REVIEW ARTICLE

Kinetic models in reverse micelles

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INTRODUCTION

Biological studies at the molecular level usually follow a process which starts with the isolation from the cell of the macromolecules of interest, for instance proteins and nucleic acids, which are then studied in an *in vitro* system composed of a buffered aqueous solution. In the study of the catalytic activity of proteins it may be apparent that the isolated enzyme lacks the structured environment in which it usually acts *in vivo*.

The discipline that emerged in the late 1970s, baptized as Micellar Enzymology by Martinek's group [1–3], tries to approach the above problem by using a model system based on the self-organizing properties of amphiphiles in solution; this model system is named reverse micelles. It is hypothesized that this system reliably resembles the microenvironment that enzymes find in the cell: reverse micelles consist of micropools of water lined by a monolayer of an amphiphile, all dispersed in an apolar solvent. For a hydrophilic enzyme, which is usually found in the cytosol or the matrix of an organelle, reverse micelles provide the waterpools as a mimetic environment, where the properties of the water resemble the properties of the water closely associated with the cell [4–7]. Since the size of the protein and the size of waterpools are similar, the latter can resemble, better than a bulk buffered aqueous solution, the structured and viscous environment of the cytosol [8]. Enzymes which interact with membranes as either peripheral or integral proteins also find the appropriate environment in reverse micelles [9–14], provided by the monolayer of surfactant and its closely associated water. Not only can the environment of the protein be modelled in a reverse micellar system, so can that of the substrate [15]: reverse micelles can host all kinds of substrate molecules whether hydrophilic, hydrophobic or amphiphilic, and this is an important advantage over an aqueous medium.

It seems, therefore, that reverse micelles may be an appropriate model system for biological studies at the molecular level. Indeed, studies such as those on the conformational properties of peptides and proteins [16–20], protein folding [21,22], enzymological studies regarding reactivity and specificity [23–30], limited proteolysis catalysed by proteinases [31], DNA splicing by restriction enzymes [32], and so on, have been carried out in reverse micelles and have yielded substantially different results from those obtained in an aqueous medium. In addition, reverse micelles have opened up the possibility of developing new biotechniques to carry out bioconversions of apolar compounds [33–40] and to extract proteins from a liquid medium [41–45]. Many of these topics have already been reviewed [46–53], but one of the more controversial aspects of Micellar Enzymology, namely the explanation at the theoretical level of enzyme kinetics in reverse micelles, has not yet been reviewed and will be the topic of this paper.

Throughout this review we will describe physicochemical aspects of the reverse micellar system which are relevant to enzymology. Certain aspects of enzyme kinetics in reverse micelles which differ from those in bulk water will be highlighted, with special attention being paid to the so-called 'bell-shape' and 'superactivity' phenomena, and we will show how enzyme- and substrate-containing reverse micelles are described by each of the different theoretical approaches. Next, each of the different models proposed will be presented, stressing mainly their theoretical basis and assumptions rather than the algebra inherent in the theories (whose main formulae will be appended at the end of the review). Likewise, experimental results which support one or other model and the results that each model can adequately explain will be presented. We will conclude the review by suggesting, from our point of view, the type of information that it is still necessary to obtain from enzyme-containing reverse micellar systems in order to formulate a theory on enzyme kinetics in such a medium that is more accurate than the models proposed to date.

STRUCTURE AND DYNAMICS OF REVERSE MICELLES

Reverse micelles possess some macroscopic properties that make them an ideal system for enzymological studies. First of all, a reverse micellar solution is thermodynamically stable and optically transparent, and large amounts of host molecules can be accommodated without disturbing these macroscopic properties [53]. For instance, aqueous solutions of concentrated protein (1–20 mM), buffer (200–300 mM) and water-soluble substrates (100 mM) dispersed in a surfactant solution in an organic solvent are commonly used in routine kinetic experiments. In addition, apolar substrates can be delivered as a solution in an organic solvent.

These macroscopic properties have permitted the normal use of the techniques of CD [13,16,17,54–57], polarization and time-resolved fluorescence [54,55,58–62], phosphorescence [63,64], UV–visible spectroscopy [55,65], ESR [66–68] and NMR [69] to investigate the structure and active centre environment of proteins solubilized in the waterpools, while Fourier-transform IR spectroscopy [70], luminescence [71–73] and UV–visible spectroscopy [24,74–76] have been applied to the monitoring of the reactions occurring in reverse micelles.

Structural characterization of reverse micelles

While the above provide reliable information concerning the enzyme/substrate system in reverse micelles, no less important are the studies concerning the microscopic structural and dynamic properties of reverse micelles for the correct interpretation of the kinetic data and the implementation of a theory able to predict the behaviour of enzymes in reverse micelles.

The structure of individual surfactant-stabilized reverse micelles, together with their mutual interactions, have been well

Abbreviations used: AOT, dioctyl sodium sulphosuccinate (Aerosol-OT); CTAB, cetyl trimethylammonium bromide; Np, *p*-nitroanilide; GPNA, glutaryl^{-}-Phe-NH-Np.

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characterized over recent years by a range of physical methods, principally photon correlation spectroscopy and small-angle neutron and X-ray scattering [77,78]. It is widely accepted that reverse micelles consist of spherical nanometre-sized water droplets coated with a close-packed surface monolayer of surfactant molecules, orientated in such a way that the surfactant head-groups are hydrated at the surface of the water droplet, with the apolar tails protruding out into, and solvated by, the organic solvent.

Eicke and co-workers [79] have demonstrated, through calculating the Gibbs free energy of a single reverse micelle, that the droplet radius has a very low dispersity, and describe the size distribution of a population of reverse micelles as nearly monodisperse.

This well defined structural organization, together with the near monodispersity of the droplet radius [79,80], has permitted, in the case of dioctyl sodium sulphosuccinate, the establishment of a linear relationship between the droplet radius (r_w), in nm, and the externally controlled parameter ω_0 [77,78,81,82]:

$$\omega_0 = [\text{water}]/[\text{surfactant}] \quad (1)$$

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

The hydrodynamic radius, r, is the water droplet radius plus the length of the surfactant molecule:

$$r(\text{hydrodynamic})(\text{in nm}) = r_w + 1.5 \quad (3)$$

Thus ω_0 controls the size of the droplets and can be routinely varied from zero to several tens, although the upper limit depends on the particular ternary system being studied. The most widely used system is that formed by water, an alkane (normally isooctane) and the anionic surfactant dioctyl sodium sulphosuccinate, better known as AOT. [Aerosol-OT is a trademark substance from American Cyanamide that contains dioctyl sodium sulphosuccinate as a surface active agent. This compound, even when chemically pure, is usually abbreviated to AOT.] Other systems which have been used in micellar enzymology are hexanol/water/cetyl trimethylammonium bromide (CTAB) [83,84] and iso-octane/chloroform/water/CTAB [85] where the surfactant is cationic, and cyclohexane/water/Brij 96 [76,86] where the surfactant is non-ionic.

When water and surfactant are varied simultaneously so that ω_0 remains constant, reverse micelles vary in concentration but not in size. In this case, the nomenclature is not very uniform as this variable is sometimes called θ, but also appears as '[surfactant]', indicating in the legends that ω_0 is fixed, or as '[reverse micelles]'. We will use the Greek letter θ for consistency with the nomenclature of the reverse micelle size, ω_0. Figure 1(a) illustrates the structural changes occurring when water and surfactant are varied.

The water confined within reverse micelles has been the subject of intensive physicochemical study. Below the hydration requirements of the surfactant polar heads, the waterpool water has properties which are substantially different from those of normal bulk water. The anomalous water at low hydration will obviously influence the chemical properties of guest molecules, although of greater interest is the fact that this water resembles the water adjacent to biological membranes [87].

Infrared data have provided direct evidence for the existence of different populations of water in the waterpools. Bulk water exhibits a band at 1670 nm, while water that is not involved in a tetrahedral array of hydrogen bonds absorbs at approx. 1400 nm. At $\omega_0 = 1.5$ in AOT reverse micelles only the 1400 nm band is seen, shifting to longer wavelengths as ω_0 increases. The 1670 nm band is first clearly present at $\omega_0 = 8.3$ [18]. These findings led to the proposal of a layer of structured water of reduced motion

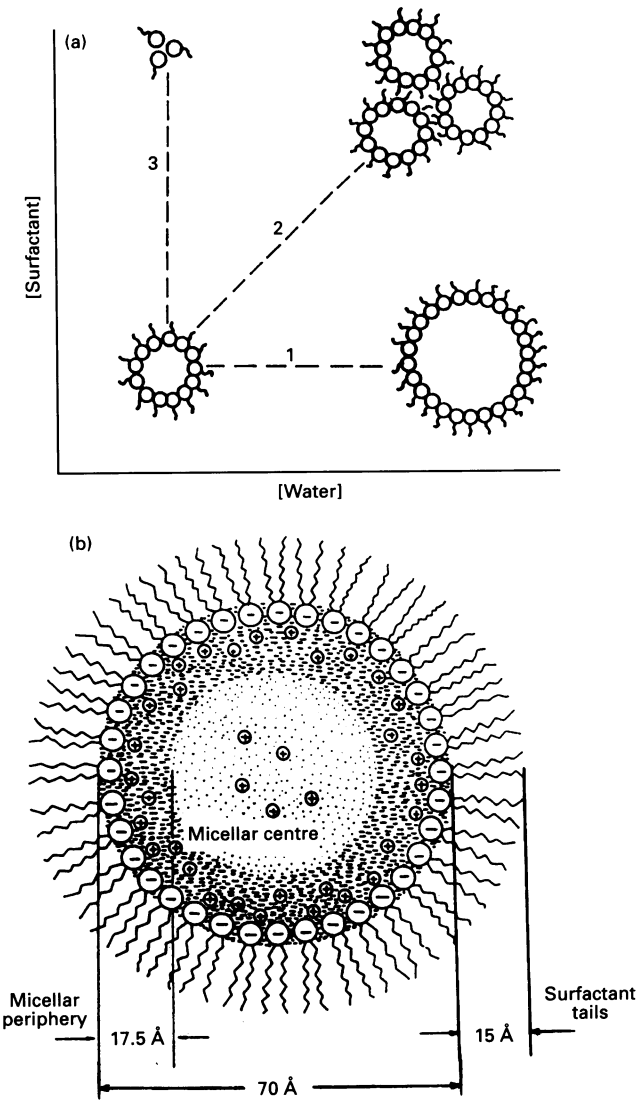

Figure 1 (a) Structural changes occurring in reverse micelles upon variation of water and surfactant and (b) microstructure of a reverse micelle

(**a**) 1, Increase in water leads to an increase in micelle size (ω_0); 2, increase in water and surfactant simultaneously leads to an increase in micelle concentration (θ) of a fixed size; 3, increase in surfactant leads to decrease of micelle size. (Redrawn from [2] with permission.) (**b**) The three domains of the reverse micelle are indicated (redrawn from [95] with permission). Dimensions correspond to a reverse micelle of $\omega_0 = 20$ having ten water molecules solvating each surfactant polar head. For comparison, the diameter of α-chymotrypsin is approx. 44 Å (4.4 nm).

induced by the hydration of the surfactant polar heads, with clearly different properties from those of the freely rotating bulk water [88–93].

Structural models have been proposed particularly for aqueous pool systems confined by a charged wall, e.g. water/AOT/n-heptane [94,95]. A qualitative picture is shown in Figure 1(b).

Dynamic properties of reverse micelles: the exchange of solubilized molecules

Figure 1(b) may induce the erroneous belief that reverse micelles are rigid structures dispersed in an organic medium and behave as hard spheres which simply collide by Brownian motion and

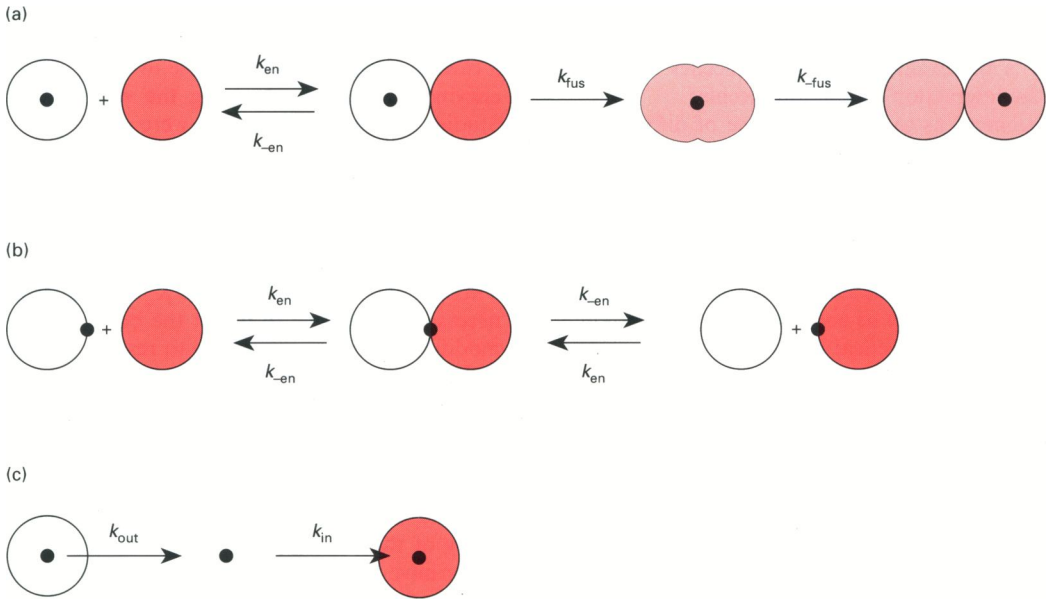

Figure 2 Exchange of solubilisates between reverse micelles

(**a**) Exchange of hydrophilic molecules: fusion of reverse micelles and formation of a transient dimer is compulsory; (**b**) exchange of amphiphilic or interface-bound molecules: micelle collision is sufficient for exchange, and so fusion is not compulsory; (**c**) exchange of oil-soluble molecules through interfacial transport: collision is not even required. Subscripts en and fus denote encounter and fusion respectively.

undergo diffusion-controlled encounters only. Nothing is further from reality, because reverse micelles exchange their contents very rapidly, as has been demonstrated in a variety of experiments. It was found that a number of different solubilized ions were exchanged with essentially the same rate constant in a reverse micellar system of given composition [96–98]. The exchange rate did not depend on the size or the charge of the transferred ion. These results indicate a passive role for the ion in the exchange process.

For molecules confined to the water-pool of reverse micelles, it is generally accepted that there is an exchange mechanism mediated by a transient dimer. This originates from the existence of short-range attractive forces when micelles make contact, giving rise to the fusion of surfactant shells and thus to content exchange [97,99]. The process is reversible and two rearranged (in solubilizate and in surfactant) reverse micelle 'daughters' are formed from the dimer [15]. The exchange constant for the whole process, $k_{ex.}$, is of the order of 10^6–10^8 $M^{-1} \cdot s^{-1}$ [97], indicating that one collision in 1000–10000 results in content exchange.

If the solubilized molecule is not completely confined to the waterpool, but can partition into the interface or even the organic solvent, a different exchange mechanism can operate [15]. For instance, mere contact between micelles without transient dimer formation is sufficient for there to be an effective exchange of molecules at the interface, whether they are solubilized molecules or molecules of the surfactant itself [100,101]. Sometimes, contact is not necessary for molecules to be transferred from micelle to micelle if such molecules are distributed to any great extent in both water and organic solvent. These two last mechanisms are faster than the first, as the slow formation of a transient dimer is not necessary for exchange to take place. These exchange processes are illustrated in Figure 2.

In a homogeneous aqueous medium, mass transfer is a process which does not affect the enzyme reaction rate because the supply of substrate and the removal of product by diffusion in the vicinity of the enzyme are much more efficient than the transformation of substrate into product by the enzyme.

The efficiency of substrate supply and product removal is obviously lower in reverse micelles than in the aqueous solution because of the exchange process which takes place, especially in the case of molecules confined to the waterpool. The effect that the lower efficiency of mass transfer may exert on the enzyme reaction rate also depends on how fast the enzyme turns over its catalytic cycle. In the case of the very fast enzyme catalase ($k_{cat.} = 3.8 \times 10^7$ s^{-1}; $k_{cat.}/K_m = 3.5 \times 10^7$ $M^{-1} \cdot s^{-1}$ [65,102]), it has been reported that the reaction rate can be limited by substrate exchange in reverse micelles [65]. However, normal values for the $k_{cat.}$ of most enzymes studied in reverse micelles range from 1 to 100 s^{-1} [15,53], and the rate-limiting step of the enzymic reaction is quite unlikely to be the exchange of substrate.

The exchange rate determined for small ions and small molecules in reverse micelles is still fast ($k_{ex.} \simeq 10^7$ $M^{-1} \cdot s^{-1}$) compared with enzyme-catalysed reactions [97,101], but it should not be forgotten that one of the interacting species, namely the enzyme, is a macromolecule and that in some cases both interacting species may be such macromolecules. In the first case, for the quenching of the triplet-state decay of Zn-porphyrin cytochrome *c* by small molecules such as potassium ferricyanide and Methyl Viologen [103], it was shown that the exchange rate is comparable to that for the exchange of two small molecules. The interaction between macromolecules in reverse micelles was studied by Bru and García-Carmona [104], who showed that the inhibition of trypsin by its protein inhibitor from soybean occurs slowly in reverse micelles, even at high inhibitor/trypsin ratios, while in bulk water the inhibition is instantaneous in equimolar conditions or when there is a deficiency of inhibitor. From the time-dependent inhibition, a first-order constant could be obtained which was related to the fusion rate of macromolecule-containing reverse micelles. As a result, the exchange rate between protein-containing reverse micelles was three orders of magnitude

less than the exchange rate between small molecule- and macromolecule-containing reverse micelles. It seems likely, therefore, that it is the exchange between micelles that is the limiting step in the interaction of macromolecules.

There is disagreement as to the importance of diffusion and mass transfer when drawing up a theoretical basis for the different proposed models. These factors gain importance as the rate of the enzyme reaction increases, and in a few cases, such as that of catalase, this rate is so high that diffusion and mass transfer are relevant to the enzyme kinetics in reverse micelles [65]. This suggests that more than one theoretical treatment for enzyme kinetics in reverse micelles may be needed, i.e. models should be developed from different theoretical bases.

FEATURES OF ENZYME KINETICS IN REVERSE MICELLES

Performing enzyme kinetic studies in an *in vitro* system may become a very complicated task as the reaction mechanism becomes more complex, even in such a simple system as a buffered aqueous medium. Since reverse micelles form a system structurally more complex than bulk water which influences the enzyme kinetics, it seems obvious that the simpler the enzyme mechanism is, the better will be our understanding of the response of the enzyme in this apparently alien medium. So it is worth searching for an enzyme composed of a single polypeptide chain with a simple reaction mechanism, if possible monosubstrate, about which much is known in buffered aqueous media and which is easy to assay. Proteinases and, in particular, serine proteinases, were the models chosen to carry out pioneer studies in reverse micelles because they fulfil almost all the above conditions [24,105–107]. Oxidases that use O_2 as substrate and which are simple to assay have been found to be equally attractive, although their mechanisms are more complex [75,76,108,109]. Other hydrolases, such as lipases [38,54,70,110], have also been used, but mostly for their applied interest. Some dehydrogenases have also been kinetically studied although their bi-substrate mechanism makes the results more difficult to interpret [111,112].

There are several good candidates which might be taken as models for enzyme kinetic studies using reverse micelles. However, they always have some imperfection, and even in the case of serine proteinases, which are good models, their substrates are normally charged and interact with the charged surfactant heads [106].

Kinetic studies of the transition phase

A comparison of the product accumulation curves of the enzymic reactions performed both in an aqueous medium and in reverse micelles using similar conditions of pH, temperature and enzyme and substrate concentrations is the first step to be taken in the search for particular aspects of enzymic reactions in reverse micelles.

At first sight, there is no indication that the reactions in reverse micelles progress any differently from the way in which they progress in aqueous medium; in particular, the reverse micellar medium does not abolish or induce any transition phase, whether lag or burst, that did or did not already exist in the reaction progress curve. Luisi and co-workers have carried out fast kinetics studies of the hydrolases α-chymotrypsin and trypsin by using the stopped-flow technique. In a first study on the trypsin-catalysed hydrolysis of benzyloxycarbonyl-LysO-Np at acidic pH [113], where deacylation of the acyl–enzyme complex is the rate-limiting step in aqueous solution, they observed that the burst which characterizes this reaction disappeared when the reaction was performed in CTAB/iso-octane/chloroform reverse micelles. Thus they concluded that the rate-limiting step in reverse micelles is no longer the deacylation step, but the step previous, namely the acylation of the free enzyme, and therefore a reverse micellar system is able to bring about a change in the enzyme kinetics by changing the step which is rate-limiting. Studies of the isotopic effect were consistent with their observations.

A more detailed stopped-flow investigation of the α-chymotrypsin-catalysed hydrolysis of *p*-nitrophenyl ester substrates [114] showed that the burst phase also occurred in reverse micelles but, because of a drastic decrease in the acylation rate constant and an increase in the K_m, the experimental conditions necessary to be able to detect the burst had to be substantially modified in reverse micelles with respect to those in bulk water. Indeed, when using the same overall substrate and enzyme concentrations in reverse micelles as in water, no burst could be detected, thus explaining the results previously obtained for trypsin. Despite the fact that the acylation rate decreases, the deacylation step is still the rate-limiting step.

There are not many more examples in which a comparative study of the reaction rate constants in reverse micelles and in water have been carried out, but in those studies which do exist, the kinetic parameters, $k_{cat.}$ and K_m, are determined from steady-state measurements. Thus the results obtained in both water and reverse micelles can be compared.

Kinetic studies under steady-state conditions

In this section we will look at those features of the kinetics of enzymes in reverse micelles which differentiate this system from classical enzymology and which a theoretical model should be able to explain and predict. These features are basically the phenomenon of superactivity, the overall versus waterpool substrate concentration, and the bell-shaped and multiple bell-shaped profile for $k_{cat.}$ versus ω_o.

Superactivity of enzymes in reverse micelles

In carrying out this kind of study, it was noted from the very beginning of micellar enzymology that sometimes the reaction rate was higher in reverse micelles than in bulk water, despite the fact that the overall concentrations of enzyme and substrate were the same. Likewise, the pH-independent $k_{cat.}$ was shown to be greater in reverse micelles [24,105,106]. Such a phenomenon was named 'superactivity'. Since such superactive behaviour might be the consequence of pH profile shifts or the concentration of reagents in the waterpools of reverse micelles [115], the term 'superactive' is used to designate those enzymes that display a greater substrate- and pH-independent catalytic constant, $k_{cat.}$, in reverse micelles than in bulk water. Several reports can be found in the literature concerning superactive enzymes and, although the α-chymotrypsin catalytic constant is only modestly enhanced [24,105,106], peroxidase has been reported to be accelerated 100 times [116,117], acid phosphatase 200 times [118] and laccase 60 times [119].

The overall versus waterpool substrate concentration: influence on K_m

As regards K_m, it was pointed out at an early stage [105] that if the waterpool was the solubilization site of the substrate, then two sorts of substrate concentration might be considered. For instance, 'overall' concentration would refer to the whole volume of the reaction medium, and 'waterpool' concentration to the volume of water contained in reverse micelles. Hence the terms $K_{m,overall}$ and $K_{m,waterpool}$ were also created to distinguish to which volume this parameter was being assigned, in contrast to

$K_{m,bulk\,water}$, the Michaelis constant in the mother buffered aqueous solution.

As regards hydrophilic substrates, a decrease in $K_{m,overall}$ with respect to bulk water values might be expected due to the concentration of reagents in the waterpool, although it is necessary to ascertain that the substrate in question is completely soluble in the waterpool, otherwise 'unexpected' K_m values might be obtained in reverse micelles.

Such unexpected K_m values were observed for both α-chymotrypsin and trypsin in reverse micelles: when the surfactant has an opposite charge to that of the substrate, $K_{m,overall}$ was much higher than $K_{m,bulk\,water}$, but when the electrostatic effect was repulsive, $K_{m,overall}$ decreased, although it was not as different from $K_{m,bulk\,water}$ as would be expected if the substrate were all concentrated in the waterpools [106].

Again, in the case of α-chymotrypsin it was found that $K_{m,overall}$ for the negatively charged substrate succinyl⁻-Ala-Ala-Pro-Phe-NH-Np in negatively charged AOT reverse micelles was close to $K_{m,bulk\,water}$, and so $K_{m,waterpool}$ should be much higher than $K_{m,bulk\,water}$ if the substrate is located only in the waterpool. However, when α-chymotrypsin was inhibited by the doubly charged reaction product succinyl⁻-Ala-Ala-Pro-Phe⁻, $K_{i,bulk\,water}$ was, as expected, much higher than $K_{i,overall}$ and similar to $K_{i,waterpool}$ [120]. These authors discussed the possibility that the substrate might undergo strong partitioning between the waterpool and surfactant layer, the partition coefficient being dependent on ω_0, while the inhibitor remained totally in the waterpool because of the double charge, leading to strong product inhibition.

Finally, it might be expected that if the substrate and surfactant charges are attractive, the waterpool will be devoid of substrate and $K_{m,overall}$ will increase enormously. If, on the other hand, the charges are repulsive, $K_{m,overall}$ would be expected to decrease to well below $K_{m,bulk\,water}$, although other factors, such as partitioning between the waterpool and surfactant tails, would gain in importance and make $K_{m,overall}$ larger than expected.

The validity of each K_m definition, overall and waterpool, is beyond doubt in the theoretical case that the substrate is water-soluble. In practice, however, substrates are distributed among waterpool, interface and organic solvent according to certain partition coefficients. The term $K_{m,waterpool}$ lacks physical meaning if it is the result of multiplying $K_{m,overall}$ by the water fraction in the system without taking into consideration the distribution of the substrate. While the distribution between bulk water and oil is easily determined, the experimental determination of the partition coefficients between waterpool–interface and interface–oil is rather complex [109,110,121], and no general technique has been developed.

The controversy surrounding the K_m in reverse micelles arose principally from the microheterogeneity of the system, but with our present knowledge this point has been clarified, and changes in substrate specificity when passing from bulk water to reverse micelles observed in the case of alcohol dehydrogenase [116] are easily explained. In bulk water, the K_m for two different substrates simply reflects the microscopic affinity of the enzyme for each one, but in reverse micelles the observed K_m also reflects the interaction of each substrate with the surrounding medium. In this sense, enzymes acting in reverse micelles might be regarded as a simplified version of what may occur in a living cell, i.e. every enzyme is ultimately controlled by the supply, not by the abundance, of substrate.

$K_{m,overall}$ is the observed experimental variable which actually reflects an average value to which several factors may contribute (namely the partition coefficients and relative volumes of the phases that comprise the reverse micelles [110,121–123], although for some authors the exchange rate between micelles also contributes to the observed $K_{m,overall}$ [124,125]) and to which theoretical approaches should refer. The problem is really to know what concentration of substrate is accessible to the enzyme.

Effect of the micellar parameters ω_0 and θ on $k_{cat.}$ and the bell-shaped phenomenon

The other kinetic parameter that can be obtained through the determination of the reaction rate under steady-state conditions, $k_{cat.}$, also shows a particular behaviour pattern with respect to the micellar parameters ω_0 and θ. The $k_{cat.}$ values observed in reverse micelles at different waterpool sizes, that is at different ω_0 values, are usually compared with the values observed in bulk water under the same conditions of pH, temperature and ionic strength as in the waterpool or, more correctly expressed, the values in reverse micelles are compared with those obtained in a mother aqueous medium that served to create the waterpools. This distinction has to be made, because the local concentrations of H^+ and salts in waterpools can be influenced by the charged surfactant palisade [126–130]. From the above-mentioned comparison, a typical representation of $k_{cat.}$ versus ω_0 emerges in micellar enzymology. The shape of the $k_{cat.}$ versus ω_0 profile has been the focus of numerous studies with a large number of enzymes [1,48]. From the pioneer studies with α-chymotrypsin acting on glutaryl⁻-Phe-NH-Np (GPNA) [105] to the most recent studies, bell-shaped profiles have often been found, i.e. there is an optimum size of the waterpool to express maximal catalytic power. However, recently there has been some controversy concerning these bell-shaped profiles [120,131,132] because it was not clear whether the parameter plotted versus ω_0 in some reports was the true $k_{cat.}$ or a reaction rate (obtained at a particular, supposedly saturating, substrate concentration) which had been normalized with respect to the enzyme concentration used in that particular experiment [113,122,133–138]. If the latter is the case, apparent bell-shaped profiles can be obtained at non-saturating substrate concentrations which turn into hyperbolic curves at saturating substrate concentrations [119]. It has also been demonstrated recently that bell-shaped profiles may become pH artifacts. Because of the low overall buffer concentration in the micellar solution, the pH may fall when acidic substrates such as the typical α-chymotrypsin substrate GPNA are used, giving rise to a bell-shaped dependence of initial velocity on ω_0 [139]. Another finding, which has yet to be fully understood, is that the shape of the $k_{cat.}$ versus ω_0 profile in the case of α-chymotrypsin depends on the substrate used in the determination of $k_{cat.}$ [131]. When designing a kinetic model, then, it is important to keep in mind that $k_{cat.}$ versus ω_0 profiles may not always be bell-shaped.

In line with the finding that profiles for $k_{cat.}$ versus ω_0 can be bell-shaped, there is an interesting hypothesis that suggests that, at the optimum ω_0, the protein dimensions match those of the waterpool of the reverse micelle [106,140–143]. In support of this hypothesis, data on a dozen different enzymes have been presented where the optimum ω_0 correlates, with some exceptions, with the molecular mass of the protein [2,48]. Furthermore, multimeric enzymes have been shown to display multiple bells according to whether the waterpool size matches the size of the monomer, the dimer, the tetramer, etc. [144], but no evidence was presented that these forms are catalytically active in bulk water. On the other hand, several other enzymes [65,108,120,123,132] have been shown to display a profile that is not bell-shaped, and thus the above hypothesis would not apply in these cases. The most studied and at the same time most controversial example is that of α-chymotrypsin, to which some reports assigned a bell-shaped profile [105,140] and others did not [107,120,131]. When

pH artifacts are controlled and esters or amides are used as substrates it seems that the profile for α-chymotrypsin is not bell-shaped, independently of whether the rate-limiting step is acylation or deacylation of the enzyme [131]. In contrast, in a study of the hydrolysis of the acyl–enzyme complex *N*-transcinnamoyl–α-chymotrypsin in AOT reverse micelles, the profile was shown to be bell-shaped and related to the conformational rigidity of the protein [140]. In this case the deacylation step was measured independently of the acylation reaction and the bell-top appeared when water was of the 'bound water' type. Thus both bell-shaped and non-bell-shaped profiles can be possible for α-chymotrypsin in AOT reverse micelles.

CLASSIFICATION OF THEORETICAL MODELS FOR ENZYME KINETICS IN REVERSE MICELLES

In a previous section we described the structural and dynamic features of reverse micelles which might be important to enzyme kinetics in these systems. In this section, we will use these concepts as a basis for the classification of the models proposed and will show some pictorial representations of a reverse micellar system taking into account the principles of each theory.

The first broad criterion for classifying models emerges from the dynamic nature of reverse micellar systems and refers to the importance of diffusion. Some models consider that diffusion effectively controls the enzymic reactions which occur inside reverse micelles, while others completely ignore such diffusional contribution to the observed reaction rate. Thus we can speak about diffusional and non-diffusional models [40] (Table 1).

Diffusional models

Within the category of diffusional models, that proposed by Maestro et al. [145–147] can be considered purely diffusional since theirs is not a kinetic model in the proper sense. Its principal aim is to check to what extent the intermicellar diffusion of reverse micelles and the intramicellar diffusion of substrates can influence the enzyme kinetics in reverse micelles, taking the simplest case of a Michaelian reaction scheme. For this model, the reverse micelle and the enzyme behave as hard spheres and the substrate as an object of a known geometry, so that their respective diffusion coefficients can be easily calculated. The reaction takes place at the surface of the enzyme, which occupies a central position in the reverse micelle, only after certain diffusional events occur: an enzyme-containing and a substrate-containing reverse micelle merge, their contents are mixed and substrates reach the enzyme. The enzyme reaction rate is affected by the rate constants of the diffusion steps.

In this group of diffusional models, two more models can be included: the model proposed by the Dutch group of Verhaert, Hilhorst and Veeger [124] and that proposed by Oldfield [125]. Because the principles of both are very similar and Oldfield's model compares very well with one particular case of the Dutch model, we will focus on the latter and point out their main differences when appropriate.

The importance of diffusion in these models is shown mainly by the special definition of the substrate concentration which is relevant to the enzyme. They both consider that substrate molecules confined to the aqueous environment are randomly distributed among the waterpools according to a Poisson distribution. The slow diffusion of substrate between waterpools means that an enzyme entrapped in a reverse micelle experiences the concentration of solutes in that particular waterpool, and so the concentration experienced by the enzyme is the average concentration of substrate in those waterpools which contain one or more substrate molecules. This is the so-called intramicellar approach [148]. Note that when the concentration of

Table 1 Classification of kinetic models in reverse micelles

Model	Observations	References
Diffusional		
Purely diffusional	● There is intermicellar diffusion of micelles and intramicellar diffusion of substrate	[145–147]
Intramicellar approach		
Occupancy number = 1	● Exchange rate between micelles limits the catalytic cycle turnover	[125]
Any occupancy number	● As above	[124, 148, 155]
Non-diffusional		
Polydisperse	● There is a dispersion of micelle sizes; thus there is a coexistence of small, large and optimal micelles	[149]
Pseudophasic approach	● Solutes distribute according to partition coefficients between the micellar pseudophases and the continuous oil. Reverse micelles formed by:	
to the substrate	– one pseudophase: micellar	[122]
	– two pseudophases: interface and aqueous	[121]
	– three pseudophases: interface, bound water and free water	[123]
to the enzyme	– three pseudophases: interface, bound water and free water	[150]
Electrical potential distribution	● Ions, including the substrate, distribute inside reverse micelle aqueous pool according to the electrical potential generated by the charged surfactant layer	[126, 152]

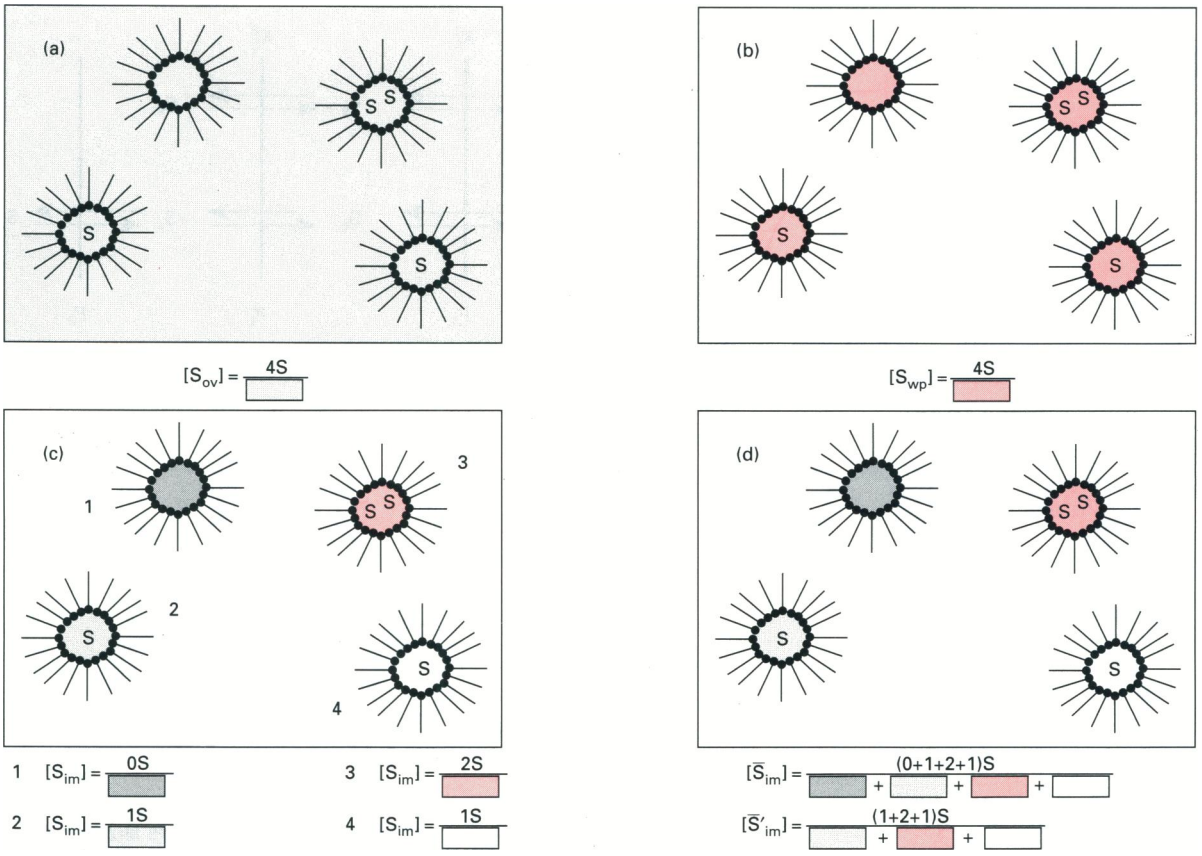

Figure 3 Different ways of expressing substrate concentration in reverse micelles

(**a**) Overall concentration; (**b**) waterpool concentration; (**c**) intramicellar concentration; (**d**) averaged intramicellar concentration and averaged intramicellar concentration in substrate-filled reverse micelles. [Redrawn with permission from Verhaert, R. M. D., Hilhorst, R., Visser, A. J. W. G. and Veeger C. (1992) in Biomolecules in Organic Solvents (Gomez-Puyou, A., ed.), p. 133. Copyright CRC Press, Boca Raton, FL.]

substrate is low compared with the concentration of waterpools, a large number are unoccupied. According to both models, the empty waterpools do not contribute to the concentration of substrate experienced by the enzyme. Oldfield's model restricts the maximal occupancy number to 1, i.e. waterpools contain one substrate molecule or none. Figure 3 illustrates the ways in which substrate concentration can be defined: overall concentration, $[S_{ov}]$, where the whole medium is considered as a valid volume for calculating the concentration (Figure 3a); waterpool concentration, $[S_{wp}]$ (Figure 3b), which, in the case of amphiphilic substrates, is an interfacial concentration, $[S_{inph}]$, coinciding with the definition of concentration for the pseudophase approach (see below); intramicellar concentration (Figure 3c), $[S_{im}]$, where the concentration inside each particular waterpool is considered according to their degree of occupancy and, when averaged (Figure 3d), $[\bar{S}_{im}]$ is the same as $[S_{wp}]$; and average intramicellar concentration in filled waterpools (Figure 3d) is $[\bar{S}'_{im}]$, where the volume of empty waterpools is not used in the calculation of substrate concentration. The last definition is the one adopted in these models.

Non-diffusional models

The non-diffusional models consider that the flow of substrate to the enzyme in reverse micelles is not limited by mass transfer, because the enzymic reaction is slow compared with the exchange of solutes between waterpools (remember that this is the slowest exchange mechanism). Using this as a starting point, several models have been proposed to explain the different phenomena observed in reverse micelles, such as superactivity and bell-shaped profiles.

The so-called polydispersed model [149] developed by the group of Martinek focuses on these aspects. Starting from the observation that the $k_{cat.}$ versus ω_o profiles are bell-shaped, they consider the possible existence of several populations of micelles due to the statistical dispersion of sizes, although to enzyme kinetics only three sorts of micelles are relevant: small, optimal and large. Assuming a normal distribution of sizes, depending on the average size (the externally set ω_o) and the standard deviation, the frequency of each population of micelles is estimated. Because the $k_{cat.}$ of the enzyme in optimal micelles is high, whereas in sub-optimal micelles (small and large) it is much lower or null, a bell-shaped curve of $k_{cat.}$ versus average ω_o can be obtained according to the evolution of the frequency of the optimal micelle population. In addition, a factor of inactivation associated with surfactant concentration was introduced in the model in order to explain the response of certain enzymes to the surfactant concentration at fixed ω_o, i.e. those which display 'surface activity'. An illustrative picture of the 'enzyme in reverse micelle' concept of this model is shown in Figure 4(a). For a particular enzyme there is an optimum waterpool size, ω_o^{opt}, which accepts a certain degree of dispersion for optimal activity, $\Delta\omega_o$. Below $(\omega_o^{opt} - \Delta\omega_o)$,

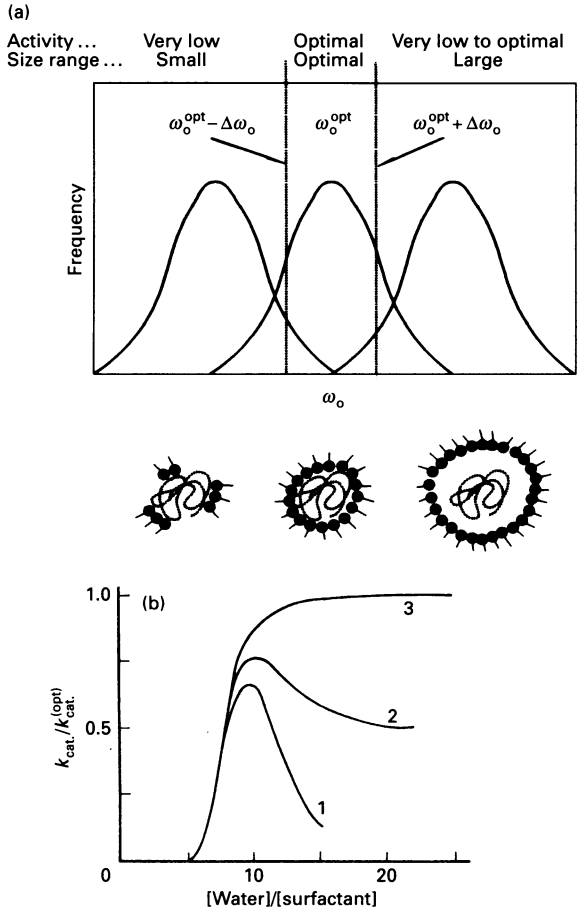

Figure 4 Polydispersed model

(**a**) Hypothetical normal distribution of three reverse micellar preparations of small, optimal and large average radius. The population of optimal micelles for the depicted protein is that between the vertical broken lines. The picture shows the situation, at microscopic level, of protein with respect to micelles. The catalytic power of the enzyme in each micelle population is also indicated (for an explanation, see the text). (**b**) The relative catalytic constant, $k_{cat.}/k_{cat.}^{(opt)}$, of an enzymic reaction in reverse micelles as a function of the hydration degree, ω_o. Simulations were made by using Appendix 2 eqn. (A2.11). The ratio $k_{cat.}^{(i)}/k_{cat.}^{(opt)}$ is varied: 1, 0; 2, 0.5; 3, 1.0. $1/K_4$, K_1/K_3 and K_2/K_5 were set to be low in order to make the effect of surfactant concentration negligible. (Reprinted from [149] with permission.)

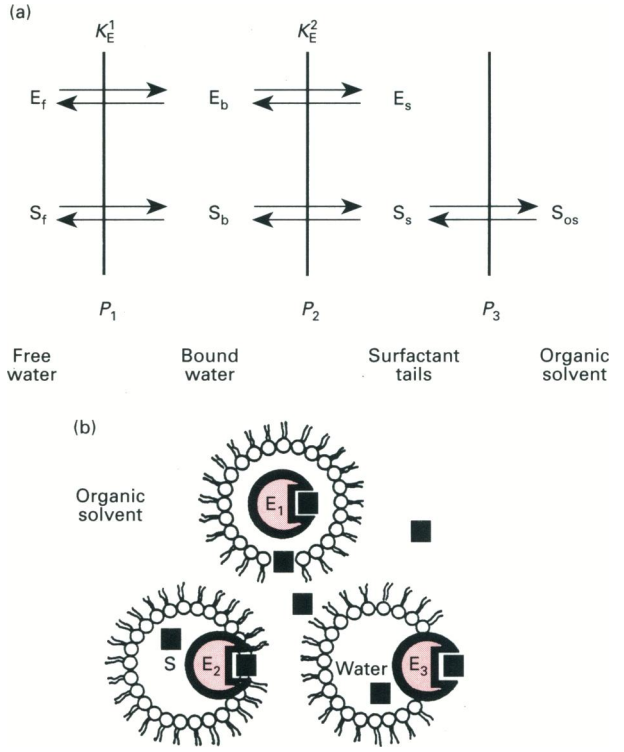

Figure 5 (a) Distribution of enzyme and substrate among the micellar pseudophases and the continuum oil; (b) possible locations of the enzyme in reverse micelles

(**a**) K_E^1 and K_E^2 are partition coefficients for the enzyme, and P_1, P_2 and P_3 those for the substrate. (**b**) E_1, enzyme in free water; E_2, enzyme in bound water; E_3, enzyme in interface. (Redrawn from [116] with permission.)

$k_{cat.}$ is practically null and above $(\omega_o^{opt}+\Delta\omega_o)$, $k_{cat.}$ may vary from the optimal value to zero. When a ω_o value is set externally, the population of micelles which falls with the range of optimal sizes, $\omega_o^{opt}\pm\Delta\omega_o$, together with the concentration of enzyme incorporated in such optimal micelles, will determine the extent of activity expressed by the enzyme.

The model proposed by our group uses the pseudophase approach for both the enzyme [150] and the substrate [123,151]. The pseudophase approach can be used when the rate of the reaction studied is much slower than the time scale for the exchange between solubilizates [15]. In such conditions, all the dispersed phases of the system can be regarded as a pseudo-continuum in equilibrium with each other. Thus the volume that plays a role in the concentration of solutes is the whole volume of the pseudophase in which that solute resides. The expression which pseudophasic models use to calculate the substrate concentration is as follows:

$$[S_{psph}] = \text{mol in } S_{psph}/\text{litres of pseudophase} \quad (4)$$

Thus the concentration of substrate in a particular pseudophase ($[S_{psph}]$) is the number of mol of substrate in that pseudophase divided by the volume of that pseudophase.

The pseudophase approach was first used by Levashov et al. [122] to describe enzyme kinetics in reverse micelles. They considered the simple case of the micellar pseudophase in a continuous oil phase, and established a partition of substrate between both. Because this model only has a structural basis to explain the behaviour of hydrophilic or hydrophobic substrates, it was further extended by Khmelnitski et al. [121], who considered that the micellar pseudophase was actually composed of the surfactant and the aqueous pseudophases, thus giving structural support to amphiphilic substrates such as fatty alcohols. We [123,150] considered yet another pseudophase derived from the aqueous pseudophase based on the structural model of reverse micelles proposed by El Seoud [95] shown in Figure 1. In this model, the waterpool consists of a region of water hydrating the surfactant polar heads, the so-called bound water, and a core of free water in the micellar centre. In addition to the distribution of substrate among the pseudophases and the continuum oil, the idea of the distribution of enzyme among the micellar pseudophases was introduced in order to explain such phenomena as the bell-shaped curves and superactivity. Figure 5(a) shows the distribution of substrate and enzyme with the corresponding distribution equilibrium constants, and Figure 5(b) shows the possible locations of enzyme and substrate according to a pseudophasic scheme.

The model of Ruckenstein and Karpe [126,152] refers to the

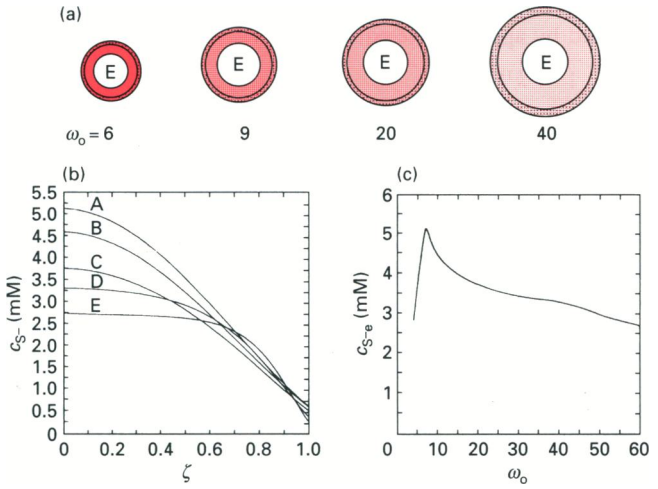

Figure 6 The electrical potential distribution model

(**a**) Distribution of substrate (negatively charged) in a reverse micelle (negatively charged) filled with enzyme, at different sizes, according to the electrical potential distribution model. The sizes of enzyme and micelle may not be to scale. The intensity of colour represents the waterpool concentration at a fixed overall concentration of substrate. (**b**) Radial variation of substrate concentration, c_{S^-}, in the waterpool of enzyme-filled reverse micelles simulated with the electrical potential distribution model for different ω_o values: A, 6; B 9; C, 20; D, 40; E, 60. The origin corresponds to the enzyme surface and 1.0 to the micellar periphery. (Reprinted from [152] with permission.) (**c**) Variation of the substrate concentration near the surface of the solubilized enzyme with hydration ratio, ω_o. 'e' stands for 'in enzyme-containing reverse micelle'. (Reprinted from [152] with permission.)

phenomena of bell-shaped curves and superactivity and neglects the contribution of diffusion to the enzymic reaction, as do the other non-diffusional models. According to this model, the factor that really controls the enzyme reaction rate is the concentration of substrate at the surface of the enzyme. These authors consider the case of a charged substrate in a reverse micellar system formed by a charged surfactant. Assuming an electrical potential distribution in equilibrium in the waterpool, originated by the surface of the charged surfactant heads, the model estimates the substrate concentration as a function of the distance from the waterpool periphery. This concentration is maximal at a certain distance, thus giving rise to bell-shaped profiles and superactivity. It also takes into consideration enzyme dimensions in order to estimate the distance from the micellar periphery to the enzyme surface and its contribution to the electrical potential distribution, assuming a central location of the protein. Figure 6(a) is a pictorial representation of substrate distribution inside enzyme-filled reverse micelles of the same charge sign according to this theory, which, for the sake of clarity, we will call here the electrical potential distribution model. This model pays no attention to uncharged substrates or surfactants.

To conclude this section, it is important to point out that the explanation of the behaviour pattern of $k_{cat.}$ in reverse micelles and with respect to its value in bulk water given by each model is based on essentially different principles. Some models, whether diffusional or not, consider that the catalytic-centre activity does not change upon entrapment in reverse micelles and that the differences observed can be explained by taking into consideration the diffusion of substrate to the enzyme (purely diffusional model [145–147]), the exchange of solubilizates between waterpools (intrawaterpool approach model [124,125,148]) or the distribution of substrate inside the waterpool (electrical potential distribution model [126,152]). On the other hand, some non-diffusional models, namely the polydispersed model [149] and the enzyme pseudophase approach model [150], assume that the catalytic-centre activity expressed by the enzyme is not affected by diffusional processes, and may differ in bulk water and reverse micelles due to the conformation of the protein in these different environments.

THE MODELS: BRIEF PRESENTATION AND CRITICAL ANALYSIS

Purely diffusional model

This model was created to explain the bell-shaped profiles observed for α-chymotrypsin-catalysed reactions in reverse micelles, although the principles of the model are applicable to other enzyme-catalysed reactions. However, the results that this model tried to explain [105] have been shown to be inaccurate, since some acidic impurities were present in the waterpools [15,153]. In addition, the profiles of $k_{cat.}$ versus ω_o were shown to be not bell-shaped for a variety of substrates [131]. However, using the concentration-dependent second-order rate constant $k_{cat.}/K_m$, as this model does, bell-shaped curves can be obtained and experimental data can be theorized.

The basis of the model is the diffusion theory. Two consecutive diffusion processes must take place before the true chemical step of the enzymic reaction can occur, as illustrated in Figure 7(a). In the first intermicellar diffusion step, the micelles which contain substrate and those which contain the hydrated enzyme must collide. In the second process, the substrate molecules must reach

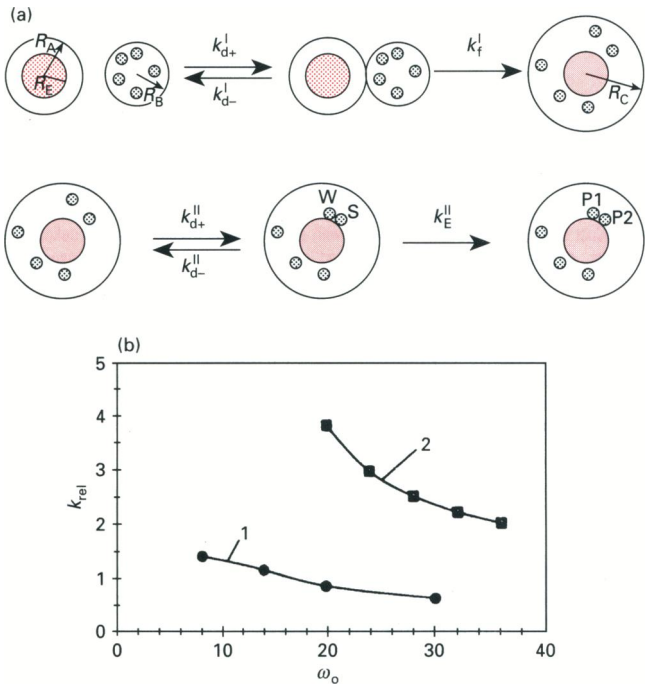

Figure 7 The purely diffusional model

(**a**) The two steps of enzyme catalysis in reverse micelles in the purely diffusional model. I, Intermicellar step; II, intramicellar step; R_A, radius of an enzyme-filled reverse micelle; R_B, radius of a substrate-filled reverse micelle; R_C, radius of a fused enzyme-filled reverse micelle; R_E, enzyme radius. S and W stand for the first and second (water) substrate respectively, and P1 and P2 are the two products formed. The constants for the individual steps are also represented. (Redrawn from [147] with permission.) (**b**) Simulations of the behaviour of α-chymotrypsin in AOT reverse micelles with the purely diffusional model. α-Chymotrypsin in 50 mM AOT/iso-octane at 25 °C; Gr-Phe-NH-Np as substrate. 1, Experimental data; 2, theoretical curve: simulation of experimental data for k^I_f varied between 10^7 and 10^2 s^{-1}. (Redrawn from [147] with permission.)

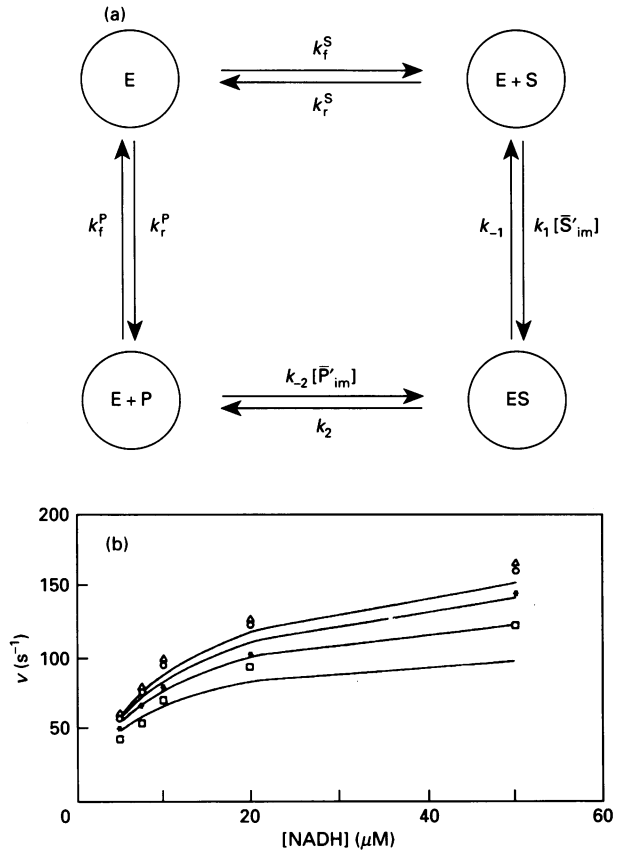

Figure 8 The intramicellar approach model

(**a**) Proposed catalytic cycle for the reaction between enzyme and water-soluble substrate in reverse micelles according to the intramicellar approach model. Substrate is supplied to the enzyme-containing reverse micelle by exchange with substrate-containing reverse micelles and the product formed is relieved by exchange with empty reverse micelles. For the definition of constants, see Appendix 1; subscripts f and r denote forward and reverse respectively. [Redrawn with permission from Verhaert, R. M. D., Hilhorst, R., Visser, A. J. G. W. and Veeger, C. (1992) in Biomolecules in Organic Solvents (Gomez-Payou, A., ed.), p. 133. Copyright CRC Press, Boca Raton, FL.] (**b**) Comparison of the rates of the enoate reductase reaction in AOT reverse micelles experimentally determined (symbols) and calculated (lines) by using the intramicellar approach model. Simulation was carried out using the same exchange rate for both NADH and 2-methylbutenoic acid. Concentrations of 2-methylbutenoic acid: □, 0.075 mM; *, 0.15 mM; ○, 0.3 mM; △, 0.6 mM. (Reprinted from [111]; permission from the authors is acknowledged.)

the enzyme's active site by a second, intermicellar, diffusion process. Bell-shaped curves originate from two opposite effects, both of which depend on the waterpool radius: the larger the waterpool, the higher the intermicellar diffusion rate; and the further the substrate has to travel (i.e. the larger the radius), the longer it takes for the substrate to diffuse to the enzyme surface. The diffusion processes are thus controlled by the distance the substrate has to travel, and the radii and shape factors of the interacting particles (waterpools, substrates and enzyme). The only parameter which can be manipulated to make simulations is the first-order fusion rate constant, k_f^I, of the two colliding reverse micelles, one containing substrate and the other containing the enzyme.

Experimental and simulated results are compared through a dimensionless parameter named k_{rel}. The equation:

$$k_{rel} = (k_{cat.}/K_m)_{reverse\ micelles}/(k_{cat.}/K_m)_{bulk\ water} \quad (5)$$

is used to treat the experimental data, while theoretical data are obtained by applying the equation:

$$k_{rel} = \frac{1}{k^b_{exp.}} \cdot \frac{k^I k^{II}}{k^I + k^{II}} \quad (6)$$

where the terms k^I and k^{II} contain the rate constants of the intermicellar (k^I_{d+}, k^I_{d-} and k^I_f) and intramicellar (k^{II}_{d+}, k^{II}_{d-} and k^I_E) events respectively, calculated on the basis of the shape and size of the reverse micelles, the enzyme and the substrate (see Figure 7a). The term $k^b_{exp.}$ contains the rate constant of the diffusional events in bulk water.

Typical results of simulations with this model are presented in Figure 7(b). The authors of the model recognize that the results are not satisfactory as far as the quantitative aspect is concerned, although they claim a reasonable agreement in qualitative aspects. Certainly the system is too complex to be explained through one sole variable such as k^I_f which, as these authors state, must contain all the missing variables that would be necessary to describe the process correctly. In addition the values obtained for k^I_f which produce the best fittings to the experimental results are far (several orders of magnitude distant) from the fusion rate constant value experimentally determined in similar systems [97,154].

The intramicellar approach model

The first problem addressed by this model is the definition of the substrate concentration that is relevant to the enzyme. This point was considered and explained in the previous section, it being concluded that it is the average intramicellar concentration in substrate-filled reverse micelles, $[\bar{S}'_{im}]$.

The enzymic reaction which takes place in the reverse micellar medium is considered to occur in two steps, as described in Figure 8. In the first step the substrate-containing micelles diffuse to the enzyme-filled reverse micelles, and this is followed by fusion and an exchange of contents; all the reagents are concentrated inside the core of the reverse micelle. In the second step, which is confined to the waterpool of the reverse micelle, the actual enzymic conversion takes place (substrate–enzyme interaction and catalytic reaction). The reverse reaction, i.e. the conversion of enzyme and product into the enzyme–substrate complex, was later considered to be important even at the beginning of the reaction, due to the high local concentration of product which is found before the reverse micelle in which the reaction takes place is relieved of product [125,155] by means of exchange with an empty micelle. In the case of apolar or amphiphilic substrates, the first step of the process described above would be the transfer of mass across the surfactant interface.

The principal point made by this model is that the steps prior to the enzymic conversion itself may limit the overall rate of the catalytic cycle. As droplet exchange is slower than transport across the interface, we will focus mainly on the former.

Essentially, this model distinguishes two limiting situations: one in which the overall substrate concentration is low compared with the concentration of reverse micelles ($[S_{ov}]/[RM] < 0.2$), and the other in which the concentration of substrate is high ($[S_{ov}]/[RM] > 5$). In these limiting cases, the equation that describes the initial reaction rate can be simplified considerably (see Appendix 1). Interestingly, the model predicts that the observed K_m will vary from one extreme case (low substrate-to-micelles ratio) to the other (high substrate-to-micelles ratio). Nevertheless, this conclusion is not surprising if we consider that, according to this model, at a low substrate occupancy level (one or no substrate molecules per micelle) the 'effective' substrate

concentration, $[\bar{S}'_{im}]$, is constant and equal to one molecule over the waterpool volume, independently of the overall substrate concentration (mol of substrate per litre of micellar solution). To date, no enzyme which shows hyperbolic behaviour in bulk water has displayed the feature of substrate-concentration-dependent K_m when studied in reverse micelles. The model was first tested with enoate reductase [111] and hydroxysteroid dehydrogenase [112] in different reverse micellar systems, namely AOT, CTAB and Triton X-100. In these systems, the authors used a range of micelle concentrations from 1 to 2.7 mM and a range of hydrophilic substrate (NADH) concentrations from 5 to 146 μM, so that the highest substrate-to-micelle ratio was 0.146. According to the model, this should be regarded as a case of a low concentration range, and the data can be simulated by using a simplified equation. Some typical results are presented in Figure 8(b). Note that if the concentration relevant to the enzyme is the intramicellar concentration in filled reverse micelles, $[\bar{S}'_{im}]$, by definition it should be constant over the range used (5–146 μM overall). However, both enzymes responded to substrate concentration in this range.

Until now, there has been no reference to the typical phenomena occurring in reverse micelles: those of bell-shaped curves and superactivity. The latter feature is not explained in any way by this theory. The problem of the bell-shaped curves for $k_{cat.}$ versus ω_o, as well as the effect of reverse micelle concentration, was further considered by Verhaert and Hilhorst [155]. These authors introduced the parameter ω_o using the terms reverse micelle concentration ([M]) and fraction of water in the reaction medium (θ), which appear in the general equation of velocity (see Appendix 1) and which are related to ω_o through simple algebraic expressions of reverse micelle geometry once the surfactant concentration is fixed. The model then supported a bell-shaped dependence of the initial reaction rate on ω_o, although no experimental data were fitted or simulated. The effect of micelle concentration is implicit in the model, since the exchange rate between waterpools depends on the micelle concentration ($v_{ex} = k_{ex}[M]^2$) and both the exchange rate constant and the micelle concentration are present in the general equation. It should also be expected that, when the substrate-to-micelles ratio is kept constant, any increase in the overall micelle concentration would involve a concomitant acceleration of the reaction rate, since the exchange rate would increase. This type of experiment led to a slight increase in AOT reverse micelles of the activity of catalase [65], which is a very fast enzyme. The exchange between micelles may have been the rate-limiting step, although it was not clear which $[H_2O_2]$ was constant, the overall or the waterpool concentration. It would be interesting to perform this kind of test in order to ascertain whether the micelle concentration, and thus the exchange between micelles, have any effect on the reaction rate.

The polydispersed model

This model basically tries to explain the changes occurring in the catalytic constant of enzymes entrapped in reverse micelles provoked by changes in both reverse micelle size and concentration. Any effect produced by the substrate is not considered in this model, as it is assumed to be saturating.

Reverse micelle size, which in this theory is computed as the radius of the inner cavity, affects the catalytic constant of enzymes because an 'optimal' radius exists, where enzymic activity is highest, as well as other 'small' and 'large' radii, where enzymic activity either decreases or disappears. For the sake of simplicity, $k_{cat.}$ in small-radius reverse micelles was set to zero (see Figure 4a). The larger the proportion of optimal micelles (always assuming a normal distribution of sizes), the higher the $k_{cat.}$ observed. The model assumes a broad distribution of reverse micelle sizes, although this assumption contrasts with the results of Eicke et al. [79], who concluded that the standard deviation of the reverse micelle radius is so small that, from the practical point of view, the population of reverse micelles can be considered as monodisperse: for a typical value of a 2 nm radius ($\omega_o = 11.5$), the half-width of the distribution curve frequency versus ($R_{av.}$–R) will be about 0.2 nm.

The concentration of micelles affects the catalytic constant because an equilibrium is assumed in which an optimal, small or large enzyme-containing reverse micelle interacts with an empty micelle to yield an optimal, small or large temporarily inactivated enzyme-containing reverse micelle called a 'deformed' micelle. The deformed micelle may compare with the transient dimer in the scheme of solubilizate exchange (Figure 2). The larger the number of empty micelles, the higher the amount of temporarily inactivated enzyme and the lower the activity. However, sometimes the collision of a filled micelle with an empty micelle may result in a simple transfer of the protein from one micelle to the other, thus transforming the optimal $k_{cat.}$ into $k_{cat.}$ in small or large micelles, and vice versa. Likewise, the enzyme could be transferred between small and large micelles, although this equilibrium was neglected in the model for no apparent reason. Some typical results of simulations are presented in Figure 4(b).

It is apparent that this model contains strong structural components since it describes many different kinds of reverse micelles, which can be empty, enzyme-filled or deformed enzyme-filled, each type being small, large or optimal. However, not enough experimental data have been obtained to support the existence of all these kinds of micelles. Recent studies on the structural features of α-chymotrypsin in AOT reverse micelles [156] indicate that, when the degree of hydration is sufficiently high, the protein may create its own micelle or, at least, the enzyme-containing micelle does not grow in a similar way to empty micelles when ω_o increases. Presumably, such a micelle might be the optimal one. Enzymes have been classified as surface-active and non-surface-active [142], on the basis of whether micelle concentration affects their activity or not. The most striking example is that of α-chymotrypsin, which in the native form behaves as a non-surface-active enzyme, while it is surface-active when covalently modified with a stearoyl group [157]. As the formation of deformed micelles is an equilibrium process, a shift towards them implies longer-lived deformed micelles. In the model, $k_{cat.}$ is controlled by micelle concentration, which in turn is exclusively modulated by means of the equilibrium constants of the deformed micelle formation process, since $k_{cat.}$ in such micelles is, by definition, zero. Since for a given reverse micellar system some enzymes are surface-active and others are not, the hypothesis can be formulated that it must be the very enzyme molecule (in this case the stearoylated α-chymotrypsin) that modifies the equilibrium constant in the formation of deformed micelles.

The main algebraic expressions of this theory are shown in Appendix 2.

Pseudophasic models

The feature common to all the proposed pseudophasic models is the definition of solute concentration, whose expression is given in eqn. (4). To resolve this simple expression it is necessary to know both the number of mol of the solute in a particular pseudophase and the volume of that pseudophase.

The latter is relatively easy to estimate, as the parameters ω_o and θ are directly related to the radius of the spherical micelles

[77,78,81,82]. As for the aqueous pseudophase, it is sufficient to know the amount of water added to the surfactant solution in the organic solvent. The interface volume is usually estimated by using the molar volume of the surfactant and its concentration, although this value is even more accurate if the volume of the surfactant tail portion which protrudes into the organic solvent [79,150] is subtracted. The remaining volume is that of the continuum oil.

In the model proposed by Bru et al. [150], where two aqueous pseudophases are considered, the hydration requirements of the surfactant polar head are used to estimate how much water belongs to the bound water pseudophase, and the volume of the free water pseudophase is estimated by subtraction.

The main disadvantage of the pseudophasic models concerns the estimation or determination of the number of mol of the solute (substrate and enzyme) present in each pseudophase, i.e. the partition coefficients. Because of the difficulties inherent in experimentally determining the partition coefficients in reverse micelles, it has been a common practice to assume a certain behaviour of the substrates based on their chemical structure and on their distribution in a standard two-phase system composed of water and octanol [158]. In the case of the enzyme, the partition coefficients are simulated by taking into account the shape of the $k_{cat.}$ versus ω_o profiles and the $k_{cat.,\,reverse\,micelles}/k_{cat.,\,bulk\,water}$ ratio [123].

Substrate partitioning

Khmelnitski et al. [121] measured the partition coefficient of aliphatic alcohols between AOT reverse micelles and the continuum oil by flow microcalorimetry. They then took from the literature the partition coefficients of these alcohols between water and the erythrocyte membrane, and assumed they were the same as between the waterpool and the interface. By using these partition coefficients, they calculated the partition coefficients both between interface and organic solvent and between waterpool and organic solvent, the latter being in reasonable agreement with those determined experimentally in an aqueous/organic solvent system. Thus this last comparison served to validate the partition coefficients taken from the literature. By using all the determined and calculated partition coefficients, they were able to describe satisfactorily the kinetics of alcohol dehydrogenase in AOT reverse micelles. However, it is not always possible to determine all the independent partition coefficients experimentally. For instance, Fletcher et al. [110] approached the problem of estimating the partition coefficients by comparing the base-catalysed rate of hydrolysis of esters in bulk water and in reverse micelles. They assumed one sole partition coefficient between the continuum and the waterpool, which was shown to be different from that obtained directly from a bulk water/oil two-phase system. As they did not consider that esters located at the interface could also have been attacked and hydrolysed, what they would actually have obtained was the partition coefficient between oil and micelles (interface + waterpool). Kurganov et al. [109] also determined the partition coefficients of fatty acids between oil and micelles by separation of the micellar pseudophase and the continuum oil by ultracentrifugation.

Enzyme partitioning

In all of the above cases it was assumed that the enzymic reaction only took place in the waterpool. However, it should not be forgotten that the reaction can also take place at the interface and even in the water associated with the surfactant polar heads, the so-called bound water.

The enzyme partitioning model [150] takes into account the latter considerations and therefore defines the distribution of enzyme between the three pseudophases (free water, bound water and interface) as possible locations of the enzyme (Figure 5b). This distinction is made not only to make the substrate in each pseudophase directly accessible to the enzyme but also because the $k_{cat.}$ expressed by the enzyme may change when the polarity, viscosity, ionic strength, etc., of its surrounding medium changes. Active centre titration of horse liver alcohol dehydrogenase [121] revealed that the concentration of active centres per protein concentration does not change upon incorporation in AOT reverse micelles, i.e. there is no partial inactivation, although the $k_{cat.}$ is lower than in bulk water. For the deacylation of *N-trans*-cinnamoyl-α-chymotrypsin, the $k_{cat.}$ was even higher than in bulk water, thus giving rise to superactivity. These results were simulated by using the enzyme pseudophase model as seen in Figure 9(c).

By combining the enzyme partitioning with the environment-modulated $k_{cat.}$, the enzyme partitioning model is able to predict different types (bell-shaped and non-bell-shaped) of $k_{cat.}$ versus ω_o profiles. The main algebraic expressions of this model are given in Appendix 3.

Although a number of enzymes have been described as generating bell-shaped curves when entrapped in reverse micelles, others such as α-chymotrypsin [107,120,131], tyrosinase (Figure 9a) [123], cholesterol oxidase [108], lipoxygenase [151], catalase [65] and elastase [132] do not exhibit such behaviour.

To date, no attempt has been made to estimate the partition coefficients of proteins between the three pseudophases of free water, bound water and interface, but even if the pseudophasic distribution of an enzyme is known, the assignment of a $k_{cat.}$ value to a particular enzyme-in-pseudophase should be simulated.

Although pseudophasic models describe quite satisfactorily the behaviour of enzymes in reverse micelles, the inherent difficulty in ascertaining the large number of parameters they use (especially the partition coefficients of substrate and enzyme) reduces their applicability. When complex behaviour is displayed by the enzyme, such as that found for lipoxygenase in AOT/iso-octane reverse micelles, a graphic tool named an 'active-phase plot' has been reported to help in determining these parameters [151]. This plot represents the changes in concentration of substrate and amount of enzyme in the pseudophases where the enzyme expresses catalytic activity as the micellar parameters are varied.

When it is assumed that the enzyme is only active in the waterpool, and no great distinction is made between enzyme in free and bound water (something assumed by most models), then the description of the effect of micelle concentration, θ, on the reaction rate can be quite satisfactorily described by the pseudophasic models: for trypsin [122], alcohol dehydrogenase [121] and tyrosinase (Figure 9b) [76,123], for example. In all of these examples $k_{cat.}$ did not depend on θ, while K_m was linearly dependent, as predicted. The underlying reason for this behaviour is that micellar substrates, whether hydrophilic or amphiphilic, undergo micellar dilution when θ increases [123].

The electrical potential distribution model

This model [126,152] provides a theoretical basis for explaining the two typical phenomena of reverse micelles: bell-shaped curves and superactivity. It is seemingly built on sound foundations, although it is unfortunate that, as in the early model of Maestro et al. [145,146], the data used to test the model [105] have been shown to be erroneous because of acidic

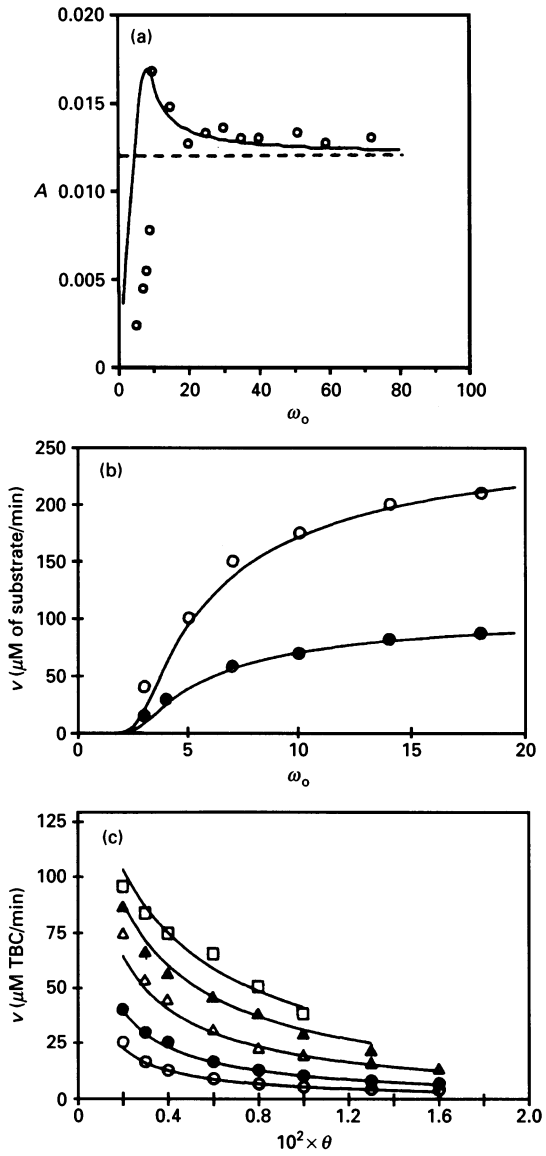

Figure 9 Simulations with the enzyme pseudophase model

Firstly, by taking into account the information provided by the experimental data, the $k_{cat.}$ boundaries can be set so as to discount a certain range of $k_{cat.}$ values. Then, possible values are tuned until a satisfactory simulation is obtained [123]. (**a**) Simulation of *N-trans*-cinnamoyl-α-chymotrypsin deacylation in reverse micelles with the enzyme pseudophase model. The enzyme was assumed to be active both in bound water and in free water, with a higher $k_{cat.}$ being displayed in bound water than in bulk water. *A* denotes maximal velocity. (Reprinted from [150] with permission.) (**b**) Simulation of the dependence of $V_{max.}$ on ω_o of mushroom tyrosinase in reverse micelles with the enzyme pseudophase model. ○, t-Butylcatechol as substrate; ●, 4-methylcatechol as substrate. (Reprinted from [123] with permission.) (**c**) Simulation of the dependence of reaction rate of θ of mushroom tyrosinase in reverse micelles with the enzyme pseudophase model. t-Butylcatechol (TBC) overall concentrations: 2.5 mM (○), 5 mM (●), 10 mM (△), 17.5 mM (▲) and 25 mM (□). (Reprinted from [123].)

impurities present in the AOT preparations [15,153]. As the model establishes a solid basis for calculating the distribution of a charged substrate in an electric field generated by the charged interface, the fact that AOT is contaminated would only affect the calculation of the electrical potential distribution, i.e. charged species other than counterions, co-ions, substrate, buffer, protons and enzyme should be computed.

The concept of superactivity that this model tries to explain is that described formerly for enzymes whose initial velocity was higher in reverse micelles than in water with similar overall concentrations of substrate and enzyme. Likewise, the bell-shaped phenomenon is understood by these authors to be the consequence of the dependence of initial velocity on ω_o, and not of the dependence of $k_{cat.}$ on ω_o.

The authors explain these phenomena as follows. The enhanced activity is due to the enhanced substrate concentration near the enzyme surface, which is caused by the substrate being pushed away from the similarly charged reverse micellar surface towards the enzyme surface, and by the higher average substrate concentration in the much smaller volume of the waterpools compared with that defined on the basis of the entire micellar solution. The increase in the absolute value of the reverse micelle surface charge with increasing ω_o and the increased waterpool width act in opposite directions. These effects, together with the decrease in the average substrate concentration in the waterpool as ω_o increases at constant $[S_{ov}]$, are responsible for the bell-shaped dependence on ω_o of the substrate concentration at the enzyme surface which, in turn, causes the bell-shaped dependence of the activity on ω_o.

The enzyme is set in a fixed central location within the waterpool, and the enzyme solubilization model used for estimating the size parameters of the enzyme-containing reverse micelles is the simple core and shell model [159]; in other words, the volume of the enzyme-containing waterpool is the sum of the volume of the empty waterpool plus the volume of the enzyme. Thus, as the micelle grows, the distance from the waterpool periphery to the enzyme surface is readily calculated. The Boltzmann distribution is utilized to calculate the ionic distributions in the waterpool assuming the equilibrium condition. The electrical potential distribution, and therefore the distribution of charged species (among them the substrate), can thus be calculated as a function of the radius. The substrate concentration calculated at the enzyme surface is used in the Michaelis–Menten equation, and since this concentration is much smaller than the K_m, the concentration of substrate is the factor that controls enzyme activity. The repelling force of the charged interface is, however, overestimated as the authors state that such a force increases with ω_o, the reason given being that the degree of dissociation of the AOT increases with ω_o. Since $pK_{AOT} = 0.475$ [160], a change from a pH_{st} ($_{st}$ = stock solution) of 8.0 to a pH_{local} of 7.2–7.4 (ω_o = 6 to 60) may not be sufficient to produce a substantial change in the degree of AOT dissociation; in any case, a fall in pH_{local} would involve protonation of AOT and therefore a decrease in surface charge density.

Although the data presented for the dependence of the substrate concentration at the enzyme surface on ω_o clearly indicate that it passes through a maximum (Figure 6b), the radial dependence of the substrate concentration in enzyme-filled reverse micelles at different ω_o values (Figure 6c) does not seem to point to such a maximum at the enzyme surface, but suggests that it probably occurs near the micellar periphery. Indeed, intuitively, and bearing in mind that calculations have been made which assume that the partition coefficient of substrate does not depend on ω_o, it might be concluded that at any given ω_o value the substrate is simply pushed towards and concentrated in the micellar centre (where the enzyme is located) due to the electrostatic repulsions mentioned above; the larger the micelle, the more diluted the substrate will be at a fixed $[S_{ov}]$. In conclusion, the reasons given by the authors to explain the bell-shaped dependence are not well supported by their results.

The principal disadvantage of this model, as with the purely diffusional model, is its structural rigidity: to apply the equations

properly, a fixed central location of the enzyme has to be assumed, and so, when the average enzyme position in the waterpool is eccentric or located at the interface, the calculations are no longer valid and a still more complex computation or even a different theoretical basis is required. On the other hand, the mathematical development formulated to estimate the substrate concentration in the waterpool as a function of the distance could be beneficially applied to the pseudophasic models as a way of mathematically expressing the structure of the entrapped water and its effect on substrate concentration and enzyme reactivity.

CONCLUDING REMARKS

In this review we have presented several models, some of which only focus on particular features of micellar enzymology, while other important aspects are overlooked. The purely diffusional model and the electrical potential distribution model are too restrictive in their assumptions, which makes them extremely rigid and not applicable to most real situations. The intramicellar approach model also makes restrictive assumptions, since it denies that any change takes place in the $k_{cat.}$ or K_m of the enzyme when it is entrapped in reverse micelles. Instead, the hypothesis is offered that the differences observed in reverse micellar systems with respect to bulk water arise from the reduced mass transfer efficiency in such systems. This hypothesis may hold for purely hydrophilic substrates which are transformed by very fast enzymes. An example would be the decomposition of H_2O_2 by catalase, although catalase is perhaps an exception among the huge majority of 'slow' enzymes because of its substrate and catalytic-centre activity. Other features, such as superactivity, are beyond the scope of the intramicellar approach model and no example of an enzyme with a bell-shaped profile has ever been simulated. It is fair, therefore, to conclude that this model might only be applied under very special and limiting conditions.

The polydispersed model and the enzyme pseudophase model may be applied more generally. For both models the enzyme is the target and the change in the catalytic-centre activity is the origin of the singular features of micellar enzymology. However, the mechanisms by which such changes in activity take place differ substantially. At the time that these models were proposed there was no strong experimental evidence concerning the structure of enzyme-containing reverse micelles, and so each kinetic model was based on its own enzyme-in-reverse-micelle hypothetical model. As far as substrate is concerned the models seem to be in agreement, as the pseudophasic approach is utilized in both cases. The development of strategies for the determination of partition coefficients for substrates in reverse micellar systems, as well as their dependence on ω_0 and θ, would be of great help for the correct interpretation and theorization of the observed kinetic parameters.

The direct determination of the concentration of solutes in each pseudophase has not yet proved possible, and such a determination is certainly difficult due to the macroscopic homogeneity of the reverse micelles. Other approaches to the problem of determining the partition coefficients of solutes should use an indirect method that permits their calculation through a mathematical procedure. For instance, if the determination of the concentration of a solute in equilibrium in one of the phases (i.e. the organic continuum) is possible, the concentration of the solute in the other phases (and thus the partition coefficients) might be deduced through a system of equations whose coefficients might be obtained by systematically changing the relative pseudophase volumes (R. Bru, A. Sánchez-Ferrer and F. García-Carmona, unpublished work).

Likewise, clarification is needed as to which model of protein-containing reverse micelles is the operative one: the water-shell, the induced fit or the fixed size. Some studies have been carried out recently that indicate that the enzyme might create its own micelle, although it may grow as ω_0 increases, thus supporting an intermediate situation between the water-shell and the fixed size models. In any case, these studies put forward a certain enzyme–reverse-micelle interaction that may not correspond to the situation depicted by the kinetic models proposed. New experiments need to be designed in order to collect more information concerning the enzyme-in-reverse-micelle structure which should serve to unify the criteria for building kinetic models in reverse micelles.

It can be concluded that the models proposed so far may not have been based on well understood systems, probably due to the lack of appropriate structural information on enzyme-containing reverse micelles and sometimes due to the masking of kinetic results by undesired artifacts (i.e. pH problems). On the positive side, it should be stated that some of the proposed models have helped in the understanding of certain aspects of enzyme kinetics in reverse micelles, although new ideas supported by solid kinetic and structural information are necessary in order to refine the actual models and to fully understand the principles of micellar enzymology.

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APPENDIX 1

Main equations of the intramicellar approach model

For the meaning of the constants, the reader is referred to Figure 8 of the main paper. In the general case that substrate can be located anywhere in the reversed micellar system, i.e. in the waterpool, interface or bulk oil, the equation for velocity is:

$$\frac{v}{[E_o]} = \frac{k_2}{1 + k_2 \cdot \left(\frac{1}{k_t^S} + \frac{1}{k_t^P}\right) + \frac{[M]k_{-2}}{\phi k_t^P} + \frac{1}{[\overline{S'_{im}}]} \cdot \left(K_m + \frac{k_{-1}k_{-2}[M]}{k_1 k_t^P \phi}\right) \cdot \left(1 + \frac{k_r^S}{k_f^S}\right)} \tag{A1.1}$$

There are two limiting cases for substrates located exclusively in the waterpool. When $[S_{ov}]/[M] < 0.2$, then the equation for velocity is:

$$\frac{v}{[E_o]} = \frac{k_2}{1 + \frac{2k_2}{k_{ex}[S_{ov}]} + \frac{2k_{-2}}{\phi k_{ex}} + \frac{\phi K_m}{[S_{ov}]} + \frac{2k_{-1}k_{-2}}{k_{ex}k_1[S_{ov}]}} \tag{A1.2}$$

and the expressions for kinetic constants are:

$$k_{cat.}^{app.} = \frac{k_2}{1 + \frac{2k_{-2}}{\phi k_{ex}}} \tag{A1.3}$$

$$K_m^{app.} = \frac{\phi K_m + \frac{2k_2}{k_{ex}} + \frac{2k_{-1}k_{-2}}{k_{ex}k_1}}{1 + \frac{2k_{-2}}{\phi k_{ex}}} \tag{A1.4}$$

When $[S_{ov}]/[M] > 5$, then the equation for velocity is:

$$\frac{v}{[E_o]} = \frac{k_2}{1+\frac{3k_2}{k_{ex}[M]}+\frac{k_{-2}}{\phi k_{ex}}+\frac{\phi K_m}{[S_{ov}]}+\frac{2k_{-1}k_{-2}}{k_{ex}k_1[S_{ov}]}} \tag{A1.5}$$

and the expressions for kinetic constants are:

$$k_{cat.}^{app.} = \frac{k_2}{1+\frac{3k_2}{k_{ex}[M]}+\frac{k_{-2}}{\phi k_{ex}}} \tag{A1.6}$$

$$K_m^{app.} = \frac{\phi K_m+\frac{2k_{-1}k_{-2}}{k_{ex}k_1}}{1+\frac{3k_2}{k_{ex}[M]}+\frac{k_{-2}}{\phi k_{ex}}} \tag{A1.7}$$

In the case of apolar substrates, the equation for velocity is:

$$\frac{v}{[E_o]} = \frac{k_2}{1+\frac{\phi K_m}{[M]}+\frac{\phi k_2}{k_{out}^P[M]}+\frac{k_{-2}}{k_{out}^P}+\frac{\phi k_{-1}k_{-2}}{k_{out}^P k_1[M]}+\frac{K_m P^S}{[S_{ov}]}+\frac{k_{-1}k_{-2}P^S}{k_{out}^P k_1[S_{ov}]}+\frac{k_2}{k_{in}^S[S_{ov}]}} \tag{A1.8}$$

where P^S is the partition coefficient of compound S between the organic phase and the water phase ($P^S = [S_{org.}]/[S_{wph}]$), and the expressions for kinetic constants are:

$$k_{cat.}^{app.} = \frac{k_2}{1+\frac{\phi K_m}{[M]}+\frac{\phi k_2}{k_{out}^P[M]}+\frac{k_{-2}}{k_{out}^P}+\frac{\phi k_{-1}k_{-2}}{k_{out}^P k_1[M]}} \tag{A1.9}$$

$$K_m^{app.} = \frac{P^S K_m+\frac{k_{-1}k_{-2}P^S}{k_2 K_{out}^P}+\frac{k_2}{k_{in}^S}}{1+\frac{\phi K_m}{[M]}+\frac{\phi k_2}{k_{out}^P[M]}+\frac{k_{-2}}{k_{out}^P}+\frac{\phi k_{-1}k_{-2}}{k_{out}^P k_1[M]}} \tag{A1.10}$$

APPENDIX 2

Main equations for the polydispersed model

The enzyme can be transferred from micelle to micelle according to the following equilibria:

$$\bar{M}_{opt} + M_s \overset{K_1}{\rightleftarrows} M_{opt} + \bar{M}_s \tag{A2.1}$$

$$\bar{M}_{opt} + M_l \overset{K_2}{\rightleftarrows} M_{opt} + \bar{M}_l \tag{A2.2}$$

where M_{opt}, M_l and M_s are optimal, large and small reverse micelles respectively. The line over M denotes enzyme-filled reverse micelle. In addition, there may be some collisions between enzyme-containing and empty micelles (M) that result in temporarily inactivated enzyme in deformed micelles (indicated by *):

$$\bar{M}_s + M \overset{K_3}{\rightleftarrows} \bar{M}_s^* \tag{A2.3}$$

$$\bar{M}_{opt} + M \overset{K_4}{\rightleftarrows} \bar{M}_{opt}^* \tag{A2.4}$$

$$\bar{M}_l + M \overset{K_5}{\rightleftarrows} \bar{M}_l^* \tag{A2.5}$$

The equilibrium constants that govern these reactions are defined as follows:

$$K_1 = \frac{[M_{opt}][\bar{M}_s]}{[M_s][\bar{M}_{opt}]} \tag{A2.6}$$

$$K_3 = \frac{[\bar{M}_s][M]}{[\bar{M}_s^*]} \tag{A2.7}$$

$$K_5 = \frac{[\bar{M}_l][M]}{[\bar{M}_l^*]} \tag{A2.8}$$

$$K_2 = \frac{[M_{opt}][\bar{M}_l]}{[M_l][\bar{M}_{opt}]} \tag{A2.9}$$

$$K_4 = \frac{[\bar{M}_{opt}][M]}{[\bar{M}_{opt}^*]} \tag{A2.10}$$

By relating these equilibrium constants to the catalytic constant expressed by the enzyme in each kind of micelle (optimal, large or small), the following expression can be derived for the observed catalytic constant:

$$k_{cat.} = \frac{k_{cat.}^{(opt)} + k_{cat.}^{(l)} K_2 B}{1 + K_1 A + K_2 B + C\left(\frac{1}{K_4} + \frac{K_1}{K_3} A + \frac{K_2}{K_5} B\right)[SURF]} \tag{A2.11}$$

where [SURF] is the concentration of surfactant and A, B and C are statistical factors (in the original paper [149], these factors are denoted as α, β and γ respectively. Here we use A, B and C to avoid confusion with other models).

APPENDIX 3

Main equations of the enzyme pseudophase model

The volumes of the pseudophases are estimated as follows:

$$\alpha = (V_{H_2O} - V_b)\frac{1}{V} \tag{A3.1}$$

$$\beta = V_{H_2O}\left[\frac{(1-\xi)n}{\omega_{o(m)}} + \frac{\xi n}{\omega_{o(d)}}\right]\frac{1}{V} \tag{A3.2}$$

$$\gamma = V_S^M \cdot \tau \cdot \text{mol of S}\left(\frac{1-\xi}{f}\right)\frac{1}{V} \tag{A3.3}$$

$$\frac{V_{os}}{V} = 1 - (\alpha + \beta + \gamma) \tag{A3.4}$$

where α, β, γ and V_{os}/V are the volume fractions of free water, bound water, surfactant tails and organic solvent respectively. These volumes, together with the corresponding partition coefficients (see Figure 5 of the main paper), are used to calculate the concentrations of enzyme and substrate in each pseudophase:

$$[E]_f = \frac{[E]_T}{\alpha + K_E^1\beta + K_E^1 K_E^2 \gamma} \tag{A3.5}$$

$$[E]_b = K_E^1 [E]_f \tag{A3.6}$$

$$[E]_s = K_E^1 K_E^2 [E]_f \tag{A3.7}$$

$$[S]_f = \frac{[S]_T}{\alpha + P_1\beta + P_1P_2\gamma + P_1P_2P_3(1-\alpha-\beta-\gamma)} \tag{A3.8}$$

$$[S]_b = P_1 [S]_f \tag{A3.9}$$

$$[S]_s = P_1P_2 [S]_f \tag{A3.10}$$

$$[S_{os}] = P_1P_2P_3 [S]_f \tag{A3.11}$$

where subscripts f, b, s, os and T stand for free water, bound water, surfactant tails, organic solvent and total respectively.

Considering that the enzyme in each pseudophase follows a Michaelian reaction scheme, the kinetic parameters are given by:

$$k_{cat.}^{app.} = \frac{\sum_{i=f,b,s} k_{cat.}^i [E_i] j}{[E_T]} \tag{A3.12}$$

where $k_{cat.}^i$ is the catalytic constant expressed in the pseudophase i, and j is the fraction volume of that pseudophase, α, β or γ.

$$K_{m_f}^{app.} = K_{m_f}[\alpha + P_1\beta + P_1P_2\gamma + P_1P_2P_3(1-\alpha-\beta-\gamma)] \tag{A3.13}$$

$$K_{m_b}^{app.} = K_{m_b}\frac{[\alpha + P_1\beta + P_1P_2\gamma + P_1P_2P_3(1-\alpha-\beta-\gamma)]}{P_1} \tag{A3.14}$$

$$K_{m_s}^{app.} = K_{m_s}\frac{[\alpha + P_1\beta + P_1P_2\gamma + P_1P_2P_3(1-\alpha-\beta-\gamma)]}{P_1P_2} \tag{A3.15}$$

APPENDIX 4

Main equations of the electric potential distribution

The ionic distributions inside the reverse micelles can be assumed to be governed by the equilibrium distribution, assumed here to be the Boltzmann distribution. Then:

$$c_p = \bar{c}_p e^{\frac{-z_p e \Psi^*}{kT}} \tag{A4.1}$$

where k is the Boltzmann constant, T is the absolute temperature, c_p is the concentration of the pth ion at the position where the electrical potential, $\Psi^* = 0$, and z_p is its charge number. Defining:

$$\Psi = \frac{e\Psi^*}{kT} \tag{A4.2}$$

as the reduced (dimensionless) electrical potential, and:

$$\zeta = \frac{r - R_e}{R_{wp,f} - R_e} \tag{A4.3}$$

and

$$\eta = \frac{r}{R_{wp,u}} \tag{A4.4}$$

as the reduced (dimensionless) positions in the enzyme-filled (f) and unfilled (u) reverse micelles respectively, the following expression for the radial distribution of the charged substrate in the waterpool of an enzyme-filled reverse micelle can be obtained after a series of intermediate steps:

$$\bar{c}_s = \frac{\Omega_{SRM}[S_o]_{ov}}{3\Omega_{wp}\int_0^1 e^{\Psi}\eta^2 d\eta + \dfrac{e^{\Psi_{PM}}\Omega_{iph}}{P_{wp/iph}}} \tag{A4.5}$$

where Ω_{SRM}, Ω_{wp} and Ω_{iph} stand for the volume fractions of solution of substrate-containing reverse micelles, volume fraction of solubilized water and volume fraction of surfactant interface, and $P_{wp/iph}$ is the partition coefficient of substrate between waterpool and surfactant layer. This expression is then used to calculate the substrate concentration at the enzyme surface and the reaction rate is obtained by applying the Michaelis–Menten equation.