measuring the UV absorbance at 280 nm using an UV/visible spectrophotometer (Shimadzu UV-2400 PC). The water content of the reverse micellar organic phase was measured by Karl-Fischer titration with Hiranuma AQV-5S. Circular dichroic (CD) spectra were recorded with a Jasco J-820, using a 1 mm cell at wavelengths from 200 to 250 nm. The ellipticity is expressed in mean residue ellipticity. All the experiments were carried out at 298 K.

RESULTS AND DISCUSSION

Effect of pH

Figure 1 shows the effect of initial pH of the feed solution on the hemoglobin extraction using a 200 mM AOT/isoctane reverse micellar organic solution. The forward extraction percentage of hemoglobin was dependent on the initial pH of the feed solution, indicating that forward extraction of hemoglobin can be controlled by the feed solution pH. Highest extraction percentage of ca. 100% was achieved at pH 6.0. Forward extraction percentage decreased greatly at the pHs higher than the isoelectric point of the protein (pI = 7.0). Thus, the pH dependency of the forward extraction indicates that the hemoglobin is forward-extracted mainly by the electrostatic interactions between the protein and the polar head group of the amphiphiles (Goklen et al., 1987).

Effect of Salt Concentration
Figure 2 shows the effect of salt concentration on the hemoglobin extraction using a 100 mM AOT/isooctane reverse micellar organic phase. NaCl and KCl were used as an additive salt, respectively. In both salt systems, the forward extraction percentage of hemoglobin decreased with increasing of the salt concentration. The reduction on the extraction indicates that there is electrostatic interaction between the protein and the AOT reverse micelles. However, even at salt concentrations above 1 M, especially in the NaCl system, higher extraction percentages more than 80 % were obtained. This implies that the hemoglobin extraction in this system is also affected by other interactions other than the electrostatic interaction.

Figure 2 also shows the water contents in the micellar organic phase at each salt concentration. The water content in the micellar organic phase is expressed as the molar ratio of water to AOT in the organic phase, $W_0 = [\text{H}_2\text{O}]/[\text{AOT}]$. In both salt systems, the water content decreased steeply with increasing salt concentration. Since the radius of water pool of reverse micelle ($r_w$) is linearly proportional to the $W_0$ value represented as $r_w = 0.15W_0$ (Pileni et al., 1985), it is indicated that the micellar size declined steeply with increasing salt concentration. In particular, at higher salt concentrations above 0.5 M the $W_0$ values of the micellar organic phase were less than 15 in both salt systems. Using the linear correlation, the water pool’s diameter at $W_0 = 15$ is calculated as 4.5 nm. Judging from the fact that the diameter of hemoglobin molecule is 6.1 nm (Squire et al., 1979), it is suggested that hemoglobin is extracted in the organic phase by the reverse micelles smaller than hemoglobin itself. Clusters of small micelles may extract hemoglobin molecules in the organic phase.

**Effect of Amphiphile Concentration**

Figure 3 shows the effect of AOT concentration on the extraction of 3 µM hemoglobin in AOT reverse micellar organic system. The forward extraction percentage of hemoglobin increased with increasing AOT concentration and reached a plateau of ca. 100% at an AOT concentration of 100 mM. In the cases of cytochrome c, lysozyme, and ribonuclease A, the minimal concentration of AOT required for 100% forward extraction into reverse micelles were reported (Ichikawa et al., 1992; Naoe et al., 1996; Imai et al.,...
1997). This critical amphiphile concentration is called ‘minimal AOT concentration,’ and is a significant operating condition for the practical design of micellar extraction processes (Ichikawa et al., 1992). The AOT concentration of 100 mM obtained in this study corresponds to the minimal AOT concentration for 3 µM hemoglobin.

**Effect of Initial Protein Concentration in Feed**

Figure 4 shows the effect of initial protein concentration in feed on the hemoglobin extraction using AOT reverse micellar organic phase at each AOT concentration. At all the initial protein concentration, the forward extraction percentage increased with increasing AOT concentration and a minimal AOT concentration for hemoglobin was found. The minimal AOT concentration for hemoglobin increased with increasing the initial hemoglobin concentration in the feed. This indicates that a larger amount of AOT is required for the extraction of hemoglobin at higher concentrations in the feed.

**Backward Extraction of Hemoglobin from Reverse Micellar Organic Phase**

Figure 5 shows the effect of salt concentration on backward extraction of hemoglobin from 200 mM AOT/isoctane reverse micellar organic phases. NaCl and KCl were used an additive salt, respectively. In both salt systems, the backward extraction percentage of hemoglobin was drastically reduced by only a small increase in the salt concentration. Using “distilled water” as a stripping phase, almost 100 % hemoglobin recovery from the reverse micellar organic phase was achieved. At higher salt
concentrations above 0.5 M, red aggregates of hemoglobin appeared at the O/W interface. As a consequence, remaining percentages of hemoglobin in the raffinate organic phase decreased at higher salt concentrations.

**Higher Order Structure of Hemoglobin**

Higher order structure of hemoglobin in this system was examined by CD measurement. Figure 6 shows the far-UV CD spectra of hemoglobin in the micellar organic and recovered aqueous phases. The CD spectrum of hemoglobin in the feed is shown for comparison. The backward extraction of hemoglobin was carried out by contacting the micellar organic phase containing the protein with fresh distilled water. In general, CD spectra in the far-UV (200 to 250 nm) region indicate the secondary structure of a protein. In particular, the mean residue ellipticity at 222 nm is used as an indicator for a protein’s α-helix content (Chen et al., 1971). The CD spectrum of the recovered hemoglobin is very similar to that in the reverse micellar organic phase but is not similar to that in the feed. The ellipticity value at 222 nm of the recovered protein was higher than that of the feed one, indicating that the recovered hemoglobin had less α-helix content than the feed one. These results imply that the recovered hemoglobin interacts strongly to the AOT molecules.

**CONCLUSION**

In this study, extraction behavior of hemoglobin using AOT reverse micellar system was carefully investigated. Highest extraction percentage of ca. 100% was achieved at pH 6.0. The forward extraction percentage of hemoglobin decreased with salt concentration. The water content in the reverse micellar organic phase also decreased steeply with increasing salt concentration. The forward extraction percentage of 3 µM hemoglobin increased with increasing AOT concentration and reached a plateau of ca. 100% at an AOT concentration of 100 mM. AOT concentration required for extraction increased with increasing initial hemoglobin concentration. The backward extraction percentage of hemoglobin from the reverse micellar organic phase was drastically reduced by only a small increase in the salt concentration. Using “distilled water” as a stripping phase, almost 100% hemoglobin was successfully recovered from the reverse micellar organic phase. Higher order structure of the recovered hemoglobin is very similar to that in the reverse micellar organic phase but is not similar to that in the feed. An investigation of higher order structure of the recovered hemoglobin by CD measurement indicated that the recovered hemoglobin interacts strongly to the AOT molecules.

**NOMENCLATURES**

Eb: backward extraction percentage [%]
Ef: forward extraction percentage [%]
Re: remaining percentage in raffinate organic phase [%]
$W_0$: molar ratio of water to AOT [mol-H$_2$O/mol-AOT]

$[\theta]$: mean residue ellipticity [deg·cm$^2$/dmol]

REFERENCES


