^{13}C NMR OF STUDY OF ENTRAPPING PROTEINS (α-CHYMOTRYPSIN) INTO REVERSED MICELLES OF SURFACTANTS (AEROSOL OT) IN ORGANIC SOLVENTS (n-OCTANE)

Yurii E. SHAPIRO[a], Nikolai A. BUDANOV[a], Andrei V. LEVASHOV[b], Nataliya L. KLYACHKO[b], Yurii L. KHMELNITSKY[c] and Karel MARTINEK[d]

[a] *Polytechnic Institute, SU-150 053 Yaroslavl, U.S.S.R.*
[b] *Moscow State University, Chemistry Department, SU-117 234 Moscow, U.S.S.R.*
[c] *Institute of Biochemistry, U.S.S.R. Academy of Sciences, SU-117 071 Moscow, U.S.S.R. and*
[d] *Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, CS-166 10 Prague, Czechoslovakia*

Received October 26, 1988
Accepted November 9, 1988

Hydrated reversed micelles of Aerosol OT (AOT) in octane have been studied by ^{13}C NMR spectroscopy. The changes of spin-lattice relaxation times (T_1) for individual segments of the AOT molecule, induced by entrapping a protein (α-chymotrypsin) into the micelle, have been determined by the inversion-recovery technique. The dramatic (three-fold) increase of T_1 found for the α-CH_2 groups in the AOT molecules indicates that (unlike in the unfilled micelle) in the protein-containing micelle the boundary of the water cavity is shifted outward (0·5—0·7 nm, under the given experimental conditions), the alkyl chains of the surfactant being "flooded" by water molecules. This observation explains why the outer size of the reversed micelle does not change on insertion of a bulky protein molecule.

Micellar enzymology investigates enzymes (proteins) entrapped into hydrated reversed micelles of surfactants in organic solvents[1-6]. One of the crucial problems of this discipline is the question of structure: to understand structure-function relationships studied by micellar enzymology, it is important to know whether there is any structural change in a reversed micelle (aggregation number, hydration degree, geometric dimensions and conformation) upon entrapping protein molecule, and if so, what is the extent of such change.

The literature offers two diametrically different models of protein-containing reversed micelles. According to the water-shell model (Fig. 1*a*), suggested by Luisi and collaborators[2,7,8], the inclusion of a protein molecule is invariably accompanied by an increase of the micelle size, and the filled micelle contains more surfactant and water molecules than before the inclusion (as the result of redistribution of these micellar components between the filled and unfilled micelles). An alternative model, suggested by us[9,10], assumes that such increase in size on entrapping a protein takes

place, of course, when the inner cavity of the unfilled micelle is smaller than the protein molecule (the induced fit model, see Fig. 1*b*); in this case inclusion of a protein can, in principle, increase the aggregation number of the surfactant as well as the degree of hydration, depending on the character of the protein. In contrast with the water-shell model (Fig. 1*a*), if the size of the unfilled water cavity exceeds (or approximately equals) that of the protein molecule, the protein entrapping may not lead to any substantial increase of the reversed micelle (the fixed size model, see Fig. 1*c*).

Sedimentation measurements[9,10] have confirmed the fixed size model for water-soluble (hydrophilic) proteins such as trypsin, chymotrypsin, lysozyme, egg white albumin, horse liver alcohol dehydrogenase and γ-globulin. It has been found[9,10] that the increase in volume, if any, of the protein-containing micelle (approximated as a sphere) is at most 10% (even when the protein molecule and the water cavity in the unfilled micelle are of the same size). The protein-containing micelle contains practically the same number of both surfactant and water molecules as the unfilled one; strictly speaking, the observed mass of the new aggregate is equal to the sum of masses of the protein and the empty micelle.

Although these (at the first glance surprising) findings[9,10] met with some doubts[2,8], the recent studies based on direct physical methods such as quasi-elastic neutron scattering[11,12] and photon correlation spectroscopy[12] have confirmed the validity of our fixed size model. It is worth notice that the confirmation experiments[11,12] were performed with the same hydrophilic protein (chymotrypsin) which had been used in our earlier investigations[9,10].

On the other hand, solubilization of myelin basic protein (MBP) in reversed micelles of Aerosol OT in iso-octane is accompanied by some deviations from an ideal fixed size model, as found[13] by the quasi-elastic light scattering method. We assume that the observed[13] deviations are due to the capability of MBP to interact with lipid

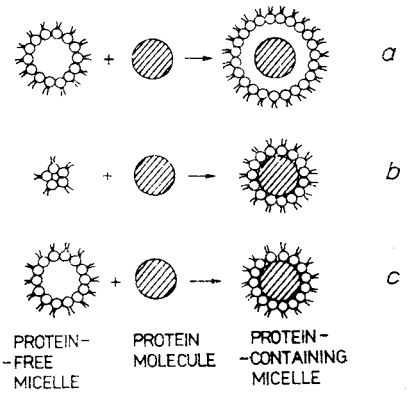

FIG. 1

Hypothetical models for solubilization of protein molecules (radius r_P) in reversed micelles (outer radius R_M, inner cavity radius r_M) under formation of protein-containing micelles (outer radius R_{MP}, inner cavity radius r_{MP}): *a* water shell model: $R_{MP} > R_M$ and $r_{MP} > r_P$, independent of the r_P/r_M ratio; *b* induced fit model: $R_{MP} > R_M$ and $r_{MP} \approx r_P$, when $r_P > r_M$; *c* fixed size model: $R_M \approx R_{MP}$, when $r_P \leqq r_M$

membranes[14]. In actual fact, it has been recently found by French authors[15] that (in contrast with an unfilled micelle) the hydrodynamic radius of a reversed MBP-containing micelle depends substantially on the surfactant concentration. Extrapolation of these data to zero concentration of the surfactant gives[15] again an only one value of hydrodynamic radius for both the protein-containing and the empty micelles (naturally, at a sufficiently high degree of surfactant hydration, enabling free insertion of the protein molecule into the water cavity; see Fig. 1*c*).

The validity of the fixed size model can be regarded as proven at least for the above-mentioned proteins[9-12,15]; yet there is still an unsolved problem: what change in the *inner* structure of a reversed micelle occurs, under retention of the *outer* size, when a bulky protein molecule is entrapped into the micelle (Fig. 1*c*)? Without answer to this question doubts[2,8,16] might still persist whether the fixed size model reflects indeed physical reality.

Since much information in this respect can be gained from ^{13}C NMR spectroscopy, we used this method to follow changes in the spatial arrangement of water and surfactant molecules induced by inclusion of α-chymotrypsin in reversed micelles of Aerosol OT in octane. We chose α-chymotrypsin because it has been shown[9-12] by several methods (see above) that this protein conforms the fixed size model.

EXPERIMENTAL

α-Chymotrypsin (EC 3.4.21.1) (Biokhimreaktiv, Olaine, U.S.S.R.). was purified by gel filtration on a Sephadex G-75 column in 10^{-3}M-HCl and lyophilized. According to the active center titration with N-*trans*-cinnamoylimidazole[17], the active enzyme content in the preparation was higher than 80%.

Sodium 1,2-bis(2-ethylhexyloxycarbonyl)-1-ethanesulfonate (Aerosol OT; AOT), purchased from Merck, was purified[18] and dried in vacuo at 40°C. The purity of the obtained preparation was checked as described by Levashov et al.[18]. Octane was a Reakhim (U.S.S.R.) product.

Preparation of micellar sollutions. An aqueous solution of 0·05M Tris-HCl buffer (pH 8·0) was added to 0·4M solution of AOT in octane by means of a microsyringe until the required hydration degree was achieved; see „Selection of experimental conditions" discussed below. The molar concentration ratio [solubilized water]/[AOT] in the micellar solution was then checked by ^1H NMR spectroscopy.

Lyophilized enzyme was added with shaking to the obtained solution of hydrated reversed AOT micelles in octane as described by Menger and Yamada[19] and the mixture was incubated at room temperature for 15 h. Negligible amount of undissolved protein was separated by centrifugation. The concentration of α-chymotrypsin in the obtained solution was determined by weighing, and then checked spectrophotometrically[17] at 280 nm.

Spectral measurements. Proton NMR and broad-band proton-decoupled ^{13}C NMR spectra were obtained at 25°C in the FT mode on a Tesla BS-567A spectrometer at 100 MHz and 25·142 MHz, respectively. For the ^1H NMR measurements the pulse length was 9 μs and the acquisition time 1·069 s in one scan; for ^{13}C NMR measurements the respective values were 8 μs and 1·069 s, with 200 acquisitions. An external coaxial capillary of deuterium oxide was used to maintain lock. Tetramethylsilane was used as internal reference.

The ^{13}C spin-lattice relaxation times (T_1) for AOT in reversed micelles were determined using the inversion-recovery method (pulse sequence $180° - \tau - 90° - T$). The pulse interval τ varied from T to 0·05 s and the waiting time T was longer than $5T_1$ for full relaxation of all the ^{13}C nuclei. The values of T_1 were calculated[20] as follows:

$$\ln (A_\infty - A_\tau) = \ln 2A_\infty - \frac{\tau}{T_1}, \qquad (I)$$

where A_τ is the signal intensity after 90° pulse for the given τ, and A_∞ is the limit value of A_τ for the longest τ. The values of T_1 were obtained from the slope of the plot of $\ln (A_\infty - A_\tau)$ versus τ by the least squares refinement; the relative error did not exceed 2%.

Selection of experimental conditions. The dimensions of α-chymotrypsin molecule[21] are 4 × 4 × 5 nm. It may be approximated as a sphere of the same volume (about 41 nm^3) with a radius of 2·15 nm. The size of inner cavities of the reversed AOT micelles in octane depends[22] on the hydration degree. We have chosen the molar ratio $[H_2O]/[AOT] = 12$ at which the water cavity in the reversed micelle has a radius of 2·21 nm, according to Eicke and Řehák[22]. This means that, under the conditions of our experiment, the volume of the inner cavity in an empty micelle (45 nm^3) slightly — about 10% — exceeds that of the enzyme molecule (41 nm^3).

RESULTS AND DISCUSSION

In the discussion we shall adhere to the already ascertained fact[9,10] that (under the above-mentioned conditions) the amount of water and the aggregation number of AOT are the same both in the protein-containing and in the protein-free micelle. In such case a simple calculation shows that water molecules in the micelle form an about 0·6 nm thick layer around the entrapped enzyme (at the given volume ratio of the water cavity to the α-chymotrypsin molecule, vide supra). In terms of the fixed size model (with unchanged outer size of the protein-containing micelle; see Fig. 1c) this means that water molecules are expelled from the inner cavity, penetrating into the outer shell (as far as the hydrocarbon chains of the AOT molecules). The situation is depicted schematically in Fig. 2.

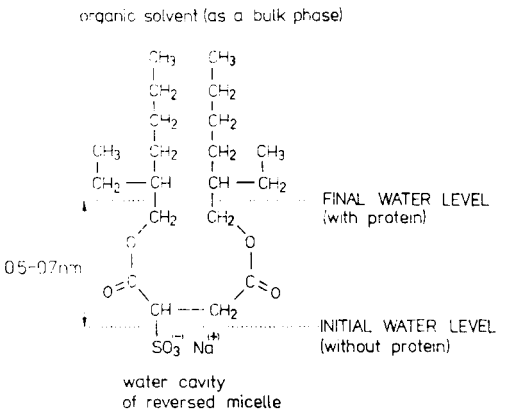

FIG. 2

Scheme of expulsion of water from the inner cavity of a reversed AOT micelle on inclusion of protein molecule (fixed size model in Fig. 1)

For detection of such process (and particularly, changes in microenvironment of the alkyl chains in the surfactant molecules) we made use of ^{13}C NMR spectroscopy which has already proven[23] its advantages in the study of colloidal systems of polymers.

^{13}C NMR *Spectra of* AOT

The observed ^{13}C NMR spectra of reversed micelles of AOT in octane are in accord with those obtained for other organic solvents[24-26]. The chemical shifts (with tetramethylsilane as internal reference) are given in Table I. To check the assignments, we calculated the chemical shift values according to the additive scheme of Lindeman and Adams[27].

As seen from Table I, inclusion of α-chymotrypsin into the micellar system affects only little the chemical shifts.

Carbon-13 *Spin-Lattice Relaxation*

The octane signals (14·26; 23·26; 30·02 and 32·63 ppm) practically obscur the signals occurring in the region 14·60–34·20 ppm (see Table I). Therefore, the relaxation times T_1 of side chains in the AOT molecules were evaluated only for the nuclei CO-2,2′, CH_2-3,3′, CH-1′, CH-4,4′ and CH_3-10,10′.

TABLE I

Chemical shifts of $^{13}C\{^1H\}$NMR signals (relative to tetramethylsilane) of reversed AOT micelles (0·4M) in octane, containing solubilized aqueous buffer solution (0·05M Tris-HCl, pH 8·0). Hydration degree, $[H_2O]/[AOT] = 12$. For numbering of the ^{13}C carbon nuclei, see Fig. 3

Signal	Chemical shift (δ, ppm)	
	without protein	with 0·8 mM α-chymotrypsin
CH_3-10,10′	11·56	11·56
CH_3-8,8′	14·60	14·60
CH_2-7,7′	23·72	23·72
CH_2-9,9′	24·20	24·18
CH_2-5,5′; 6,6′	30·80	30·80
CH_2-1	34·20	33·55
CH-4,4′	39·26	39·13
CH-1′	61·97	61·76
CH_2-3	67·18	67·18
CH_2-3′	68·83	68·83
CO-2	169·87	170·26
CO-2′	171·75	172·09

The ^{13}C NMR spectra of reversed micelles of AOT in octane, obtained by the inversion-recovery method, are depicted in Fig. 3. The values of T_1, obtained according to Eq. (*1*), are listed in Table II.

In the absence of protein, the terminal CH_3-10,10′ groups exhibit rather long spin-lattice relaxation times (Table II). The mobility of the other hydrocarbon groups in the AOT molecule is hindered by their dense packing inside the nonpolar shell region.

As evident (Table II), the CO-2,2′ groups are characterized by the longest spin-lattice relaxation times (in the absence of protein); obviously[20], the reason is that the 2,2′ carbons have no hydrogen substituents. Introduction of α-chymotrypsin into the micellar solution decreases the T_1 values of the carbonyl ^{13}C nuclei (Table II). Perhaps, their lower mobility is due to contact with the surface of the enzyme globule.

On the other hand, the segmental mobility of the α-CH_2 alkyl groups in positions 3 and 3′ increases when the protein is added. The observed change (threefold increase, Table II) of T_1 is quite dramatic (cf. ref.[20]). We assume that it results from a substantial change in the microenvironment of the segments in question.

Fine Structure of the Fixed Size Model

On the basis of the found spin-lattice relaxations for the individual carbons of the AOT molecule (Table II) we suggest the following model for protein entrapment into a micelle. In a protein-containing micelle (in contrast to an empty one) the level of water is shifted outward, so that water entirely "floods" the α-CH_2 groups at positions 3 and 3′, whereas it only slightly affects the 4- and 4′-CH groups. Finally, the 10- and 10′-CH_3 groups are completely unaffected as seen from the practically unchanged value of T_1 (Table II).

Consequently, on entrapping protein molecule into a hydrated reversed micelle the boundary of the water cavity (Fig. 2) is shifted 0·5 – 0·7 nm outward as compared with the unfilled micelle. Our estimation of this shift (about 0·5 – 0·7) nm is based on the size of the AOT molecule[26] and agrees with the value (0·6 nm) calculated by us (vide supra).

Inspection of molecular models, constructed on the basis of available data on conformation of AOT molecules in reversed micelles[24,25,28,29], confirms that the suggested expulsion of water from the micellar core is possible; in the areas where the AOT polar heads are bonded to the hydrocarbon tails (Fig. 2) there are cavities quite capable of hosting the expelled water molecules.

The thermodynamics of the mentioned change in the inner micellar structure deserves comment. In itself, the assumed forced contact of water with hydrophobic side-chains of the surfactant molecule should be thermodynamically unfavourable. However, the possible free-energy loss in this part of the micelle may be compensated by the gain resulting from interaction of AOT with the enzyme (e.g. formation of hydrogen or electrostatic bonds).

TABLE II

Spin-lattice relaxation times, T_1(s), of ^{13}C nuclei in reversed AOT micelles (0·4M) in octane, containing solubilized aqueous buffer (0·05M Tris-HCl, pH 8·0). Hydration degree, $[H_2O]/[AOT] = 12$. For numbering of the ^{13}C carbon nuclei, see Fig. 3

Signal	Relaxation time, s	
	without protein	with 0·8 mM α-chymotrypsin
CH-1′	0·113	0·155
CO-2′	1·99	1·72
CO-2	2·53	2·11
CH_2-3′	0·099	0·336
CH_2-3	0·136	0·369
CH-4,4′	0·235	0·262
CH_3-10,10′	1·45	1·44

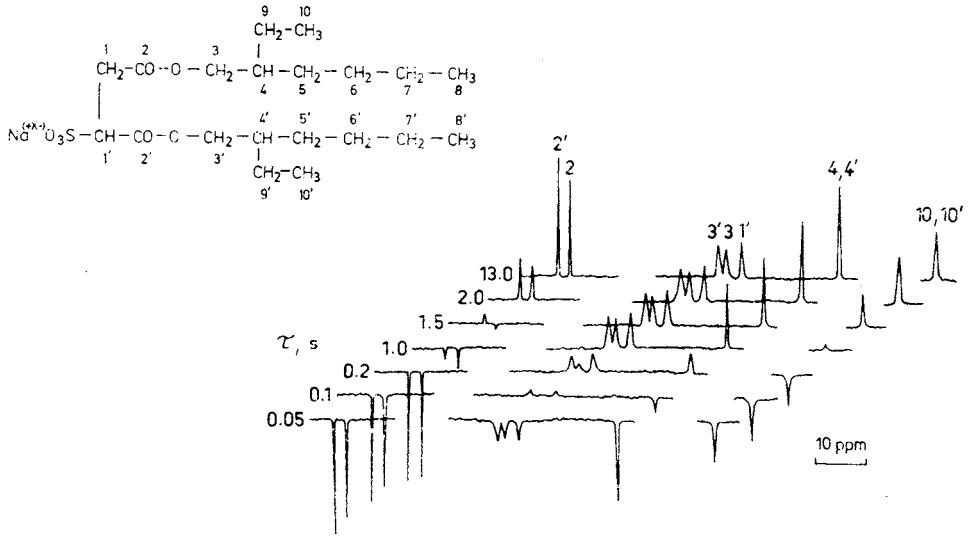

FIG. 3

Inversion-recovery $^{13}C\{^1H\}$ NMR spectrum of reversed AOT micelles (0·4M) in octane, containing solubilized aqueous buffer (0·05M Tris-HCl, pH 8·0). Hydration degree, $[H_2O]/[AOT] = 12·0$; 0·8 mM α-chymotrypsin

CONCLUSION

The presented mechanism of protein entrapping into a reversed micelle without change in its outer size may be of importance for membranology. Biological membranes consist not only of lipid bilayer[30] but also evidently contain various non-bilayer structures, particularly lipid particles of reversed micelle type[31,32]; for review, see refs[1,33]. Therefore, in view of our present results we can assume that some proteins (enzymes) incorporate into the biomembrane according to the fixed size model, without altering substantially the volume or conformation of the membrane (Figs 1c and 2).

Moreover, our description of the spatial arrangement of water and surfactant molecules in the AOT reversed micelles is in accord with recent results[34] of studies on the hydration of lipid bilayers in biological membranes. According to Scherer[34], "water is not constrained to a separate layer but is free to occupy the whole of the hydrophilic part of the bilayer. The reason for this lies in the existence of large voids in the head-group region that can accommodate water". As a result, the apparent partial molar volume of water in biological membranes depends[34] critically on the degree of lipid hydration, varying from 0·03 nm^3 (in pure water) to zero. In our model (micellar) system[9,10], the apparent partial molar volume of water drops to zero when the protein is added. This is the reason why protein entrapping into a reversed micelle proceeds without change in the outer size of the micelle.

REFERENCES

1. Martinek K., Levashov A. V., Khmelnitsky Yu. L., Klyachko N. L., Berezin I. V.: Eur. J. Biochem. *155*, 453 (1986).
2. Luisi P. L., Magid L. J.: CRC Crit. Rev. Biochem. *20*, 409 (1986).
3. Waks M.: *Proteins (Structure, Function and Genetics) 1*, 4 (1986).
4. Shield J. W., Ferguson H. D., Bommarius A. S., Hatton T. A.: Ind. Eng. Chem. Fundam. *25*, 603 (1986).
5. Luisi P. L., Laane C.: Trends Biotechnol. *4*, 153 (1986).
6. Martinek K., Berezin I. V., Khmelnitsky Yu. L., Klyachko N. L., Levashov A. V.: Collect. Czech. Chem. Commun. *52*, 2589 (1987).
7. Bonner E. J., Wolf R., Luisi P. L.: J. Solid Phase Biochem. *5*, 255 (1980).
8. Zampieri G. G., Jäckle H., Luisi P. L.: J. Phys. Chem. *90*, 1849 (1986).
9. Levashov A. V., Khmelnitsky Yu. L., Klyachko N. L., Cherniyak V. Ya., Martinek K.. J. Colloid. Interface Sci. *88*, 444 (1982).
10. Levashov A. V., Khmelnitsky Yu. L., Klyachko N. L., Cherniyak V. Ya., Martinek K.: Anal. Biochem. *118*, 42 (1981).
11. Fletcher P. D. I., Howe A. M., Perrins N. M., Robinson B. H., Toprakcioglu C., Dore J. C. in the book: *Surfactants in Solution* (K. L. Mittal and B. Lindman, Eds), Vol. 3, p. 1745. Plenum, New York 1984.
12. Fletcher P. D. T., Robinson B. H., Tabony J.: J. Chem. Soc., Faraday Trans. 1, *82*, 2311 (1986).
13. Chatenay D., Urbach W., Cazabat A. M., Vacher M., Waks M.: Biophys. J. *48*, 893 (1985)

14. Riccio P., Masotti L., Cavatorta P., De Santis A., Juretic D., Bobba A., Pasquali-Ronchetti I., Quagliareillo E.: Biochem. Biophys. Res. Commun. *134*, 313 (1986).
15. Chatenay D., Urbach W., Nicot C., Vacher M., Waks M.: J. Phys. Chem. *91*, 2198 (1987).
16. Sheu E., Göklen K. E., Hatton T. A., Chen S.-H.: Biotechnol. Progr. *2*, 175 (1986).
17. Schonbaum G. R., Zerner B., Bender M. L.: J. Biol. Chem. *236*, 2430 (1961).
18. Levashov A. V., Pantin V. I., Martinek K.: Kolloidn. Zh. (Russ.) *41*, 453 (1979).
19. Menger F. M., Yamada K.: J. Am. Chem. Soc. *101*, 6731 (1979).
20. Farrar T. C., Becker E. D.: *Pulse and Fourier Transform NMR*. Academic Press, New York 1971.
21. Squire P. G., Himmel M. E.: Arch. Biochem. Biophys. *196*, 165 (1979).
22. Eicke H.-F., Řehák J.: Helv. Chim. Acta *59*, 2883 (1976),
23. Shapiro Yu. E.: Bull. Magn. Reson. *7*, 27 (1985).
24. Ueno M., Kashimoto H., Kyogoku Y.: J. Colloid Interface Sci. *63*, 113 (1978).
25. Martin C. A., Magid L. J.: J. Phys. Chem. *85*, 3938 (1981).
26. Okabayashi H., Taga K., Tsukamoto K., Matsushita K., Kamo O., Yoshikawa K.: Colloids Surfaces *24*, 337 (1987).
27. Lindeman L. P., Adams J. Q.: Anal. Chem. *43*, 1245 (1971).
28. Maitra A. N., Eicke H.-P.: J. Phys. Chem. *85*, 2687 (1981).
29. Maitra A.: J. Phys. Chem. *88*, 5122 (1984).
30. Singer S. J., Nicholson G. L.: Science *175*, 720 (1972).
31. Cullis P. R., De Kruijff B., Hope M. J., Nayar R., Schmid S. L.: Can. J. Biochem. *58*, 1091 (1980).
32. Cullis P. R., De Kruijff B., Hope M. J., Verkleij A. J., Nayar R., Farren S. B., Tilcock C., Madden T. D., Bally B. in the book: *Membrane Fluidity in Biology* (R. C. Aloia, Ed.), Vol. 1, p. 39. Academic Press, New York 1983.
33. Klyachko N. L., Levashov A. V., Pshezhetsky A. V., Bogdanova N. G., Martinek K.: Eur. J. Biochem. *161*, 149 (1986).
34. Scherer J. R.: Proc. Natl. Acad. Sci. U.S.A. *84*, 7938 (1987).