RESEARCH COMMUNICATION

Reverse vesicles as a new system for studying enzymes in organic solvents

Alvaro SÁNCHEZ-FERRER and Francisco GARCÍA-CARMONA*

Departamento de Bioquímica y Biología Molecular, Facultad de Biología, Universidad de Murcia, E-30001 Murcia, Spain

A new approach to the study of enzyme activity in organic solvents has been developed by using optically transparent reverse vesicles. Polyphenol oxidase was incorporated in an active form into the above ternary system formed by the non-ionic surfactant tetra(ethylene glycol) dodecyl ether/n-dodecane/water. The enzyme in this microenvironment, surprisingly, showed an apparent positive co-operativity which has never before been described either in aqueous solution or in reverse micelles. In addition, the $V_{\max}$ expressed was similar to that in water and twice that displayed in reverse micelles.

INTRODUCTION

The study of proteins in amphiphilic self-organizing systems has attracted much attention over the last three decades because this represents a good experimental approach for studying the functions of proteins in biomembranes [1]. In particular, work done with liposomes or normal vesicles, since they were first described in 1964 [2], is vast. In the field of reverse micelles, since 1977 [3] over 30 enzymes have been entrapped in this system and been shown to retain their biological activity. In addition, enzymes have expressed their catalytic activity in liquid crystalline structures of lamellae, cylinders and ball-shaped micelles [4].

A method for preparing reverse vesicles has recently been described [5], using the non-ionic surfactant tetra(ethylene glycol) dodecyl ether ($R_{12}EO_4$) in dodecane. Reverse vesicles are self-closed spherical structures in which surfactant inverted bilayers (head-to-head) separate well-defined interior and exterior apolar phases (Fig. 1).

The present paper aims to show for the first time that such reverse vesicles are capable of entrapping enzymes without any loss in enzyme activity. The enzyme used was polyphenol oxidase (EC 1.14.18.1), as this oxidase has been assayed in other surfactant structures, such as liposomes [6] and reverse micelles, by our group [7–9].

MATERIALS AND METHODS

Materials

$R_{12}EO_4$ and t-butyl catechol (99%) were purchased from Fluka (Madrid, Spain). Mushroom polyphenol oxidase was from Sigma (St. Louis, MO, U.S.A.) and was used without further purification. n-Dodecane and sodium metaperiodate were from Merck (Darmstadt, Germany). The latter can oxidize the o-diphenol to o-quinone [10], and was used to prepare t-butyl o-benzoquinone in order to determine its spectral properties ($\lambda_{\max}$, ϵ) and its influence on the structure of reverse vesicles.

Preparation of reverse vesicles

Reverse vesicles were prepared using the method reported by Kuneida *et al.* [5]. The vesicles are formed by microinjection of

1% (w/w) 50 mM-Mes buffer, pH 6.0, into a 99% (w/w) dodecane solution containing 2.5% (w/w) $R_{12}EO_4$, while being gently shaken at 15 °C. These reverse vesicles are oligolamellar and range in size from < 1 μ m to 10–15 μ m [5].

Enzyme assay

In the presence of molecular oxygen, polyphenol oxidase catalyses the o-hydroxylation of monophenols (cresolase activity). It also catalyses the oxidation of the o-diphenols produced to the corresponding quinones (catecholase activity). The substrate used was t-butyl catechol, whose corresponding t-butyl o-quinone has an ϵ of $1570 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 370 nm. Unless otherwise stated, the reverse vesicle standard reaction medium contained (final concentrations) 24 mM-t-butyl catechol, 52 mM- $R_{12}EO_4$ and polyphenol oxidase (1.5 μ g/ml) dissolved in 50 mM-Mes buffer, pH 6.0, at 20 °C.

Enzymic activity at different temperatures was studied by incubating the enzyme in the standard reaction medium for 15 min. The total amount of t-butyl o-quinone produced was evaluated at 370 nm, after cooling the surfactant solution to 15 °C.

Fig. 1. Schematic structure of a reverse vesicle

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; GST, glutathione transferase; L_{50} , half-saturation concentration of ligand; I_{50} , inhibitor water/surfactant molar ratio, representing micelle size.

* To whom correspondence should be addressed.

Thermostability was measured by incubating the enzyme reverse vesicles at different temperatures without substrate for 15 min. The enzyme solution was then cooled to 20 °C and assayed with *t*-butyl catechol in the standard reaction medium.

Polyphenol oxidase activity was also measured in 50 mM-Mes buffer, pH 6.0, at 20 °C and in Brij 96/cyclohexane reverse micelles [water/surfactant molar ratio (W_o) = 12] by using previously described methods [7,9].

RESULTS AND DISCUSSION

The non-ionic surfactant $R_{12}EO_4$ dissolves in dodecane to form isotropic solutions without any liquid crystal structures. When a small amount of water is added, this binary system splits into a lamellar liquid crystalline phase, in which the space between the reverse bilayers depends on the amount of water [5].

Fig. 2. Effects of temperature and substrate concentration on reverse vesicle turbidity

(a) Changes in the turbidity of reverse vesicles with temperature at different substrate concentrations: ○, 12 mM; ●, 18 mM; □, 36 mM. The reaction medium consisted of 1% (w/w) 50 mM-Mes buffer, pH 6.0, in a 99% (w/w) dodecane solution containing 2.5% (w/w) $R_{12}EO_4$, to which *t*-butyl catechol was added to reach the desired concentration. (b) Effect of *t*-butyl catechol (TBC) concentration on the temperature at which the reverse vesicle phase became turbid (T_p).

These structures are transformed into optically transparent reverse vesicles by simple shaking by hand. In fact, the addition of 1% (w/w) pure water to a 99% dodecane solution containing 2.5% (w/w) $R_{12}EO_4$ readily results in the formation of these reverse vesicles [5].

In our case, the addition of neither polyphenol oxidase nor Mes buffer had a significant effect on the system, unlike the substrate *t*-butyl catechol. This substrate did not alter the global reverse vesicle spherical structure at different substrate concentrations (1–36 mM) at 20 °C, although it modified the temperature limits of the reverse vesicle phase. The reverse vesicle spherical structure was confirmed by polarized light microscopy [11] (results not shown).

It is well known that there is a very pronounced change in the appearance of the Gibbs phase diagrams of oil/non-ionic surfactant/water systems with increases in temperature [12]. Movement induced by temperature in the relative positions and extension of the phases present in the oil/ $R_{12}EO_4$ /water system have been reported by Friberg & Lapczynska [13]. These changes can be shown by repeated measurements of the temperature at which the mixture becomes either clear or turbid [14], and are easily followed at 500 nm [15]. The typical curve obtained against temperature is sigmoid (Fig. 2a). From this curve, the temperature at which the changes in the reverse vesicle phase occur (T_p) can be estimated (Fig. 2a) as the intercept of the extrapolation of the first part of the curve, before the onset of turbidity, and the straight line defined by the slope before the maximum in turbidity. This turbid phase should correspond with the isotropic liquid phase described for this ternary composition [13]. Fig. 2(b) shows a decline in T_p when the *t*-butyl catechol concentration is increased. This dependence may be the result of changes in the hydrophilic-hydrophobic interaction between surfactant molecules owing to the presence of *t*-butyl catechol molecules, which may act as a co-surfactant, altering the phase equilibrium. In fact, *t*-butyl catechol has been characterized as an interfacial substrate for polyphenol oxidase in reverse micelles [9].

The effect on the system of the *o*-quinone formed during the reaction was studied, since oxidation of the substrate, *t*-butyl catechol, should lead to products displaying affinities for the $R_{12}EO_4$ phase distinct from those of the non-oxidized substrate molecules. In particular, the final products (more hydrophobic than the parents) might desorb from the hydrophilic part of the bilayer, thus altering its physical organization and hence the enzymic activity. This has been checked by adding *t*-butyl *o*-benzoquinone produced in water by the oxidation of the substrate with metaperiodate. However, no effects were detected on the T_p of the system using quinone concentrations up to 2 mM, which is four times higher than the levels of quinone obtained with the enzyme (see below).

Once the effect of the reactants on reverse vesicles had been tested, the enzyme was placed in the presence of its substrate, showing it to be catalytically active. This activity was linear with time and was proportional to polyphenol oxidase concentration.

Activity was also influenced by temperature (Fig. 3). The maximum activity after 15 min was found in the temperature range (15–25 °C) corresponding to reverse vesicles producing a final quinone concentration of 0.5 mM. This activity decreased progressively near the phase boundary until it disappeared in the turbid phase. This different enzymic behaviour cannot be explained by a straightforward effect of temperature on the enzyme, since the enzyme remains catalytically active at up to 45 °C in water and 35 °C in reverse micelles [8]. Instead, such behaviour might be due to an alteration in the physical properties of the enzyme microenvironment (dielectric permeability, polarity and/or microviscosity) [4] and/or to the fact that the enzyme may be forced to work in greater hydrophobic contact

Fig. 3. Dependence of enzymic activity on temperature
●, Activity expressed by polyphenol oxidase, estimated by the production of t-butyl o-quinone at 370 nm, after 15 min in the standard reaction medium at each temperature. ○, Turbidity of the triphasic system R₁₂EO₄/n-dodecane/t-butyl catechol (24 mM) in Mes buffer at each temperature, measured at 500 nm. The activity expressed at 20 °C in the standard reaction medium by the enzyme reverse vesicle solution is shown after incubation for 15 min at 20 °C (△) and at 35 °C (▲).

Fig. 4. Dependence of polyphenol oxidase activity on substrate concentration
The double-reciprocal plot (●) shows apparent positive co-operativity which fits the Hill equation (dashed line) with $h = 2.1$. TBC, t-butyl catechol.

with surfactant molecules [4]. This microenvironment effect leads to an irreversible inactivation of the enzyme above 25 °C (Fig. 3). This inactivating effect was tested by incubating the reverse vesicles containing the enzyme for 15 min at these temperatures ($t > 25$ °C), and then measuring the activity at 20 °C.

The dependence of polyphenol oxidase activity on substrate concentration was also examined in reverse vesicles (Fig. 4). The enzyme, surprisingly, exhibited non-Michaelis behaviour in this system, even though polyphenol oxidase has been described as a

Table 1. Kinetic parameters of polyphenol oxidase in water, reverse micelles and reverse vesicles

The enzyme concentration used in each medium was 1.5 μg/ml.

	$V_{\max}$, (μM/min)	K_m (mM)	EC_{50} (mM)	h
Water*	300	4	—	1
Reverse micelles†	175	13.5	—	1
Reverse vesicles	318	—	31.5	2.5

* Activity measured in 50 mM-Mes buffer, pH 6.0, at 20 °C according to [7].
† Activity measured in Brij 96/cyclohexane reverse micelles ($W_0 = 12$) by using previously described method [9].

Michaelis enzyme in water [16] and in reverse micelles [7–9]. This apparent positive co-operativity of the enzyme fits the Hill equation [17], with $h = 2.1$ and an EC_{50} of 31.5 mM (Table 1). In addition, it should be noted that the $V_{\max}$ expressed in reverse vesicles (Table 1) is similar to that expressed in buffer and twice that exhibited in reverse micelles [9].

This positive co-operativity found in reverse vesicles might be explained by conformational changes in the proteins induced by a more structured microenvironment. However, this possibility seems to be very unlikely, since the enzyme expressed the same $V_{\max}$ as in water, and did not exhibit this behaviour in the reverse micellar medium [7–9]. Another explanation could stem from the effect of t-butyl catechol as co-surfactant, increasing the fluidity of the reverse vesicular medium as the concentration of the substrate rises. Consequently, the substrate itself is more accessible to the enzyme as its concentration increases, showing an apparent enzymic co-operativity.

In conclusion, the fact that catecholase activity in reverse vesicles resembled that in aqueous solution suggests that the catalytic activity was not drastically altered by using this new optically transparent self-organized surfactant system in an apolar medium. This work opens up new possibilities for studying enzymes in apolar media, since this ternary system protects the enzyme against the denaturation produced by the surrounding organic solvent, and the activity can be easily monitored by conventional spectroscopic techniques.

The potential applications of the system range from basic membrane research to bioconversion of water-insoluble compounds in fine organic synthesis. One example of the former would be the study of the proteins Folch-Pi and myelin basic protein, which are closely associated with an inverted bilayer environment in the multilamellar architecture of the myelin sheath [18]. As for the second possible application, the low water content of the system, less than in reverse micelles and close to that of neat organic solvents [19], permits the study of reverse chemical reactions in which water acts as a reactant, such as peptide synthesis by the use of proteinases [20].

We thank Dr. F. López-García for his kind advice on the polarized light microscopy. This work was partially supported by CICYT (Proyecto BIO91-0790). A. S.F. is a holder of a reincorporación grant from Ministerio de Educación y Ciencia.

REFERENCES

1. Martinek, K., Levashov, A. V., Klyachko, N., Khmel'nitski, Y. L. & Berezin, I. V. (1986) *Eur. J. Biochem.* **155**, 453–468
2. Bangham, A. D. & Horne, R. W. (1964) *J. Mol. Biol.* **8**, 660–668
3. Luisi, P. L., Giomini, M., Pileni, M. P. & Robinson, B. H. (1988) *Biochim. Biophys. Acta* **947**, 209–246

4. Klyachko, N. L., Levashov, A. V., Pshezhetsky, A. V., Bogdanova, N. G., Berezin, I. V. & Martinek, K. (1986) *Eur. J. Biochem.* **161**, 149–154
5. Kuneida, H., Nakamura, K. & Evans, D. F. (1991) *J. Am. Chem. Soc.* **113**, 1051–1052
6. Miranda, M., Amicarelli, F., Poma, A., Ragnelli, A. M. & Arcadi, A. (1988) *Biochim. Biophys. Acta* **966**, 276–286
7. Sánchez-Ferrer, A., Bru, R. & García-Carmona, F. (1988) *FEBS Lett.* **233**, 363–366
8. Bru, R., Sánchez-Ferrer, A. & García-Carmona, F. (1989) *Biotechnol. Bioeng.* **34**, 304–308
9. Bru, R., Sánchez-Ferrer, A. & García-Carmona, F. (1990) *Biochem. J.* **268**, 679–684
10. Cabanes, J., García-Cánovas, F. & García-Carmona, F. (1987) *Biochim. Biophys. Acta* **914**, 325–327
11. Papahadjopoulos, D. & Miller, N. (1967) *Biochim. Biophys. Acta* **135**, 624–638
12. Attwood, D. & Florence, A. T. (1983) in *Surfactant Systems: Their Chemistry, Pharmacy and Biology* (Attwood, D., ed.), pp. 40–72, Chapman and Hall, New York
13. Friberg, S. & Lapczynska, I. (1975) *Progr. Colloid Polymer Sci.* **56**, 16–20
14. Lichterfeld, F., Schmeling, T. & Strey, R. (1986) *J. Phys. Chem.* **90**, 5762–5766
15. Alonso, A., Urbaneja, M. A., Goñi, F., García-Cánovas, F., García-Carmona, F. & Gómez-Fernandez, J. C. (1987) *Biochim. Biophys. Acta* **902**, 237–246
16. Galindo, J. D., Pedreño, E., García-Carmona, F., García-Cánovas, F., Solano, F. & Lozano, J. A. (1983) *Int. J. Biochem.* **15**, 1455–1461
17. Hill, A. V. (1910) *J. Physiol. (London)* **40**, iv–vii
18. Brinks, B. P., Chatenay, D., Nicot, C., Urbach, W. & Waks, M. (1989) *Biophys. J.* **55**, 949–955
19. Klibanov, A. M. (1989) *Trends Biochem. Sci.* **14**, 141–144
20. Luthi, P. & Luisi, P. L. (1984) *J. Am. Chem. Soc.* **106**, 7285–7286

Received 9 April 1992/11 May 1992; accepted 15 May 1992