A theoretical study on the expression of enzymic activity in reverse micelles

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The present work deals with a theoretical model of catalysis by enzymes entrapped in reverse micelles. Three aspects of the enzyme–reverse-micelle system have been considered: structure, dynamics and enzyme distribution and catalysis in reverse micelles. A proposed structural model of reverse micelles [El Seoud (1984) in Reverse Micelles (Luisi, P. L. & Straub, B. E., eds.), p. 81, Plenum Press, New York] consists of three domains: surfactant apolar tails, bound water and free water. Dynamics are based on a dynamic equilibrium of association–dissociation that lead one to consider the dispersed polar phase as a pseudo-continuous phase [Luisi, Giomini, Pileni & Robinson (1988) Biochim. Biophys. Acta 947, 207–246]. Enzyme is distributed among the reverse-micelle domains and it expresses a catalytic constant for each one of them. The overall activity is calculated taking into account the volume in which enzyme is solubilized, and expressed as a function of the whole volume (V). The characteristic parameters of reverse micelles, $\omega_0 (= [H_2O]/[surfactant])$ and $\theta (= \%$ water, v/v), were investigated as modulators of enzymic activity. Three basic patterns of modulation by ω_0 were found depending on which domain the enzyme expressed the highest catalytic constant. Combinations of those basic patterns lead to other modulation types that can be found experimentally, such as superactivation. Other combinations predict behaviour patterns not described to date, such as superinhibition. Dependence of catalytic activity on θ was only stated at ω_0 values around a critical value, which coincides with the appearance of free water.

INTRODUCTION

The number of entrapped enzymes in reverse micelles (Martinek et al., 1986) that have been investigated has been increasing since Hanahan (1952) studied the catalytic activity of phospholipase A_2 in ethereal solutions of phosphatidylcholine. Up to now, more than 30 enzymes have been studied in reverse micelles. Most of them are hydrolases [α-chymotrypsin (Likhtenshtein et al., 1983), trypsin (Walde et al., 1988), lysozyme (Steinmann et al., 1986), lipase (Han & Rhee, 1986)], dehydrogenases [alcohol dehydrogenase (Malakhova et al., 1983; Samama et al., 1987), 20β-hydroxysteroid dehydrogenase (Hilhorst et al., 1984)] or oxidases [peroxidase (Martinek et al., 1981), lipoxygenase (Kurganov et al., 1985), polyphenol oxidase (Sánchez-Ferrer et al., 1988)].

It has been exhaustively shown that enzymes in reverse micelles not only retain their biological activity (Martinek et al., 1986; Luisi & Magid, 1987) but also preserve it for long periods of time (Han & Rhee, 1986; Lee & Biellmann, 1986; Sánchez-Ferrer et al., 1988) under certain conditions. Moreover, their kinetic parameters have been determined, studies with inhibitors have been made (Samama et al., 1987), and the results have been compared with those obtained in bulk water.

Because of the considerable number of enzymes that are catalytically active in reverse micelles and the fact that none has been noticed to be unable to express its activity in this medium, it can be considered that reverse micelles are a useful system for studying enzyme behaviour in organic solvents.

The reverse-micelle system offers several possibilities for studying: (a) the use of substrates poorly soluble in water (Hilhorst et al., 1984; Han & Rhee, 1986); (b) membrane proteins (Delahodde et al., 1984; Nicot et al., 1985); (c) enzymes working at interfaces such as lipid/water, where the water structure is different from that of bulk water (Drost-Hansen & Clegg, 1979).

These capacities of reverse micelles are closely related to their structure and the overall properties of the system: they form transparent and homogeneous solutions, with an average micelle size of low dispersion (Eicke et al., 1984). A dynamic equilibrium of fusion and dissociation (Fletcher et al., 1987) maintain the homogeneity and monodispersity of the system.

One of the most remarkable features of enzymic catalysis in reverse micelles is the dependence of catalytic activity on water/surfactant molar ratio: $\omega_0 (= [H_2O]/[surfactant])$. For more than 15 enzymes studied the dependences are very similar; normally it is bell-shaped, but it has been shown that in other enzymes it is hyperbolic (Douzou, 1980) and even decreasing (Hilhorst et al., 1984). However, hitherto no general rule has been proposed to explain the experimentally observed phenomena.

At present there are a large amount of physicochemical and enzymological data (Martinek et al., 1986; Luisi & Magid, 1987) on reverse micelles, but a link between them has not been well established.

The present paper proposes a model to match physicochemical properties of reverse micelles with the expression of catalytic activity of the enzyme entrapped in them.

Abbreviations used: AOT, dioctyl sodium sulphosuccinate; CTAB, cetyltrimethylammonium bromide.
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The model allows us to explain the dissimilar results appearing in the bibliography on the modulation of catalytic ability of enzymes in reverse micelles by parameters such as ω_0 and degree of hydration, θ. Moreover, some modulation features can be foreseen that have not yet been experimentally revealed.

THEORY

In this section we deal with three aspects of the solubilized enzymes in the reverse-micelle system: structure of reverse micelles; dynamics of reverse micelles; distribution and catalytic expression of the enzyme in reverse micelles.

The parameters used are defined immediately below.

List of parameters

ω_0:	micelle size $[H_2O]/[\text{surfactant}]$.
θ:	% water (v/v). If ω_0 is held constant this parameter represents the micelle concentration.
r:	hydrodynamic radius of the micelle.
r_w:	water-droplet radius.
$K_{eq.}$:	equilibrium constant of the dimerization process.
$[D]$:	dimeric micelle concentration.
$[M]$:	monomeric micelle concentration.
$[M]_i$:	micelle concentration as if all the micelles were in the monomeric state.
α:	degree of micelle association.
f:	shape factor.
N_m:	number of micelles.
V_{H_2O}:	overall water volume.
N_A:	Avogadro's number.
V:	overall volume of the micellar solution.
V_i:	volume of each domain, where i indicates the domain: $i = $ s, surfactant apolar tails; $i = $ b, bound water; $i = $ f, free water; $i = $ as, apolar solvent.
ρ:	penetration factor of surfactant apolar tails into the apolar solvent.
n:	number of water molecules bound per surfactant polar head.
V^M_s:	volume occupied by 1 mol of surfactant.
mol of S:	number of mol of surfactant.
K^1_E, K^2_E:	enzyme partition coefficients between domains.
$[E]_i$:	enzyme concentration in each domain.
k_i:	catalytic constant in each domain.

Structure of reverse micelles

Reverse micelles are spherical aggregates consisting of a water core separated from a continuous apolar phase by a surfactant shell. It is well known that in the absence of water some surfactants such as AOT are able to form these aggregates, whereas other such as CTAB or SDS need a co-surfactant (short-chain alcohol, cholesterol) to generate such structures.

The presence of water increases the reverse-micelle size, which is determined by ω_0. There is an empirical relationship betwen ω_0 and reverse-micelle radius (Nicholson & Clarke, 1984) for AOT as surfactant:

$$r \text{ (hydrodynamic radius)/nm} = 0.175\,\omega_0 + 1.5 \quad (1)$$

If we assume that AOT molecule length is approx. 1.5 nm, the water-droplet radius is given by:

$$r_w \text{ (water droplet)/nm} = 0.175\,\omega_0 \quad (2)$$

and it can be systematically varied between 0 and 20 nm. The presence of a polar head in the surfactant molecule, which can be charged, leads us to assume that water molecules that hydrate the polar head are subject to different forces from those belonging to bulk water. The former can form hydrogen-bonding with polar heads and could undergo electrostatic attractions when the polar head is charged. As a result, the hydration water becomes more structured than the bulk water. Three solubilization sites have been found in reverse micelles made of AOT in heptane by using three fluorescent probes of increasing polarity derived from naphthalene (Bardez et al., 1985).

Previously, an AOT reverse-micelle structure was outlined when the amount of water exceeds the hydration requirements of the surfactant; this consists of three different domains (El Seoud, 1984), as follows.

(1) Surfactant apolar tails, whose penetration into the apolar solvent depends on surfactant and apolar solvent type. Studies on interaction between apolar solvent and surfactant apolar tails have been carried out (Eicke et al., 1984) through measurements of domain spectroscopic dielectric loss versus time in water/AOT/apolar solvent systems. It has been found that penetration degree of the apolar tail into the apolar solvent and the molecular volume of the latter are in an inverse relationship.

(2) Bound water, through hydrophilic interactions with polar head of surfactant. As mentioned above, this water possesses properties qualitatively different from those of bulk water (Kuntz & Kauzmann, 1974), such as the extent of hydrogen-bonding, effective dielectric constant and viscosity, mobility etc.

(3) Free water, whose properties tend towards those of bulk water as ω_0 increases. Measurements of water activity versus ω_0 show a hyperbolic shape, reaching a value higher than 0.98 at $\omega_0 = 10$ (Higuchi & Misra, 1962).

In accordance with this structural model, reverse micelles can possess up to three different microenvironments when water in the system exceeds the hydration requirements of surfactant polar heads (three-domain reverse micelles). If we either decrease water or increase surfactant amount, reverse micelles will consist of only two microenvironments, without free water (two-domain reverse micelle). Reverse micelles will consist of only one microenvironment with a further decrease of water (dry reverse micelles). In practice we start from dry reverse micelles and water is added to obtain a desired ω_0 value.

Dynamics of reverse micelles

Amphiphilic molecules of surfactant in water/apolar solvent mixtures self-assemble to form spontaneously not only spherical or elipsoidal aggregates but continuous liquid-crystal structures having cylindrical or lamellar arrangement depending on the composition of the medium. In particular the concentrations of the medium components (water/surfactant/apolar solvent) determine the mode of aggregation. In consequence, starting from a composition that yields a continuous liquid-crystal aggregate, reverse micelles can be obtained by increasing the concentration of the appropriate component, in this case the apolar solvent.

With these interconvertible modes of aggregation there is the underlying idea that such stable aggregates are ruled by a dynamic equilibrium. In the case of reverse-micellar aggregate the dynamic equilibrium involves a fusion and dissociation process (Fletcher *et al.*, 1987) that can be represented through the following equation:

$$O + O \underset{}{\overset{K_{eq.}}{\rightleftarrows}} O \qquad (3)$$
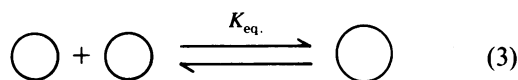
in this way:

$$K_{eq.} = \frac{[D]}{[M]^2} \qquad (4)$$

and

$$\alpha = \frac{2[D]}{[M]_i} \qquad (5)$$

In the dimerization process the water-droplet volume is twice that of a monomeric micelle, and, if we consider that dimeric micelles tend to a spheric shape, there is a loss of surface and so that $\omega_{0(d)} < 2\omega_{0(m)}$:

$$\omega_{0(d)} = \omega_{0(m)} \cdot f \qquad (6)$$

f can be estimated approx. 1.33 for a sphere.

In consequence, the dimerization process involves a microdispersion of the micelle size with a standard deviation depending on micelle concentration, so that water-droplet radius of monomeric micelles (r_w) is an estimate by extrapolation to infinite dilution of the micelles.

The number of micelles can be evaluated as:

$$N_m = \frac{3V_{H_2O}}{4\pi r_w^3} \qquad (7)$$

where r_w is correlated with ω_0 through eqn. (2). The micelle concentration as if all the micelles were in the monomeric state is given by:

$$[M]_i = \frac{N_m}{N_A \cdot V} \qquad (8)$$

Taking into account the micelle balance:

$$[M]_i = [M] + 2[D] \qquad (9)$$

then eqn. (5) can be expressed as function of eqns. (4), (8) and (9):

$$\alpha = 1 + \frac{1 - (8K_{eq.}[M]_i + 1)^{\frac{1}{2}}}{4K_{eq.}[M]_i} \qquad (10)$$

Distribution and catalysis of enzymes in reverse micelles

The enzyme molecules, like any other molecule, in the presence of a heterogeneous medium tend to be distributed among the different phases that make up the system. Reverse micelles can be thought of as a micro-heterogeneous medium where solubilized molecules are subject to a partition among the different phases.

By relating diffusion-controlled rate constant (approx. 10^{10} $M^{-1} \cdot s^{-1}$) with exchange rate constant (approx. 10^7 $M^{-1} \cdot s^{-1}$) it has been shown (Fletcher *et al.*, 1987) that 1 in 1000–10000 encounters between micelles results in solubilizate exchange. When the rate of the chemical or enzymic reaction we are dealing with is much lower than the time scale for exchange, the dispersed phase can be regarded as a pseudo-continuous phase, since transport

of solubilizates between micelles is not rate-limiting (Luisi *et al.*, 1988).

On this basis the enzyme distribution can be described through some simple relationships.

The volume filled by every microenvironment in the micellar solution is given by:

$$V_s = V^M_s \cdot \rho \cdot mol \text{ of } S(1 - \alpha/f) \qquad (11)$$

where α/f represents a surfactant fraction loss by dimerization. If a sphere is formed about 25% of the surface is lost in the dimeric fraction α:

$$V_b = V_{H_2O} [(1 - \alpha)n/\omega_{0(m)} + \alpha n/\omega_{0(d)}] \qquad (12)$$

the term $(1 - \alpha)n/\omega_{0(m)}$ means that n per every $\omega_{0(m)}$ water molecules are bound in monomeric micelles and the term $\alpha n/\omega_{0(d)}$ that n per every $\omega_{0(d)}$ water molecules are bound in dimeric micelles. The addition of both is the overall fraction of bound water with respect to V_{H_2O}:

$$V_f = V_{H_2O} - V_b \qquad (13)$$

$$V_{\alpha s} = V - (V_s + V_b + V_f) \qquad (14)$$

Scheme 1 shows the enzyme distribution among the three micellar microenvironments where:

$$K^1_E = [E]_b/[E]_f \qquad (15)$$

$$K^2_E = [E]_s/[E]_b \qquad (16)$$

If the reaction medium contains a definite number of enzyme micromoles, its distribution will be:

$$\mu mol \text{ of } E = [E]_f V_f + [E]_b V_b + [E]_s V_s \qquad (17)$$

Applying eqns. (15), (16) and (17):

$$[E]_f = \frac{\mu mol \text{ of } E}{V_f + K^1_E V_b + K^1_E K^2_E V_s} \qquad (18)$$

$$[E]_b = K^1_E [E]_f \qquad (19)$$

$$[E]_s = K^1_E K^2_E [E]_f \qquad (20)$$

When the enzyme works in bulk water it expresses a catalytic constant k, but if it works in a different environment it will express a different catalytic constant, depending on the conformation acquired in the new medium.

In this way we can define k_f, k_b and k_s as catalytic constants expressed by the enzyme in free water, bound water and surfactant apolar tails respectively.

Because of free water's tendency to be similar to bulk water, it can be assumed that $k_f \simeq k$.

Finally, the enzymic reaction takes place in limited spaces owing to the microheterogeneity of the reverse-micelle system, but we actually make measurements of
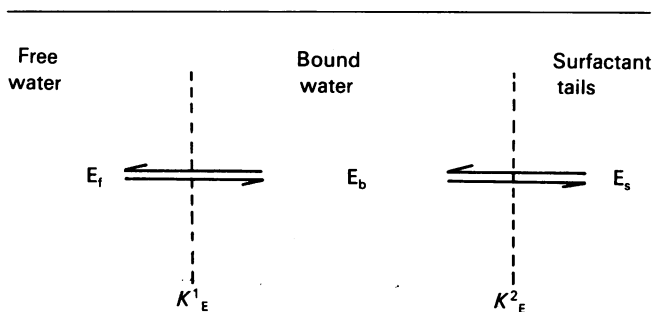
Scheme 1. **Distribution of the enzyme among the micellar microenvironments**

enzymic activity in the overall volume of the cuvette (V). Thus we can express the activity of each enzyme fraction with respect to the overall volume as:

$$A_i = \frac{k_i[E]_i V_i}{V} \qquad (21)$$

and the whole activity as:

$$A = \sum_{i=f,b,s} A_i \qquad (22)$$

RESULTS AND DISCUSSION

Eqns. (2), (7), (8), (10) and (11)–(22) were integrated in a computer program in BASIC run in an Olivetti M24 personal computer with an arithmetic unit chip (8087) to simulate the enzyme behaviour in reverse micelles.

The parameter A was studied both as a function of micelle size ω_0 and water amount contained by the system θ. ω_0 was varied by modification of surfactant concentration (θ constant), and θ was studied at constant ω_0 values.

Effect of micelle size on enzyme expression

Figs. 1(a)–1(c) show the plots of A versus ω_0 when the enzyme expresses the highest activity in only one of the three microenvironments. Three basic patterns of behaviour can be seen, having a common critical point about $\omega_0 = 8$–10. This point is expressed as: (i) a start of the activity when the enzyme is more active in the free water, (ii) a maximum of the activity when the enzyme is more active in bound water or (iii) a swerve in the fall of the activity when the enzyme works mostly in surfactant apolar tails. In the three cases the variation of enzyme behaviour is related to appearance of free water.

In accordance with the eqn. (12), it was considered that n water molecules are hydrating the surfactant polar head. This number varies depending on the kind of surfactant polar head: anionic, cationic, zwitterionic or non-ionic. The hydration number is low for cationic surfactants (CTAB will have one water of hydration per head group and perhaps two per Br^- ion) and higher for anionic (AOT has six per Na^+ ion, from two to four per sulphonate head group and perhaps one of the ester carbonyl groups is also hydrated) (Luisi & Magid, 1987).
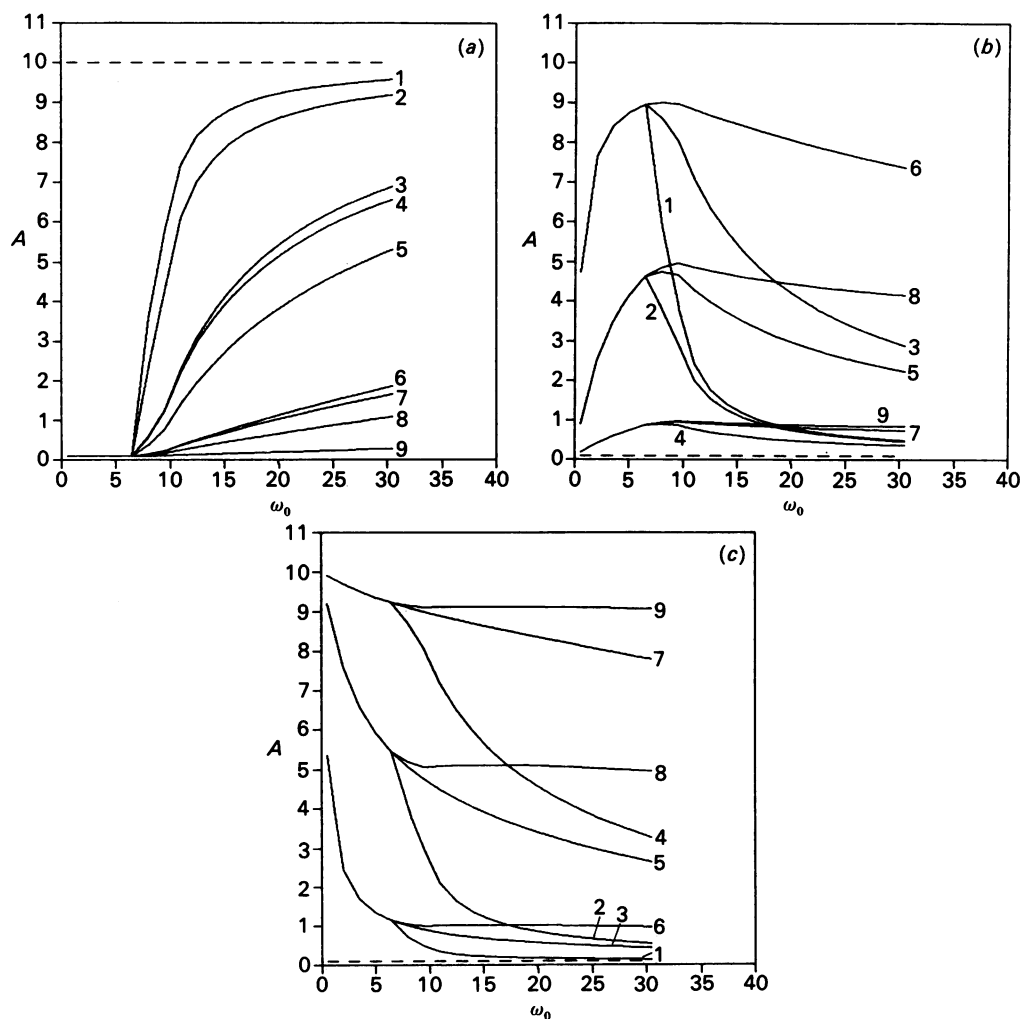

Fig. 1. Dependence of catalytic activity (A) on micelle size (ω_0)

The number of bound water molecules has been taken as 9, and the system contains 1.1% (v/v) of water. Other conditions are: $K_{eq} = 2.5 \times 10^6$ M^{-1}, $f = 1.33$, $V^M_S = 400$ ml·mol^{-1}, $\rho = 0.5$, amount of E = 10^{-5} μmol. (a) $k_f = 1000$, $k_b = 10$ and $k_s = 10$. (b) $k_f = 10$, $k_b = 1000$ and $k_s = 10$. (c) $k_f = 10$, $k_b = 10$ and $k_s = 1000$. For K^1_E and K^2_E see Table 1 (key to curve numbers). The broken line represents the activity in water.

Table 1. Key to curve numbers

K^1_E	K^2_E	Curve no.
0.1	0.1	1
0.1	1	2
0.1	10	3
1	0.1	4
1	1	5
1	10	6
10	0.1	7
10	1	8
10	10	9

The most frequently described pattern is that of Figure 1(b), where the enzyme expresses the maximal catalytic activity in bound water while it is markedly less active in the other two domains. It is usual to talk about an opimal ω_0.

Acid phosphatase and peroxidase (Martinek et al., 1986) have been shown to be superactive enzymes; the former in phosphatidylcholine reverse micelles in octane/methanol/pentanol, and the second one in several reverse-micelle systems (AOT in octane, dodecyl-ammonium propionate in diethyl ether/benzene, Brij 96 in cyclohexane and phosphatidylcholine in methanol/pentanol/octane).

N-trans-Cinnamoyl-α-chymotrypsin deacylation has also been shown to be a superactivity case (Likhtenshtein et al., 1983; Martinek et al., 1986), having an activity profile like that of Fig. 2. It can be seen that activity rises to a maximal value higher than activity in bulk water. As micelle size increases activity tends towards that in bulk water. For this type of response no coherent explanation has been found (Belonogova et al., 1983). This phenomenon can be assumed, by using the model of the present
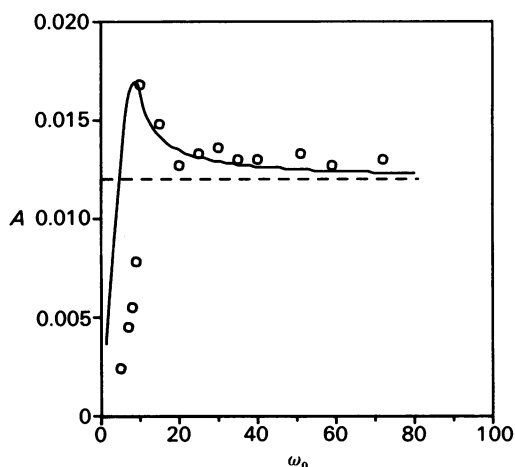
Fig. 2. Superactivity of N-trans-cinnamoyl-α-chymotrypsin deacylation

○ symbols represent experimental data taken from Martinek et al. (1986). These values have been simulated and the results are represented as the continuous line. The broken line represents the activity in bulk water. Conditions are: $K^1_E = 0.05$, $K^2_E = 5$, $k_f = 1200$, $k_b = 13500$, $k_s = 0$ and $n = 9$. Other conditions are the same as in Fig. 1.

work, to be a combination of the patterns from Figs. 1(a) and 1(b). The continuous line in Fig. 2 represents the simulation result obtained for $K^1_E = 0.05$, $K^2_E = 5$, $k_d = 1200$ s^{-1}, $k_b = 13500$ s^{-1}, $k_s = 0$ s^{-1} and $n = 9$.

Enzyme superactivity in reverse micelles can best be described as an increase in its catalytic capacity in the presence of a structured medium, which is a characteristic of the water layers near interfaces (lipid–water, synthetic surfactant–water, hydrophilic matrix–water etc.). In this medium the macromolecules are subject to different forces from those in bulk water. The use of the c.d. technique has revealed structural changes in lysozyme (Steinmann et al., 1986), trypsin (Walde et al., 1988) and myelin basic protein (Nicot et al., 1985) solubilized in reverse micelles with respect to bulk water. It has been proposed for myelin basic protein that its structural changes in reverse micelles lead to a conformation that resembles the native protein, with a more folded structure than in bulk water.

In a general sense, the treatment to which living tissues or microbial cells are submitted for extraction of proteins can involve gentle conditions (low ionic strength or a simple filtrate of the culture broth), but in many cases a drastic treatment is used (high ionic strength, detergents etc.) and afterwards the protein is characterized in buffered aqueous medium.

Obviously, the protein in vitro can be considerably different from the protein in vivo, both kinetically and structurally. In this way, the superactivation phenomenon can be understood as approaching the real properties that the protein has in vivo, since there is a low probability that a chance conformational change with respect to the native structure results in a catalytically more efficient isoform. As the synthetic medium resembles the environment where protein acts in vivo, expressed activity will be more similar to the original activity. In accordance with this hypothesis is the fact that only a few enzymes have been found to be superactive despite the great number of them that have been entrapped in reverse micelles up to now.

The proposed model predicts superactivation phenomena because it proposes that the enzyme can express higher activity in a microenvironment such as bound water or surfactant apolar tails (Figs. 1b, 1c and 2). Moreover, it also predicts superinhibition phenomena as is shown in Fig. 3, where the patterns from Figs. 1(a) and 1(c) have been combined. This inverted bell-shaped response has not been found in any case of an enzyme included in reverse micelles to date. Such behaviour requires that the enzyme be able to catalyse efficiently in surfactant apolar tails and free water, but with poor efficiency in bound water.

At the moment we have assumed that reverse micelles consist of up to three structurally different domains. Although surfactant apolar tails and free water can be thought of as homogeneous microenvironments, bound water can be strictly considered as a succession of water layers entrapped by the surfactant polar heads with several degrees of strength. Finer & Darke (1974), using ^{2}H-n.m.r. spectra, described up to four types of bound water in a phospholipid reverse-micelle system. If we consider that the bound water is heterogeneous the proposed model becomes more complex by the assignment of one catalytic constant per water shell of bound water.

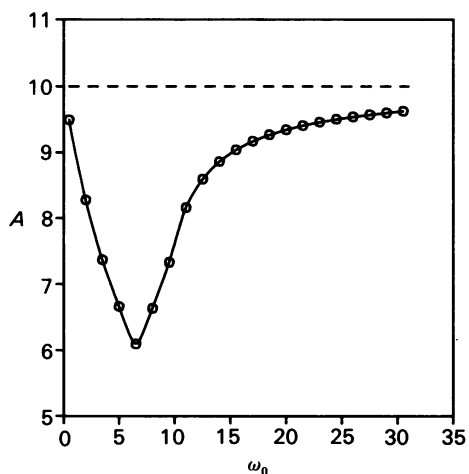Other different behaviours based on combinations of

Fig. 3. Simulation of a superinhibition case

The broken line is the activity in water. Conditions are:
$K^1_E = 0.1$, $K^2_E = 1$, $k_f = 1000$, $k_b = 10$, $k_s = 1000$ and
$n = 9$. Other conditions are the same as in Fig. 1.

the basic patterns referred to above can be proposed, and consequently plots of activity versus ω_0 can adopt several forms, but it is necessary to check how the water amount affects enzymic activity.

Effect of micelle concentration on enzyme expression

Figs. 4(a)–4(c) show the effect of θ on catalytic activity at $\omega_0 = 8$, which is the micelle size where free water appears in our simulations. Below this ω_0 value substantial variations of the activity with respect to θ do not exist because micelles are of the two-domain reverse-micelle type and dynamic equilibrium does not significantly modify the volumes of the two micro-environments and consequently the enzyme partition.

Likewise, at high ω_0 values the increase in free water by dimerization does not amount to a noticeable variation in volume with respect to overall free water, so that the effect on enzyme distribution is negligible.

However, the equilibrium between monomeric and dimeric reverse micelles strongly affects the distribution of the enzyme at ω_0 values where the third micro-environment begins to appear, and the increase in free
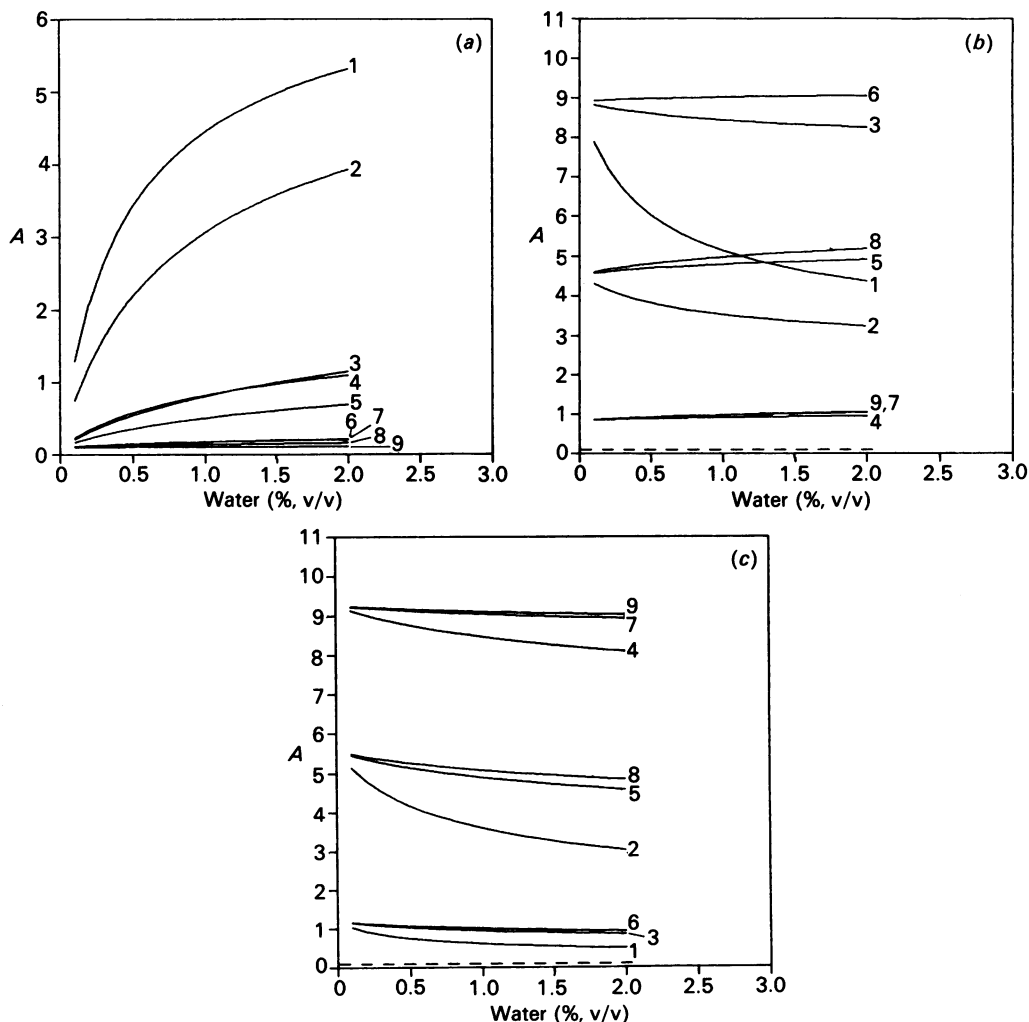

Fig. 4. Dependence of catalytic activity on percentage water (v/v) at $\omega_0 = 8$

All the other conditions are the same as in Fig. 1, including key to curve numbers. In (a) the activity in water is 10.

water represents an important fraction of the whole free water.

The effect produced by θ, which also represents the micelle concentration, on enzymic activity can be understood as a relative variation of domain volumes where the enzyme can solubilize. Such a parameter becomes important when free water begins to appear and consequently the variations among relative volumes are really significant for enzyme distribution. It can be expected that the activity (A) is not deeply affected by micelle concentration (Martinek *et al.*, 1981; Sánchez-Ferrer *et al.*, 1988) generally when the ω_0 value is far from the critical ω_0 value.

In conclusion, it can be said that the proposed model is the simplest (if we consider bound water to be homogeneous) able to explain the dissimilar results appearing in the bibliography on modulation of the catalytic activity of enzymes entrapped in reverse micelles, as well as able to predict other modulation types not described experimentally to date.

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