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MICELLAR SOLUBILIZATION OF ENZYMES IN HYDROCARBON SOLVENTS. ENZYMATIC ACTIVITY AND SPECTROSCOPIC PROPERTIES OF RIBONUCLEASE IN N-OCTANE

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Summary. Ribonuclease from bovine pancreas has been solubilized in n-octane containing the surfactant di(2-ethyl-hexyl) sodium sulfo-succinate(50 mM) and water(0.55-0.94 M).It is shown that enzymatic activity with cytidine-2':3'-phosphate and RNA is maintained in the hydrocarbon phase,and that under certain conditions it is even higher than in water solution.Absorption properties and circular dichroism of the enzyme and substrates in this new environment are investigated and compared with those in water solution.

For the last few years we have been investigating peptides and proteins solubilized in aprotic solvents.We have solubilized chymotrypsin,trypsin, pepsin,cytochrome C,glucagon,as well as shorter peptides,in cyclohexane with the aid of aqueous methyl-trioctyl ammonium chloride or di(2-ethyl-hexyl) sodium sulfo-succinate (aerosol OT, henceforth referred to as AOT). Clear and stable solutions were obtained, thus permitting spectroscopic and conformational studies of the protein or peptide in the new environment (1-4). Most probably, "inverted micelles" (5,6) are formed, i.e. micelles having a polar core in which the biomolecules are solubilized together with water.

Experiments aimed at solubilizing proteins in hydrocarbon solvents have also been described by other Authors (7-8). Recently, Martinek and coworkers (9) described preliminary enzymatic properties of α-chymotrypsin and peroxidase in the micellar system octane/AOT/H_2O, but without presenting spectroscopic or structural information.

In the present paper, we describe the spectroscopic behavior and enzymatic activity of an enzyme solubilized in n-octane with the aid of AOT. We shall show in particular that the conformation of ribonuclease and its enzymatic activity are maintained in the micellar environment, and we will point out some biophysical properties and some possible applications of enzymes solubilized in aprotic solvents via reversed micelles.

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EXPERIMENTAL

Materials: Ribonuclease from bovine pancreas, AOT and RNA from yeast were obtained from Fluka, the substrate CP from Serva. AOT was purified as described in literature (22) and kept over P_2O_5 in a dessicator under high vacuum.

Methods: Ribonuclease was solubilized in n-octane/AOT by directly injecting a small amount (30-50 µl) of a concentrated solution (14 mg/ml) of the enzyme in buffer into 3 ml of a 50 mM AOT solution in n-octane. The final water content was 1% to 1.7% (v:v), or 0.55 M to 0.94 M. A relatively low surfactant concentration was used in order to facilitate spectroscopic studies in the far uv-region. The substrate CP was solubilized in the same way, using aqueous stock solution of about 5mg/ml. RNA was also solubilized with the same technique (using an aqueous stock solution containing ca. 1.5 mg/ml). Whereas the enzyme and CP were solubilized within ca. 1 min. of gentle shaking, the solubilization of RNA appeared to require a longer time. The RNA micellar solutions were left standing overnight at room temperature and experiments for comparison were made with water solutions having the same age.

For the enzymatic activity tests with the hydrocarbon phase, a small aliquot of the enzyme in buffer (typically 2 µl) was added to 3 ml of the micellar substrate solution prepared as previously described. The reference cell contained the identical micellar solution of substrate. Kinetic runs were monitored at 286 nm, and initial velocities were calculated on the basis of difference extinction coefficients of 1150 and 1680 $M^{-1}cm^{-1}$ at pH 7.13 and 4.5 respectively. These values presume that the hydrolysis of CP in water is quantitative, an approximation which is supported by published thermodynamic data (10).

The buffers used to prepare the stock solutions of enzyme and of substrates were 0.05 M borate pH 9.8, 0.05 M tris buffer or borate buffer pH 7.13 and pH 8.0, and 0.05 M acetate pH 4.5. All experiments were carried out at room temperature ($23^{o} \pm 1^{o}C$).

UV absorption spectra and kinetic experiments were recorded with a Beckmann Acta MVI, CD spectra with a Jasco J40 AS, using 1 cm cells in the near uv and thinner cells (0.2 mm) in the far uv region (below 240 nm). The concentration of enzyme and CP in solution were determined on the basis of extinction coefficients at pH 7.0 of 9700 cm^{-1} M^{-1} at 278 (11) and 8400 $cm^{-1}M^{-1}$ at 268 (10) respectively.

RESULTS

In the investigated pH range, the uv-absorption spectrum of ribonuclease in the 250-300 nm region is practically the same in the hydrocarbon phase as in water solution. As shown in Fig. 1, there is a substantial agreement between the CD spectra of the two solvent systems, especially in the region dominated by the peptide absorption (200-220 nm). This suggests that the main chain conformation is substantially the same. Some differences are observed in the 240-250 nm region, which cannot easily be explained at the moment. In this regard, it is relevant to point out that more marked

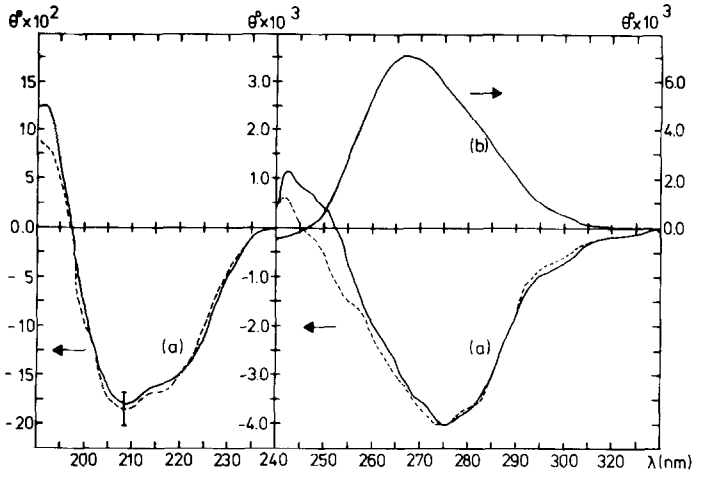

Fig. 1: a) Circular dichroic spectra of ribonuclease in tris buffer 0.05M, pH 7.13 (----) and in n-octane/AOT (50 mM)/H_2O (0.94M) (——) obtained by mixing 50 µl of a stock solution of the enzyme (14 mg/ml) in borate buffer 0.05M pH 9.8 with 3ml of the n-octane/AOT solution. The final concentration of the enzyme is the same in the two cases (15 µM). Practically the same CD spectrum is obtained in micelles prepared from pH 8.0 phosphate buffer.

b) circular dichroism of RNA in the micellar system n-octane/AOT (50 mM)/H_2O(0.55M), prepared by mixing 30 µl of RNA (1.5mg/ml) in tris buffer pH 8.0 with 3ml of the n-octane/AOT solution. Ellipticities refer in all cases to 1 cm path length.

differences between the water and the hydrocarbon solutions were observed when proteins were dissolved in cyclohexane using methyltrioctyl ammonium chloride as adjuvant (2-4).

Fig. 1 also shows the CD spectrum of RNA in a micellar solution prepared from pH 8.0. The position of the maximum and the general shape of the spectrum are the same for the water solution, but for the micelles the ellipticity is higher and the absorbance is slightly smaller. This suggests different conformations in the two solvent systems, which is now under investigation.

Fig. 2 shows the uv-absorption spectra of the cyclic substrate CP in micelles. After addition of a minimal amount of enzyme, the typical spectrum (17) of the hydrolysis product cytidine-3'-phosphate is obtained. The difference spectrum is the same as that obtained with water solution, showing that enzymatic hydrolysis in the hydrocarbon phase is analogous to that in water.

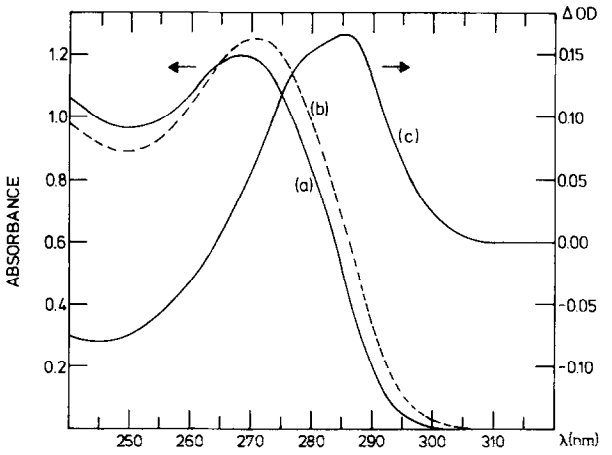

Fig. 2: Absorption spectra of cytidine-2':3'-phosphate (a) and its hydrolysis product (b) and corresponding difference spectrum (c) in the micellar system n-octane/AOT(50mM)/H_2O(0.55M). Solution a) has been prepared by adding 30 µl of a stock solution of cytidine-2':3'-phosphate (5mg/ml) in borate buffer 0.05M pH 9.8 to 3 ml of n-octane/AOT solution. Spectrum b) has been obtained by adding 2 µl of ribonuclease (14mg/ml) in the same pH 9.8 buffer to 3ml of the substrate solution.
Spectrum c) is identical to that obtained from a water solution pH 7.13.

However, for a better comparison between the water solution and the micellar system the following considerations are necessary.

Earlier studies (12) show that the absorption spectra of CP and its hydrolysis product varies with pH, mainly reflecting the ionization of the ring nitrogens. When we prepare a micellar solution of CP from a pH 9.8 buffered stock solution (see experimental) we obtain an absorption spectrum corresponding very closely to that of a ca. pH 7 aqueous solution. Analogously, when we prepare a micellar solution of CP from a pH 7.13 buffered aqueous solution, we obtain absorption spectra for CP and its hydrolysis product corresponding to those of a pH 4.5 water solution (see Table 1). Thus, it appears that inside the micelles a "local pH" is established, which is considerably lower than that present in the original water phase.

This finding is not too surprising. Fendler et al. (13) and more recently Menger and Saito (14) showed that in the aqueous core of the inverted micelles the apparent acidity is much higher than in bulk water. Thus, the pK

Table 1

Absorption properties and hydrolysis rate of cytidine 2':3' phosphate (CP) in water and in the micellar system n-octane/AOT/H_2O

Sample	λ_{max} [a], nm	λ_{min} [b], nm	$\varepsilon_{(\lambda max)}$ [c]	$\Delta OD\ (t=\infty)$ [e]	$(V/e)_{in}$ [f], sec^{-1}
in buffer pH 7.13	268 (271)	250,5 (249.5)	8400[d] (1.22)	0.148	0.165
in n-Octan/AOT from a pH 9.8 stock solution	268 (271)	250.5 (249.5)	8400 (1.24)	0.151	1.23
in buffer pH 4.5	270.5(274)	245 (246)	9200 (1.58)	0.225	0.130
in n-Octane/AOT from a pH 7.13 stock solution	270.5(273)	248.5 (247)	8960 (1.41)	0.215	0.155

a) Wavelength of the absorption maxima of CP and the hydrolysis product, respectively (± 0.5 nm)
b) Wavelength of the absorption minima of CP and the hydrolysis product, respectively (± 0.5 nm)
c) Extinction coefficient at λ_{max} of CP (in M^{-1} cm^{-1}) and, in parenthesis, the ratio of the absorbances at λ_{max} and λ_{min} for CP (± 2% ca.) d) from Reference [10] e) Total optical density change at 286 nm, after addition of enzyme (see expt.) f) initial velocity (± 5% ca.)

of p-nitrophenol increases from 7.14 to more than 11.5 in a heptane/AOT/H_2O system (14). It is then clear that the comparison of chemical properties between a water and a micellar system is not trivial. One can try to match the pH of the two different systems on the basis of spectral data, as we have shown in Table 1, but some degree of uncertainty remains because of the possible effect of other factors (dielectric constant, electrostatic effects, etc.) on the absorption spectra. With this caveat, we can compare reactivities in water and in the micelles using two different pH values (4.5 and 7.13).

Our data show a substantial agreement between the chemical reactivity of ribonuclease in pH 4.5 aqueous solution and in the micelles prepared with pH 7.13, However, for the other pair of matched experiments (aqueous pH 7.13 solution and the micelle solution prepared with pH 9.8), a much larger value for the initial velocity is obtained for the hydrocarbon phase. It is unlikely that this effect is due to a mis-match of the pHs, since pH changes have only a moderate effect on rates and K_m values (15). Initial velocities were also measured at pH 7.6 in water (borate buffer 0.05M) and found to be not significantly different from those obtained at pH 7.13. Thus, the observed enhancement seems to be due to an intrinsic property of the micelle environment, which is only arising above a certain pH.

It is also interesting to note that ribonuclease does not show any appreciable reaction in water at pH 9.8. However, when the enzyme at pH 9.8 is injected into the "hospitable" hydrocarbon phase, it rapidly regains its activity.

The chemical properties and enzymatic hydrolysis of CP as a function of pH have been extensively investigated by several authors (15-16). Our initial velocity data for aqueous solution are in good agreement with literature data (17-18).

Preliminarily, we have tested the activity of ribonuclease with RNA solubilized in micelles. Also in this case, reaction occurs readily, as judged from the decrease of absorbance at 300 nm, from the increase in absorbance at 260 nm, or from the decrease in ellipticity at 265 nm. A detailed kinetic and spectroscopic study is in progress.

In all the reactions described until now, a water solution of the enzyme is added to the micelle solution of the substrate. Reactions readily occur also when a micelle solution of the enzyme is added to a micelle solution of the substrate, but this is not as simple operationally, since the enzyme stock solution in this case cannot be higher than ca. 20 μM.

DISCUSSION

All properties of the micellar systems described here appear to be based upon two, apparently contradictory, basic features: on the one hand there is the compartimentalization of the protein in the micellar system, by which the protein is protected from the organic solvent by a layer of water and surfactant molecules. This guarantees the enzyme's stabilization and maintenance of activity. On the other hand, there is also an open system, i.e. a system which allows a fast exchange with reactants dispersed in the external organic medium. The dynamic nature of the reversed micelles has been pointed out also by other authors (19,6). While the structure of protein-containing micelles under those conditions must still be investigated, a few preliminary considerations on the system are possible on the basis of the data obtained by Eicke on similar systems (20). Thus, the molar ratio H_2O/AOT of 11.1, that we have in our enzymatic assays with CP, should produce micelles having a molecular weight of ca. 75,000, each containing ca. 116 AOT molecules and 1290 water molecules, and having a spherical aqueous pseudophase with a radius of ca. 21 Å. Thus, this inner core cannot accomodate also ribonuclease (which has per sé in solution a gyration radius of ca. 18 Å (21)), and therefore it is likely that the solubilized protein induces the formation of micelles which are larger than the "empty" ones. This view is reinforced by our data on the reaction between ribonuclease and RNA. Here, two macromolecules must be in contact, and since they are not denatured, the very large complex must be shielded from the organic solvent during the reaction, i.e. it must be encapsulated in the aqueous core of a very large reversed micelle.

Concerning the activity of the enzyme in the micelles in relation to activity in water, the conclusion is that activity is not impaired, and actually under certain conditions it tends to be higher than in water solution. It is difficult at present to give a satisfactory explanation for this enhancement effect, other than stating the obvious one, that it reflects a

microenvironment perturbation on enzyme reactivity. We are presently investigating the physical properties of the protein-containing micelles at various pHs, in order to describe more precisely the environment of the protein in the micelles.

In addition to the problem of the kinetic behavior, there are other lines of interest opened by the micellar solubilization of enzymes in aprotic solvents. For example, the method may prove useful for the characterization in solution of water - insoluble membrane proteins and it may also be technologically interesting, e.g. for the enzymatic transformation of water insoluble substrates. Also, the micellar system permits spectroscopic and enzymatic studies to be carried out at sub-zero temperatures, maintaining the enzyme in an essentially water environment (data will be shown in our next paper). Finally, the micellar system containing proteins raises a series of challenging biophysical questions, concerning for example the forces responsible for the stability of the proteic micellar structure and the state of water surrounding the solubilized protein.

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REFERENCES

1) Dossena,A., Rizzo,V., Marchelli,R., Casnati,G. and Luisi,P.L. (1976) Biochim.Biophys.Acta 446, 493-505.
2) Luisi,P.L., Henninger,F., Joppich,M., Dossena,A. and Casnati,G. (1977) Biochem.Biophys.Res.Commun. 74, 1384-1389.
3) Luisi,P.L., Bonner,F.J. and Walsoe,C. in: Proceedings of the 5th American Peptide Symposium (1977) (Goodman,M., Meienhofer,J., ed.) pp.591-593, John Wiley & Sons., New York.
4) Luisi,P.L., Bonner,F.J., Pellegrini,A., Wiget,P. and Wolf,R. (1979) Helv.Chim.Acta in press
5) Fendler,J.H. (1976) Accounts Chem.Res. 9, 153-161.
6) Eicke,H.F. (1977) in: Micellization, Solubilization and Microemulsions (Mittal K.L., ed.) Vol.1, pp. 429-443, Plenum Press, New York.
7) Gitler,C. and Montal,M. (1972) Biochem.Biophys.Res.Commun. 74, 1486-1491.
8) Gitler,C. and Montal,M. (1972) FEBS Letters 28, 329-332.
9) Martinek,K., Levashov,A.V., Klyachko,N.L. and Berenzin,I.V. (1978) Doklad. Akad.Nauk SSSR (Engl.edit.) 236, 951-953.
10) Bahr,J.T., Cathou,R.E. and Hammes,G.G. (1965) J.Biol.Chem. 240, 3372-3378.
11) Sage,H.J. and Singer,S.J. (1962) Biochem. 1, 305-317.
12) Hammes,G.G. and Schimmel,P.R. (1965) J.Am.Chem.Soc. 87, 4665-4669.
13) Nome,F., Chang,S.A. and Fendler,J.H. (1976) J.Chem.Soc. Perkins II,296-302.
14) Menger,F.M. and Saito,G. (1978) J.Am.Chem.Soc. 100, 4376-4379.

15) Findlay,D., Mathias,A.P. and Rabin,B.R. (1962) Biochem.J. 85, 139-144.
16) Herries,D.G., Mathias,A.P. and Rabin,B.R. (1962) Biochem.J. 85, 127-134.
17) Crook,E.M., Mathias,A.P. and Rabin,B.R. (1960) Biochem.J. 74, 234-238.
18) Litt,M. (1961) J.Biol.Chem. 236, 1786-1790.
19) Menger,F.M., Donohue,J.A. and Williams,R.F. (1973) J.Am.Chem.Soc. 95, 286-288.
20) Eicke,H.F. and Rehak,J. (1976) Helv.Chim.Acta 59, 2883-2891.
21) Filmer,I.D.L. and Kaesberg,P. (1962) Brookhaven Symp.Biol. 15, 210-216.
22) Wong,M., Thomas,J.K. and Grätzel,M. (1976) J.Am.Chem.Soc.98, 2391-2397.