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Kinetics of reactions catalyzed by enzymes in solutions of surfactants

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Abstract

The effect of surfactants, both in water-in-oil microemulsions (hydrated reverse micelles) and aqueous solutions upon enzymatic processes is reviewed, with special emphasis on the effect of the surfactant upon the kinetic parameters of the process. Differences and similarities between processes taking place in aqueous and organic solvents are highlighted, and the main models currently employed to interpret the results are briefly discussed.

Keywords: Enzymatic catalysis; Sufactants; Micelles

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1. Introduction

Since 1977, after the pioneering work of Martinek et al. [1], micellar enzymology has arisen as a new physicochemical line of research to approach problems in molecular biology. In fact, in the living cell, enzymes exert their function in microheterogeneous environments interacting with, or being incorporated into, membranes. Even in the cytoplasm, water plays structural and functional roles, besides being the principal component, as in laboratory assays [2]. In vitro systems that make use of the self-assembly of surfactants in aqueous solution (forming micelles or vesicles) or in organic solvents (forming reverse micelles, w/o microemulsions or reverse vesicles) are used to mimic "in vivo" conditions.

Micellar enzymology is devoted to the study of reactions catalyzed by enzymes in solutions of surfactants. Biocatalysis in apolar organic solvents is gaining increasing importance, both for academical studies and industrial applications [3,4]. The field has attracted the interest of many researchers and the relation of micellar enzymology to subjects such as bioorganic synthesis [5], and its potential applications in industrial biocatalytic processes [6,7], biodegradation of phenolic environmental pollutans [8]. The field of enzyme solubilization in hydrocarbon solvents by reverse micelles have been reviewed [9–11], but several physicochemical aspects are still not totally understood. Thus, in spite of more than two decades of intensive work, several problems remain under study in this ample and multifactorial subject. From a kinetic point of view, several aspects must be taken into consideration, such as:

(i) the microenvironment in the surroundings of the enzyme and the substrate [12], and the structural effects resulting from the enzyme-surfactant interaction [13,14];
(ii) the partitioning of the substrate between the micelles and the external solvent [15,16],
(iii) the effect of the surfactant concentration upon the kinetic parameters [16,17];
(iv) the effect of water content on the catalytic behaviour in reverse micelles [18];
(v) the substrate concentration scale that has to be employed in the evaluation of concentration dependent kinetic parameters for a meaningful comparison of the kinetic behaviour of the enzyme in the reverse micelle system [19,20];
(vi) the effect of additives [21,22];
(vii) the effect of changes in the internal [23,24] or the external solvent [21] in reverse micelles;
(viii) the mode of enzyme-surfactant interaction (cooperative, anticooperative) [25];
(ix) the effect of denaturants [26–30]; and
(x) the dependence with the surfactant type [31–35].

This review deals with the general aspects of enzymatic catalysis in solutions of surfactants focusing on the behaviour of enzymes that follows a Michaelis–Menten mechanism. Surfactants in aqueous solution, as well as in organic solvents, are considered. Emphasis is given to results reported after 1995 since former data have been exaustively reviewed by Maitra [10].

2. Surfactants, micelles and reverse micelles

Surfactants are amphiphilic molecules that posses both hydrophilic and hydrophobic parts. The hydrophilic moiety is called the head and the hydrophobic part the tail (or tails). The hydrophobic part may consist of a single chain or it may have up to four chains. The head can be a charged or uncharged polar group. According with the nature of the head groups the surfactants are classified into anionic, cationic, non-ionic and zwittterionic (amphoteric). These types of structures are capable of spontaneously aggregate in aqueous solutions to form micelles (M) and, in organic solvents, reverse micelles. In the present contribution, the term reverse micelle (RM) is applied to aqueous droplets stabilised by a surfactant, dispersed in a continuous organic medium. The term w/o microemulsion will be used in cases that the presence of cosurfactant is necessary to form the microaggregate, i.g. for surfactant/cosurfactant/oil/water systems. In these systems the cosurfactant will be considered as an additive.

Normal micelles, which will be called micelles (M), are depicted as aggregates of surfactant molecules with the hydrophilic heads oriented towards the dispersing solvent (generally water), and the hydrophobic tails oriented towards the inner part of the assembly (the micellar core). Micelles appear as the dominant form above the so-called critical micelle concentration, CMC, but free surfactant is also present in the system as monomer. The aggregation number (the average number of surfactant molecules in a micelle) is dependent on the surfactant type and its concentration. Typically, at low surfactant concentrations (below ca. 0.1 M), micelles are formed by 100–200 surfactant molecules and the aggregation number is nearly independent on surfactant concentration, ie., an increase in surfactant concentration only leads to an increase in the number of micelles [36]. The field of micellar catalysis, which explores the effect of M on the kinetics of chemical reactions, is well known and stresses the importance of these microheterogeneous media, particularly in organic reactions [37]. Also, these media have been used in enzymatic reactions, where important effects can be observed even at surfactant concentrations below the corresponding CMC. Some surfactants that are commonly used in enzymatic studies in micelles, are summarized in Table 1.

Table 1
Commonly used surfactants for the formation of micelles in studies of enzymatic reactions

Surfactant	Type
Sodium dodecylsulfate (SDS)	Anionic
Dodecyltrimethylammonium bromide (DTAB)	Cationic
Cetyltrimethylammonium bromide (CTAB)	Cationic
Cetyltriethylammonium bromide (CTEAB)	Cationic
Cetyltripropylammonium bromide (CTPAMB)	Cationic
Cetyltributylammonium bromide (CTBAM)	Cationic
Myristyldimethylammonium propanesulfonate (MDAPS)	Zwitterionic
Triton X-100	Nonionic
Polyoxyethylene 9 lauryl ether (PO9)	Nonionic

The picture of micelles is reversed when the solvent is changed from aqueous to a nonpolar medium. In order to minimize their contact with the bulk nonpolar solvents, the head groups of the surfactants associate themselves with the water molecules, protruding their hydrophobic tails into the non polar medium. The aggregates thus formed are known as reverse micelles and the aqueous core of such micellar aggregates is known as the water pool. Reverse micelles can be also formed with cores comprising other polar solvents, which have relatively high dielectric constants and are inmiscible with hydrocarbons solvents (such as ethylenglycol, glycerol, formamide, dimethylacetamide, etc.), instead of water [38–40]. The aggregation number of the reverse micelles increases with their water content, usually defined in terms of the characteristic parameter $W_0 = [H_2O]/[\text{surfactant}]$ [41]. Some surfactants that are commonly used in enzymatic studies in reverse micellar systems are summarised in Table 2.

3. Enzymes in reverse micellar solutions

3.1. Solubilization of enzymes in reverse micellar solutions

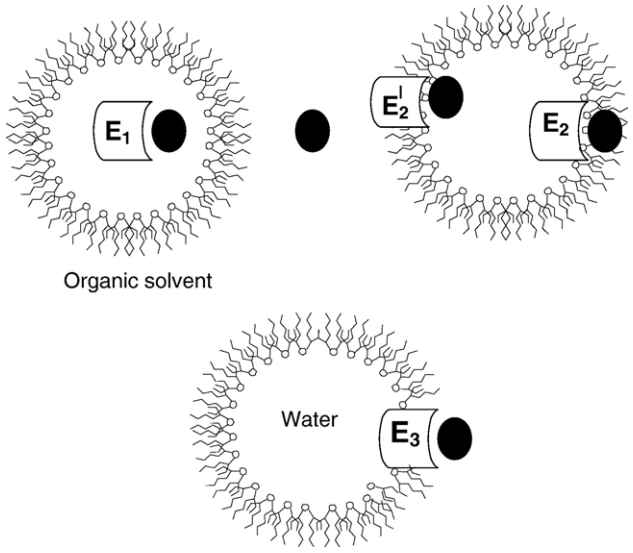
Enzymes can be solubilized in reverse micellar solutions by three procedures [42]:

(i) Injection, where an aliquot of a concentrated enzyme solution in water or buffer is added to the surfactant dissolved in the organic solvent;
(ii) dissolution, where dry, lyophilized enzyme is added to the reverse micelles already containing the desired amount of water; and
(iii) phase transfer from an aqueous phase containing the enzyme to a solution containing the surfactant in the organic solvent.

The injection method is the most frequently used due to its simplicity. The phase transfer method has the disadvantage that does not permit to regulate the water content (it is only possible to work at W_0 values corresponding to saturation) and takes a long time to reach equilibrium. The dissolution method is also more time consuming than the injection method and frequently leads to enzyme denaturation.

Table 2
Commonly used surfactant/solvent systems used for enzymatic studies in reverse micellar solutions

Surfactant	External solvent
AOT	*n*-Hydrocarbons (C_6–C_{12}), Isooctane Cyclohexane, Benzene
CTAB	Heptane: chloroform *n*-Hydrocarbons/primary alcohols
Triton X-100	Xylene
Phosphatidylcholine (PC)	*n*-Hydrocarbons (C_6–C_{12})
Lecihin	*n*-heptane; 1-propanol/iooctane
Phosphatidylethanolamine (PEA)	*n*-Hydrocarbons (C_6–C_{12})
Polyethylene glycol monododecyl ethers $C_{12}E_n$ (n=3.5; E=ether)	*n*-Hydrocarbons (C_6–C_{12})
SDS	Toluene : *n*-pentanol
β-d-glucopyranoside	Octanol



Scheme 1. Schematic representation of the different localization sites of the enzyme (E) and substrate (S) in Reverse Micelles solution. E_1: hydrophilic, E_2: surface-active and E_3 hydrophobic enzymes. S is partitioned between the microaggregates and the organic pseudophase.

The maximum solubilization capacity of reverse micelles seem to be dependent on the method employed for protein addition [43]. The dissolution of the dry enzyme is not efficient unless the micellar size is similar or larger than the enzyme size. In the injection method, a dependence of the solubilization efficiency with the micellar size has not been clearly established.

The characteristics of reverse micellar solutions offer a unique opportunity for dissolution of enzymes in the sense that they can choose an optimal microenvironment for their functioning. The localization of the enzyme inside the micelles depends upon the protein hydrophobicity [44]. In fact, hydrophilic enzymes (such as chymotrypsins) can remain localised in the micellar water pool avoiding contact with the organic solvent, a surface active enzyme (such as a lipase) can interact with the inner micellar interface, while membrane proteins (xanthine oxidase [45], atpases, CO dehydrogenase, methyl-CoM methylreductase [46] or alkaline phosphatase [47]) can be in contact with the hydrophobic region of the RM interface (Scheme 1). This scheme, that assumes that all the enzyme is associated to the RM, can be treated in terms of models comprising two (the external solvent and the RM) or three pseudophases (the external solvent, the interface and the water pool). However, it must be considered that even in this latter model, both the water pool and the interfacial pseudophase are microheterogoneous. In particular, surface active enzymes associated to the micelles can be mostly localized at the inner part of the interface or in the region of the surfactant tails (hydrophobic enzymes).

The substrate of the enzymatic reaction can be partitioned between the RM and the organic solvent. Furthermore, the RM-associated substrate can be distributed between the micellar interface and the inner pool [48]. So, the micelle incorporated

enzyme is able to interact both with hydrophilic and hydrophobic substrates. The reaction can then take place in several microenvironments, depending upon the localization of the enzyme and its activity at the different locations (Scheme 1).

3.1.1. Structural studies of enzymes in surfactant solutions

Several studies of the structural changes taking place in proteins encapsulated in reverse micelles have been performed using different techniques, such as circular dichroism (CD) spectroscopy [13,49], steady state [50] and time resolved fluorescence spectroscopy [51], small-angle X-ray scattering (SAXS) [52], electron paramagnetic resonance (EPR) [53–55], dymanic light scattering (DLS) [8,56], Fourier transform infrared spectroscopy (FTIR) [57–59], and ^{1}HNMR [49].

The conformation of three different lipases has been studied [60] in RM formed by AOT in isooctane. In the case of *human pancreatic* lipase, the conformation of the polypeptide chain is barely affected when the enzyme is transferred from a bulk aqueous solution into the RM, as revealed from far-UV CD measurements. However, the spectral changes observed in the near-UV, CD and fluorescence spectra indicate that solvation of the aromatic aminoacids side chains is considerably different in RM. On the contrary, the CD spectra of the lipases from *Candida rugosa* and *Pseudomonas sp.* are considerably different in RM, compared with the spectra in aqueous solution, indicating that both enzymes loose their native structure at the micellar interface.

CD and EPR studies [53,61] have shown that the conformation of *horse liver* alcohol dehydrogenase (HLADH) in AOT RM is altered, suggesting that AOT molecules might interact with HLADH via adsorption and active-site contamination. Similar studies on *yeast* alcohol dehydrogenase (YADH) in Brij-30 and mixed Brij-30/AOT RM show conformational changes that could explain inactivation of the enzyme in these media [62].

The secondary structure conformational changes of YADH solubilized in RM of AOT have been studied by CD spectroscopy [13]. The conformation of the enzyme is extremely sensitive to pH, temperature and water content, and a parallelism between the enzymatic activity and the fraction of α-helix conformation present in the equilibrium structure of the protein was found. Inactivation of the protein can be caused by transformation of α-helixes into either β-sheet or random coil conformations.

FTIR spectroscopy study of the incorporation of α-chymotrypsin (α-CT) in AOT RMs, showed that both the secondary and tertiary structure of the protein are not significantly perturbed by its entrapment in RM [59].

Andrade and Costa [51], from time-resolved fluorescence measurements and quenching by acrylamide, concluded that α-CT solubilised in AOT reverse micelles (W_0=20) is located in a bulk-water-like environment, a proposal compatible with the high stability of the enzyme in these RMs. When the external pH (the pH of the water employed in the RM preparation) is higher than the protein pI (i.e, 9.2) the data indicate some structural changes, with buried Trp residues becoming more exposed to solutes dissolved in the water pool. These changes would result from repulsion between the charged interface and the protein.

Structural and catalytic properties of cutinase have been studied [54] in AOT RMs. EPR spectroscopy of active site labeled cutinase, at several W_0 values, showed that the mobility of the label is higher than in an aqueous solution. Furthermore, the enzyme activity does not correlate with the mobility at the active site. In fact, there was an increase of both activity and active site mobility until W_0=9 while, at higher W_0, the mobility of the bound spin label further increased whereas the enzymatic activity dropped considerably.

From DLS experiments, it has been shown [56] that cutinase in AOT RM is attached to the micellar wall even at large W_0 values, and that this interaction can cause enzyme unfolding, even at room temperature.

The structure of cutinase from *Fusarium solani pisi* (FsC) in AOT/isooctane RM (W_0=15 ; T=30 °C) was characterized by ^{1}HNMR [49]. Both the aromatic and amide spectral regions indicated that the native structure is lost in presence of RM. In these conditions, the spectrum was characteristic of a molten globule like state with reduced spectral dispersion and broad lines. This observation was in agreement with results from fluorescence and CD measurements, which showed that the structure of the denatured state in aqueous AOT (considered as a denaturant) is similar to that in RM. However, the denatured state in aqueous AOT is more native-like than that found in guanidine hydrochloride (GdnHCl). The authors remarked that the loss of activity in RM parallels unfolding. It is concluded that the interaction between cutinase and AOT causes parallel conformational changes both in the active site region and the region around the tryptophane residues, which is far away from the active site.

The absorption spectrum of a bacterial flavoprotein, putidaredoxin reductase (PdR), solubilized in AOT/isooctane RM was significantly changed from that in aqueous buffer solution, suggesting that a strong electrostatic interaction caused a significant conformational change around the flavin prosthetic group [63]. On the other hand, it has been shown that the presence of non-ionic surfactants relieves protein denaturation caused by electrostatic interactions [64,65].

The secondary structure of horseradish peroxidase (HRP) in AOT RM, and in CTAB and SDS media, has been determined [57] utilising FTIR spectroscopy. The structure of HRP in AOT RM was the closest one to that in aqueous solution while, in CTAB and SDS systems, the spectra were much different, indicating that the enzyme structure has been partly destroyed by the microenvironment provided by the surfactants.

The conformation of RNase in RM formed by dodecylamonium butyrate in cyclohexane-water, was investigated by UV and FTIR spectroscopy. The data suggested that the structure of the enzyme in RM with high water content is similar to that in water, but larger structure differences were found at lower levels of water content [58]. Other techniques have also been applied to evaluate the protein conformation. For example, hydration and protein folding (at several W_0) has been evaluated by partial specific volume and adiabatic compressibility measurements [66].

Incorporation of oligomeric enzymes to RM can promote their dissociation. This was confirmed [67] for octopus glutatione tranferase in AOT RM, by cross-linking of the associated subunits with glutaraldehyde and separation of the monomer and dimers by electrophoresis.

Several studies have focus their attention on the characteristics/properties and structure of RM containing enzymes [52,68]. In this sense, structural comparison of AOT RM in the presence and in the absence of RNase, by using SAXS and conductivity, has not shown changes neither in the form nor in the structure of the droplet [52]. The size and intermicellar potential of filled and unfilled micelles, as well as percolation, are unchanged. Also, the properties were the same with the system containing native or hydrophobic RNase, indicating that an increase in ribonuclease's hydrophobicity does not alter the RM structure.

DLS measurements have been employed to determine the size of aggregates in three different systems: AOT RM, surfactant-laccasse complexes and surfactant-laccase complexes plus AOT RM [8]. This analysis showed that the size of RM was 10±3 nm whereas that of surfactant-laccase complex was 140±40 nm. For the third system, two different size distributions were observed: one at 320±130 nm and the other at 9±4 nm, suggesting the co-existance of surfactant-laccase complexs and RM. As the size of surfactant-complexes increased upon the addition of water (from 140±40 nm to 320±130 nm) it appeared that laccase complex was swollen by taking up water from the RM. As a result, a favourable environment for laccase catalysis towards bisphenol A oxidation could be acchieved [8].

The presence of the micelles can alter oligomerization equilibrium of the enzyme and, hence, its activity. This equilibrium can be modulated by the surfactant and enzyme concentrations (at fixed W_0) and by W_0 (at fixed surfactant) In fact, several oligomeric enzymes have been reported [69] to dissociate into subunits upon incorporation in RM at low W_0 values, and/or associate to higher oligomers in larger micelles: human prostatic acid phosphatase (PAP) [69], alkaline phosphatase [70,71], formate dehydrogenase [72], gamma-glutamyltransferase [73], D-glceraldehyde-3-phosphate dehydrogenase [71,74], glycogen phosphorylase b [75], lactated dehydrogenase [71,76], malic enzyme [77], penicillin acylase [78,79] and uridine phosphorylase [75,80]. In some systems, the dependence with W_0 is complex, with optimal catalytic activity observed both at high water content, when the volume of the aqueous core is comparable to the size of the most stable oligomer, and at low water content, were a monomeric form is present [81]. For example, in the case of calf intestinal mucosa alkaline phosphatase, two activity maxima were observed when the inner cavity was approximately equal to the sizes of the monomeric and dimeric enzyme forms, respectively [73,71].

Native and inmobilized isolipase B from *C. Rugose* show, in AOT RMs, an increase in their catalytic efficiency [82]. The crosslinked polymer modified the micellar aggregates, being the hydrodinamic radius of the particules, determined by DLS, of 400±20 Å. This radius increased with the water activity in poorly hydrated micelles (small W_0 values).

3.2. Catalytic properties of enzymes in reverse micellar solutions

Martinek et al. [1] were the first to report the retention of catalytic activity of peroxidase and α-chymotrypsin in reverse micellar solutions made of AOT in organic solvents. Since then, numerous enzymes have been studied in reverse micellar systems [83–102]. Some representative examples are summarized in Table 3. This Table mostly includes data obtained after 1995 since previous results have been discussed in Maitra's review [4], which is specifically focalised in AOT RMs.

3.2.1. Enzyme stability in reverse micelles

Incorporation of an enzyme to a RM can change, increasing or decreasing, its catalytic capacity and/or stability.

Numerous enzymes exhibe good stability in RM [103], a phenomenon explained in terms of the peculiar water structure in the micellar core. For example, *p*-nitrophenylphosphatase (pNPPase) was more stable in RM than in bulk aqueous solution under all the conditions assayed (i.e. different W_0 values and salt concentration) [104]. The stability of pNPPase depended on the size of the water pool. The higher stability found at $W_0=5.6$, compared with that at $W_0=7.5$, could result from a more rigid conformation of the enzyme in the smaller RM [103]. Lecithin-based water in olive oil microemulsions, in the presence of 1-propanol, were used for the entrapment of tyrosinase [105]. Although the monomer of the enzyme is rather unstable in aqueous media, it retained its catalytic activity in a microemulsion whose water content was 1% (v/v).

It has been reported that native α-CT is more stable in tetradecyltrimethylammonium bromide (TTAB) /heptane/octanol microemulsion than in DMF/buffer [106]. The measured half-life of the enzyme in the w/o microemulsion was higher than 30 days, while in the homogeneous mixture was only of 6.6 days [107]. However, other comparative study indicated that the enzyme is more stable in homogeneous solution (buffer) than in a CTAB/heptane/chloroform (50:50) microemulsion [90]. In this media, the stability of the enzyme changed when choroform was remplaced by a long chain alcohol, such as octanol [108]. The higher stability found for α-CT encapsulated in the microemulsion system was explained [109] by the absence of S-S bond cleavage of its cystine residues and also by the absence of auto-hydrolyses of the protein structure. Formation of small peptides and SH free groups was detected for the enzyme incubated in the aqueous and organic solvent systems.

The high stability of enzymes and their tendency towards active conformations in RM allowed Vinogradov et al. [110] to transform recombinant inactive proteins present in inclusion bodies into soluble bioactive forms. These authors found that, after extraction with RM, entrapped enzymes (*Fusarium* galactose oxidase and *Stignatella aurantiaca* putative galactose oxidase) have native-like secondary structure and catalytic activity.

However, in some systems the presence of the RM decrease the stability and activity of the enzyme. YADH has been studied in Brij-30 and mixed Brij-30/AOT RM [62]. The interaction of the enzyme with charged surfactants (AOT), both hydrophobic

Table 3
Summary of some representative enzymatic reactions that have been studied in reverse micellar solutions

Enzyme	Substrate	Medium	Observed effect	Ref
Surfactant-laccase complex	2,6-dimethoxyphenol	AOT/isooctane	Catalytic activity greatly enhanced on increasing W_0 until heterogeneity AOT RM help to deliver redox mediator	[8]
Yeast alcohol deshydrogenase (YADH)	Ethanol, NAD^+	AOT/isooctane	At W_0=28, pH 8.1 and 30 °C an hydrated YADH fits exactly the aqueous core and have the optimum α-helicity. It is in this condition that also has the highest enzymatic activity.	[13]
Desulfovibrio gigas bacterium hydrogenase	H_2-Methyl viologen	AOT/isooctane	A peak of catalytic activity is achieved at W_0=18 (50 mM imidazol, pH 9.0) The k_{cat} is slightly higher than in buffer medium. At higher W_0 activity diminishes probably because of changes in pH	[145]
DNA polymerase I and HIV reverse transcriptase	Oligonucleotides	Mixed surfactants: Brij30, Triton X-100, SDS, CTAB and Brij58 in hexanol/octane 1:6(v/v)	Polymerase activity appears in a certain range of water content and depends on the local polarity	[198]
Cutinases	*p*-nitrophenylbutyrate	AOT/isooctane	The enzyme loss activity in RM by unfolding. The interaction between cutinase and AOT causes a parallel conformational change in the active site region and the region around the tryptophan residue, which is far away from the active site. The denatured state in AOT(aq) is virtually identical to that in AOT micelles.	[49]
Fusarium solani pisi recombinant cutinase	Lauric acid and pentanol	AOT/isooctane	The enzyme activity on esterification follows a bell-shaped profile with water content. The maximum occurs at W_0=9 at pH=9. Apparent K_M and V_{max} are determined. EPR studies show that the mobility is higher than in water. The enzyme suffers a fast inactivation on incubation in anionic RMs	[54]
Cutinase	Triolein	AOT/isooctane CTAB/hexanol	A fast deactivation of the enzyme in AOT RM occurs due to a reversible denaturation process. The deactivation and denaturation is slower in small cationic RM and does not occur when the size of the cationic RM water-pool is larger than that of cutinase. 1-Hexanol exerts a stabilizing effect	[100]
Lipases	Several	Review on different surfactants, external solvent, W_0, and temperature	The importance to spread out micellar bioreactor technology over industrial processes is discussed	[21]
Lipases	Several	Review on different surfactants and external solvents	Use of lipases in hydrolytic or synthetic reactions. Illustrated biotechnological applications	[12]
Chromobacterium viscosum lipase	*p*-nitrophenyl-*n*-hexanoate	CTAB, TTAB and a series of cationic surfactants of different tail lengths (C_{10} to C_{18}) and four different hydroxyethyl head groups in water/isooctane/hexanol	The activity increases from C_{10} to C_{16} and depends on the head group. TTAB at [alcohol]/[surf]=8 gave the highest activity	[135]
Chromobacterium viscosum lipase	Olive oil	AOT/isooctane AOT/PEG/water/isooctane	Addition of low molecular weight polyethylene glycol (PEG 400) increases stability of the lipase. V_{max} was reduced significantly by the addition of PEG 400.	[22]
Rhyzopus delemar lipase	Triolein	AOT/nC_6-C_{10} alkanes, isooctane and cyclohexane/water	Highest initial rate with isooctane. Initial rate in *n*-alkanes related to hydrophobicity (log *P*). Highest k_{cat}/K_M value in isooctane at W_0=13.	[161]
Rhizopus arrhizus lipase	2-naphthyl acetate	AOT/ heptane /water at pH 7 with addition of urea	Addition of urea decreases enzymatic activity due a decrease in the association of the substrate with the micelles. The enzyme is more resistant to denaturation by urea in RM than in water solution. Urea increases K_M with a small increase of k_{cat}	[28]
Humicola langinosa lipase	Octyl decanoate	Phosphatidylcholine from egg yolk, partially purified lecithin from egg yolk or partially purified lecithin from soybean in n-heptane	The initial rate of reaction in the different systems was compared to a similar system of AOT and was superior in every case. The best were the soybean lecitin RMs. The effects of enzyme concentration, initial water concentration, water activity, surfactant concentration, initial substrate concentration and temperature are discussed	[133]
Chromobacterium viscosum lipase	Olive oil	AOT/isooctane /Water with the addition of ethylene glycol, acetone, formamide, sulfolane, DMSO, acetonitrile or ethanol to the water pool.	DMSO was found to be most effective of the solvents to enhance lipase activity (reduces K_M while V_{max} is not altered) higher lipase stability was found in AOT reverse micelles with DMSO compared with that in simple AOT systems with half-life of 125 and 33 days, respectively. DMSO molecules remain at the micellar interface and modifies the micellar interface, reducing the surface charge density creating a better environment for the enzyme.	[207]
Patatin estearase	*p*-nitrophenyl caprylate (PNP-caprylate), laurate and butyrate	AOT/isooctane	At W_0 lower than 10 the enzyme is fully inactive. Activity increases up to W_0=20, decreasing thereafter. The greatest activity was found for the PNP-caprylate, similar to results with other surfactants. The high value of K_M obtained would indicate that the substrate is partitioned between the oil and the interface	[93]
β-Galatosidase	Lactose	AOT/organic solvents	The enzyme is fairly stable. Water addition gives a bell shaped plot for product formation. Results are best at W_0=15 The RM inhibit hydrolysis in spite of an increased local substrate concentration. Best external solvents are those with higher log *P*	[112]
Hexokinase (HK)		AOT/isooctane HTAC/isooctane/octanol (85:15 v/v) $C_{12}E_8$/ isooctane/octanol (90:10 v/v)	Highly charged inner surfaces of AOT and HTAC RM were not favorable for HK catalytic activity. The activity in HTAC was 2–3 times higher that in AOT RM. Maximum activity was found in both systems at W_0=10. HK activity was much higher in $C_{12}E_8$ RM than in AOT and HTAC	[132]

Mushroom polyphenol oxidase (tyrosinase)	Alkyl catechols, phenol and *p*-cresol	AOT/cyclohexane/water	The enzyme catalyzes the oxidation of several phenols and catechols in RM. With 4-methylcatechol as substrate the behavior is similar to that in buffer. The apparent catalytic efficiency of the enzyme is strongly and directly dependent of the substrate hydrophilicity, whereas the apparent K_M is inversely dependent of it. Reveals importance of substrate partition. Optimum $W_0=20$	[92]
Agaricus bisporous tyrosinase	4-t-butylcatechol	Water pH=7/AOT and AOT/isooctane/water	The enzyme is catalytically active also in highly concentrated AOT (0.2 M), even at low water content, although its activity is reduced in these conditions. Results are explained in terms of substrate binding to the RM interface	[95]
Organic solvent resistant tyrosinase (OSRT)	t-butylcatechol and 4-methyl cathecol	AOT/isooctane/water	AOT reduces the activity of the enzyme both in aqueous and isooctane solutions. The reduction of OSRT activity in the RM is 3.8 times greater than in the case of mushroom tyrosinase. AOT concentration mainly affects K_M. This is explained considering the association of the substrate with AOT interface. Its magnitude depends on substrate polarity.	[141]
Dioleyl-*N*-D-glucono-l-glutamate-manganese peroxidase complex	2,6-methoxyphenol	AOT/ /toluene	Peroxidase activity in RM increases 10-fold with respect to the surfactant–enzyme complex in toluene	[122]
Putidaredoxin reductase	Cytochrome b_5 and putidaredoxin	AOT/isooctane/water-Tween 85	It is demonstrated the capability of RM to facilitate protein mediated electron transfer reactions. A multicomponent enzyme system is activated in organic media	[63]
α-chymotrypsin	Acetylphenylalanine ethyl ester	Tetradecyltrimethylammonium bromide (TTAB)/heptane/octanol	The enzyme is more stable in RM than in organic buffer medium or in (DMSO o DMF)/ buffer mixtures. The absence of S–S bond cleavage of its cystine residues and the absence of auto-hydrolyses of the protein structure in RM may be the cause of stability.	[109]
Bovine pancreati α-chymotrypsins types II and VII	*p*-nitrophenyl (PNP) acetate and caprylate	AOT/isooctane	SANS shows that the enzyme is entrapped in the water pool. The reactions are enhanced with the enzyme at the interface of the RM. The enzyme activity increases with W_0 until a maximum at $W_0=12$	[68]
Bovine pancreatic α-chymotrypsin	*N*-succinyl-L-phenylalanine	AOT/n-octane/water-glycerol mixtures	The presence of glycerol in the RM increases residual activity after incubation. Glycerol decreases the mobility and increases stability of the enzyme.	[23]
α-chymotrypsin	2-Naphtyl acetate	AOT/heptane/water-glycerol mixture	Comparing the corrected catalytic efficiencies ($[k_{cat}^{exp}/(K_M)_{corr}]$) obtained in both reverse micellar systems, GY-water/AOT/*n*-heptane and water/AOT/*n*-heptane, shows that the value in the former one is 5 times higher.	[24]
Vaccinium mirtyllus peroxidase	Guaiacol	AOT/isooctane/ buffer CTAB/hexanol/ isooctane/buffer l-α phosphatidylcholine/ hexanol/isooctane/buffer	The enzyme shows higher reactivity in RMs than in aqueous solution. Higher stability in CTAB/hexanol	[97]
Plant and fungal peroxidases	Several	Several	The action of these enzyme produce highly reactive radicals that in aqueous solutions start polymerization. In RM these reactions are slowed down and may be controlled. The use of these systems in the oxidation of water pollutants, biosynthesis or chemoenzymatic synthesis is discussed	[148]
Xanthine oxidase	p-substituted benzaldehydes	DTAB/heptane/hexanol/water TritonX-100/cyclohexane/hexanol/ water AOT/isooctane/water	k_{cat} and K_M where correlated by linear free energy relationships K_{cat} depends mostly on the substituents constant σ and $1/K_M$ on the partition constant. The k_{cat}/K_M ratio is smaller for RMs systems compared to water. If the partitioning of the substrate and the effective volume available to the substrate and products was restricted to be that offered for the water core, kinetics constants in water and RMs are comparable.	[45]
Soybean lipoxygenase	Bromocresol purple and phenol red	AOT/isooctane/water	Effect of addition of linoleic acid. A significant decrease of the effective pH in RM as a function of linoleic acid was found. The enzymic activity is reduced at all the effective pHs. The unfavorable partition of the substrate may be one of the mayor causes	[102]
Pyruvate kinase	A coupled system with lactate dehydrogenase and NADH	CTAB/*n*-octane, hexanol/ water with and without K^+	The k_{cat} was about 450 times larger than that in 100% water without K^+. The kinetics of the enzyme in RM was not affected by K^+. Apparently, variations in the amount of water in contact with the enzyme induce structural transitions that affect the K^+requirements of the enzyme for carrying out catalysis.	[101]
Subtilisin	Pertides	β-d-Glucopyranoside (β-OG)/water/octanol and	The activity was studied in several regions of the phase diagram. The maximum activity of the enzyme is found for RM and reverse hexagonal phase at a given W_0. The results are compared with AOT RM	[131]
Halophilic malate deshydrogenase (from *Halobacterium salinarum*)	Oxalacetic acid, NADH	CTAB/cyclohexane and 1-butanol as co-surfactant	The activity depends on W_0, surfactant concentration, and type and salt concentration. Increases with W_0 for phosphate buffer but has a bell-shaped profile for Tris/HCl. Always the activity is higher at low salt content	[193]
Acetylcholinesterase-like abzyme (monoclonal antibody) 9A8	*o*-and *p*-nitrophenylacetate	AOT/isooctane	The activity gives a bell-shaped profile with a maximum at $W_0=11.1$. Above this value, activity decreases slightly. The catalytic efficiency diminishes because of increase in K_m, due mostly to the partition of the substrate.	[138]
Hexokinase	2-Deoxy-D-(+) glucosa	AOT/isooctane; hexadecyltrimetyl ammonium chloride (HTAC)/isoctane/ octanol and Octaoxyethylene dodecyl ether ($C_{12}H_8$)/ isoctane/octanol.	The high electrostatic inner cavity potential of AOT and HTAC RMs was not favorable for the catalytic activity. This is 2–3 times higher in HTAC than in AOT RMs. The size and physical properties of the water pools, as well as the distribution of the substrate, explain the differences. The optimum W_0 was 10 for both RM. The catalytic activity was much higher in $C_{12}H_8$ and increae with the concentration of surfactant. This reveals that the enzyme activity is enhanced by the hydrated ethylene oxide chains.	[132]

and electrostatic, can lead to its deactivation, as observed frequently for alcohol dehydrogenases [87,111].

The enzymatyc activity of β-galactosidase [112] in AOT/isooctane RM decreased rapidly during one hour and at a slower rate thereafter when incubated at 45 °C [96]. The stability of the enzyme in the RM depends on its water content, being more stable at high water contents. The half-life of β-galactosidase was approximately 46 and 90 h at W_0=10 and 30, respectively. This tendency was explained as due to the fact that the properties of water in RM approach to those of bulk water at higher W_0 values.

Hydrolysis of *p*-nitrophenylphosphate, catalysed by alkaline *p*-nitrophenylphosphatase has been studied [113] in microemulsions of CTAB in cyclohexane with 1-butanol as cosurfactant. When the enzyme was microinjected into the RM containing the substrate the reaction was non-linear with time. These results were explained in terms of a kinetic model in which the enzyme is irreversibly converted from an initial form to a final stable form during the first seconds of the encapsulation process. As it has been previously mentioned, the deactivation seems to be a result of the enzyme conformational change caused by electrostatic interactions between polar head groups of the surfactant and the protein.

Acumulation of products at the RM interface, the locus of enzyme action, can also affect the rate of the process. Papadimitriou et al [105] tested olive oil microemulsions as media for the oxidation of oleuropein, the most abundant olive phenolic compound, catalysed by a mushroom tyrosinase. However, rapid inactivation, attributed to product accumulation, was observed.

Recently the influence of ethylene glycol (EG) on the kinetics of hydrolysis of *N*-α-benzoyl-L-arginine ethyl ether catalyzed by trypsin encapsulated in AOT RM was studied at different temperatures [114]. It is shown that ethylene glycol shift the range of the trypsin activity in the reverse micelles towards higher temperatures. As shown by IR and EPR the stabilization is due to the ethylene glycol which interacting with the polar heads of AOT displaces the protein from the water core to the more rigid matrix of the interface protecting it from thermoinactivation.

3.2.2. Kinetic studies in RM

In general, it is found that chemical reactions catalyzed by enzymes in reverse micellar solutions follows the same kinetics than in homogeneous solution. In cases in which the classical Michaelis–Menten mechanism applies, it can be simply represented by,

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (SE) \overset{k_{cat}}{\rightarrow} P + E \quad (1)$$

where S is the substrate, E is the enzyme, (SE) is the substrate–enzyme intermediate, k_{cat} is the catalytic rate constant, and P is the product of reaction. According with this mechanism and applying the steady-state hypothesis to (SE), it can be derived that the rate of reaction, v, is given by Eq. (2),

$$v = k_{cat}[E][S]/K_M + [S] \quad (2)$$

where K_M is the Micahelis constant defined by Eq. (3),

$$K_M = (k_{-1} + k_1)/k_{-1} \quad (3)$$

In homogeneous solutions, the meaning of [*E*] and [*S*] are straightforward. However, for the interpretation of kinetic results in microheterogeneous solution, difficulties arise due to the distribution of the substrate and the enzyme [115–117]. If the enzyme is partitioned between two (o more) environments, a pseudophase approach can be employed and the experimentally measured rate constant expressed as the sum of contributions from each pseudophase. This approach requires, at least, knowing the distribution of the enzyme. However, in most system the enzyme is associated to a single microphase and its distribution can be ignored. With regard to the substrate (and to possibly competitive and non-competitive inhibitors) the distribution between the two (or three) possible pseudophases must be known. In the case of a polar substrate that can be considered completely associated to the reverse micelles, both its concentration and its Michaelis constant have been expressed in terms of analytical concentrations (ie., in terms of the total volume of the system) or in terms of the water pool volume (local concentrations). On the other hand, when the enzymatic reaction involves apolar or amphiphilic substrates, its partitioning between the organic pseudophase and the micelles need to be considered. In this case models based in two [48] or three [118] pseudophases (the external solvent, the micellar interface and the dispersed aqueous phase) have been applied.

Two pseudophases models have been applied to interpret results obtained in xantine oxidase catalyzed oxidation of benzaldehydes in anionic, cationic and non ionic reverse micelles [40]. K_M and K_i (inhibition constant for the product of the reaction) were corrected to take into account partitioning of the substrates between the micelles and the surrounding solvent. It was concluded that this distribution can explains most of the differences in K_M constants observed in the different media.

In relation with the changes in mechanism elicited by RMs in chemical reactions catalyzed by enzymes, two contrasting examples are acid and alkaline human phosphatase. An interesting feature of human prostatic acid phosphatase (PAP) data is that the positive cooperativity in substrate binding observed in aqueous solution is also operative for the RM entrapped enzyme [69]. On the other hand, from an analysis of the effect of pH and deuteration upon the activity of human placental alkaline phosphatase, Huang [119]. proposed that the rate-limiting step of the hydrolytic reaction changes from phosphate release in bulk solution to a covalent phosphorilation step in RM.

3.2.3. Superactivity

Frequently, enzymes are more active in reverse micellar solutions than in aqueous solutions. This behavior has given rise to the concept of "superactivity" [97], and has been observed in a variety of systems. For example, laccase showed a high activity for the oxidation reaction of phenolic environmental pollutants [120,121] in RM. Also, AOT/dioleyl-*N*-D-

glucono-L-glutamate RM enhances the activity of a manganese peroxidase [122].

Some confusion still remains regarding the concept of superactivity. In particular, it must be clarified if it refers to the turnover number (k_{cat}), to the rate at a given substrate concentration, or to the catalytic efficiency (k_{cat}/K_M). Furthermore, if superactivity is referred to the rate at a given substrate concentration or to (k_{cat}/K_M) values, a comparison with results obtained in homogeneous solvents must take into account the concentration scale employed in the analysis of the data. In particular, it must be considered if the substrate concentration in RM is expressed in analytical or local scales.

Superactivity has been frequently explained [46,68,123–125] in terms of the peculiar state of water in the reverse micelles which mimics the status of intracellular water, especially water adjacent to biological membranes. However, other effects, such as the increased conformational rigidity of the enzyme promoted by the surfactant layer and the increased concentration of the substrate at the reaction site can contribute to the RM effect. From SAXS studies [68] it was proposed that the hydrolysis of the *p*-nitrophenol esters catalysed by α-CT in AOT RM (at $W_0=12$) is enhanced and optimised at the internal interface of the micelles. This fact was explained in terms of the important role that would play the presence of the interface in accelerating the metabolic turnover, (e.g. increase of the relative enzymatic activity) by increasing the apparent interfacial area of the micelles accessible to the enzymes.

On the other hand, in the synthesis of butyl butyrate catalysed by a lipase from *Mucor michei* in AOT RM, enzymatic activity decreases by a factor around two, when the enzyme concentration (analytical) increases from 0.14 to 0.70 g L^{-1} [126]. In the conditions used in this case, the esterification rate in a biphasic system was superior to that obtained in RM. The authors proposed that this is a clear indication that the interfacial area was not the limiting factor for the transfer of ester into the organic phase. The presence of the interface can favour or inhibit the process. Loss of activity due to interaction with the charged AOT heads has been invoked to explain the fact that k_{cat} for PAP-catalyzed hydrolysis reactions in AOT reverse micelles amount to only 5 to 50% of the activity displayed in aqueous solution [69]. Furthermore, it must be considered that the behaviour of a given enzyme can qualitatively depend on the surfactant employed. For example, wheat germ acid phosphatase also presented lower activity in AOT micelles than in aqueous solution, but showed superactivity in lecithin reverse micelles [86,127].

3.2.4. Effect of the nature of the surfactant and its concentration

The nature and the concentration of the surfactant play an important role on the kinetics of reactions catalyzed by enzymes in RM solutions. The effects of these parameters have been extensively investigated for lipases and α-CT catalyzed hydrolytic reactions. In most cases, it has been observed that the hydrolytic activity employing triglycerides [128–130] or *p*-nitrophenyl alkanoate esters [85–90] as substrate, is much higher in AOT RM than in cationic, non-ionic or zwitterionic-based systems.

An extensive study of xantine oxidase reactivity with several substituted benzaldehydes in water and in reverse micelles of different charges (AOT, DTAB and Triton-X100), have been reported [45]. It was found that the catalitic eficiency (k_{cat}/K_M) follows the order: DTAB > Triton-100 > water for hydroxybenzaldehydes with hydroxyl groups in meta and para positions.

Subtilisin catalysis has been studied [131] in RM of octyl β-D-glucopyranoside (β-OG), a nonionic surfactant that has been used as solubilization reagent of membrane proteins. Results obtained in octanol-water mixtures employing this surfactant were compared with those obtained in AOT/isooctane RM. The apparent rate constant observed in AOT was significantly larger than that in β-OG, but no superactivity was observed. To explain these results it was suggested that changes in the enzyme hydration/solvation or protein structure could occur in the smaller water pool present in β-OG micelles, and that the nonionic or hydrogen-bonding nature of its head group might facilitate a direct interaction with the enzyme.

The catalytic activity of hexokinase (HK) has been examined [132] in RM of AOT (anionic), HTAC (cationic), and $C_{12}E_8$ (nonionic) surfactants. It was proposed that the highly charged inner surfaces of AOT and HTAC RM were not favorable for HK catalytic activity. The activity in HTAC was 2–3 times higher that in AOT RM and the maximum activity was found in both systems at $W_0=10$. In $C_{12}E_8$ RM, the enzyme activity was much higher than in the other systems and increased with the concentration of the surfactant. These results demonstrate the dependence of HK activity with the electrostatic field, the physical properties of the water, and the hydrophobicity of the microenvironment.

Hydrated lecithin RM and mixtures of different amount of phosphatidiylcoline and lecithin in n-heptane were used [133] in the synthesis of octyl decanoate employing *Humicola langinosa* lipase. The initial rate of reaction in the different phospholipid systems was compared to a similar system stabilised by AOT and was superior in every case. Also, the rate of ester formation was higher in RM including soybean lecithin compared to those formed with pure phosphatidilcoline, indicating the influence of the surfactant structure on the enzymatic process in RM.

Differences in the behavior of a lipase in AOT and lecithin/*n*-propanol RM were attributed to differences in microdroplet structures, with widely different solute distribution and diffusion. In particular, lecithin aggregates are more rigid, a factor that could influence the enzyme activity [44].

Tyrosinase can be solubilized in the aqueous core of RM and oxidation of phenols in this medium have been studied mostly using AOT as surfactant [98,134]. For the oxidation of 4-t-butylcathecol by tyrosinase in AOT/isooctane RM it was found [134] that the surfactant concentration and water content of the system affected the enzyme activity. Tyrosinase was active in highly concentrated AOT RM even at low water contents.

The influence of the surfactant tail lengths (from C_{10} to C_{18}) in w/o microemulsions of cationic surfactants has been studied employing *Chromobacterium viscosum* lipase [135]. It was observed that, irrespective of the nature of the head group, the activity increased considerably with the length of the alkyl chain. The hydrolytic activity of the lipase in these cationic w/o

microemulsions was up to 250% higher to that observed in widely used cationic (CTAB) w/o microemulsions.

The amount of surfactant can affect the amount of solubilized enzyme and its catalytic action. It is well known that an increase of the amount of surfactant in the organic phase can lead to an increment of protein solubilization due to the increase of the amount of surfactant aggregates and/or to their larger size [136]. However, frequently these changes are not reflected in changes in enzyme solubilization. For example, in the extraction of Nattokinase (fibrinolytic enzyme) by RM, AOT concentration barely modify the efficiency of the process [137]. In fact, as the AOT concentration increased from 50 to 200 mM, protein recovery increased slowly and the activity recovery showed no significant increase.

The kinetics of enzyme catalyzed reactions can be affected by the surfactant concentration [138] (at a given W_0 value) due to:

(a) Changes in the characteristics (size, shape and microscopic properties) of the micellar microaggregates;
(b) Changes in the distribution and properties of the enzyme; or
(c) The distribution of the substrate.

Over a wide range of surfactant concentrations it can be considered that a modification of the surfactant concentration only changes the number of micelles, without changing their properties and/or their size and shape. This fact minimises factor (a).

The dependence of the enzyme distribution and properties with the surfactant concentration is less straightforward. However, it can be disregarded if,

(i) The enzyme is totally associated to the micelles, as frequently occurs (eg., for the cases of lipases [85], α-CT [88–90], alcohol dehydrogenase [86,87], peroxidase [139], etc.)
(ii) The enzyme concentration is lower than the micelles concentration (ie., the occupation number is $\ll 1$). This condition minimize enzyme–enzyme interactions and/or oligomerization processes that could be surfactant-concentration dependent and modify the enzyme behaviour.
(iii) The enzyme "fits" in the pre-formed micellar aggregates and hence does not require of special (smaller or larger) aggregates for their solubilization. If this condition is not obeyed, it is very difficult to predict how the enzyme-holding aggregates could change their characteristics with the surfactant concentration. This is a complex matter that has been barely addressed.

Commonly the dependence of the enzyme activity with the surfactant concentration is due to substrate distribution. Let as assume that the surfactant concentration [D] only changes the number of micelles. If this holds, two extreme situations can be envisaged. One is the case where most of the substrate is in the organic solvent. Under this condition the rate of the process (expressed in terms of the analytical concentration of the reactants) is independent of the surfactant concentration. The same happens with the values of k_{cat} and K_M. The other extreme situation is when most of the substrate is associated to the micelles. Under this condition the rate of the process (expressed in analytical concentrations) is independent of the surfactant concentration [D] at high (saturating) concentrations of the substrate [S], but is inversely proportional to [D] at low values of [S].

The most interesting condition applies when the fraction of the substrate associated to the micellar pseudophase changes with the surfactant concentration, ie., when the substrate is partitioned between the micellar pseudophase and the organic solvent.

If a pseudophase model applies to substrate distribution [140], a very simple procedure allows the joint evaluation of the kinetic parameters and the substrate partition constant by carrying out measurements of the rate of the process (at fixed enzyme concentration and W values) as a function of the substrate concentration at a set of different surfactant concentrations [17]. The procedure has been first applied to the hydrolysis of 2-naphthyl acetate catalyzed by lipase in reverse micellar solutions formed by AOT/buffer/heptane, and thereafter extended to other systems [24,28]. A brief description of the method is given below.

For the joint evaluation of k_{cat}, K_M (in terms of the substrate concentration in the external solvent), and the substrate partitioning it is only necessary to measure the initial rate of the process (v_0) as a function of the analytical concentration of the substrate $[S]_{analyt}$, for a set of different surfactant concentrations (at fixed [E] and W_0 values). Representative results obtained for the hydrolysis of 2-NA catalyzed by lipase in AOT/water/heptane RM at $W_0 = 10$ are shown in Fig. 1. It is seen that the v_0/[Lip] *vs* $[2\text{-NA}]_{analyt}$ profiles are dependent on the AOT concentration. This dependence could be due to:

(a) progressive inactivation of the enzyme by the surfactant, leading to lower k_{cat} and/or higher K_M values as AOT concentration increases, or
(b) Partitioning of 2-NA, leading to a lower effective (local) 2-NA concentration at the zone in which the reaction is taking place (presumably the micellar interface). This process would only increase K_M.

Assuming that a Michaelis–Menten mechanism applies, the possibilities (a) and (b) can be differentiated by analyzing the response of the data to the Lineweaver–Burk plot, Eq. (4),

$$[\text{Lip}]/v_0 = 1/k_{cat} + (K_M/k_{cat})[2\text{-NA}]^{-1} \quad (4)$$

The data of Fig. 1 plotted according with Eq. (4) are shown in Fig. 2. It is observed that a unique intercept is obtained irrespective of the AOT concentration, arguing against an effect of the surfactant upon k_{cat}, as can be expected for enzymes totally incorporated to the micelles. This suggests that the dependence of the slope of the lines of Fig. 2 with AOT concentration is due to partitioning of the substrate (possibility (b)). The slope/intercept value obtained provides the "apparent" K_M

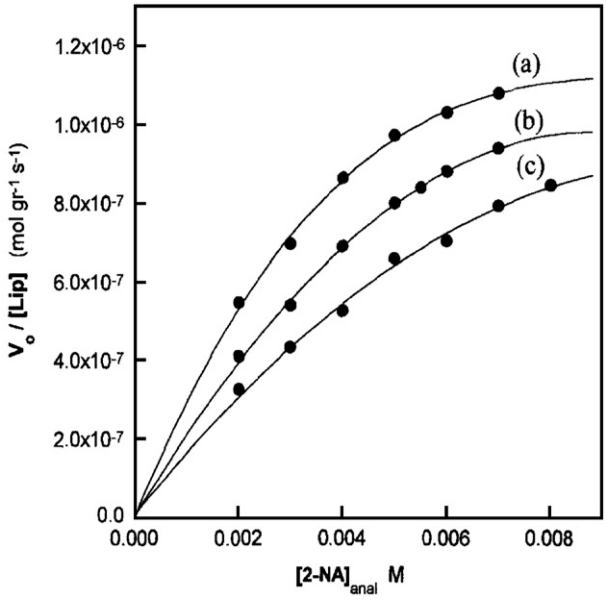


Fig. 1. Dependence of the relationship between the initial rate of reaction (per enzyme) and the analytical concentration of the substrate with AOT concentration, in RM at W_0=10. (a) [AOT]=0.025 M, $[Lip]_{anal}$=0.031 g/L; (b) [AOT]=0.2 M, $[Lip]_{anal}$=0.25 g/L; (c) [AOT]=0.4 M, $[Lip]_{anal}$=0.50 g/L.

value at each surfactant concentration. Extrapolation to zero surfactant provides the true K_M value in terms of the analytical substrate concentration. Furthermore, from the change in K_M with the surfactant concentration it can directly be derived the substrate partition constant [17].

In some systems it has been observed an increase in rate with the surfactant concentration. If changes in E distribution and size/shape of the micelles are disregarded, the increase in the rate can be explained in terms of the influence of diffusional and/or exchange processes whose rates could be enhanced by intermicellar collisions [141]. Furthermore, an increase in rate with the surfactant concentration can be expected for processes that can be inhibited by additives and/or the products.

3.2.5. Effect of the hydrofobicity of the substrate

Lipase-catalysed hydrolysis rates of several nitrophenyl alkanoate esters of varying alkyl chain length (C_4–C_{16}) have been measured [85] in aqueous solution, AOT RM and in w/o microemulsions. Reaction rates for C_4 and C_6 substrates are slower in RMs and microemulsions than in homogeneous solution, a result explained in terms of substrate partitioning to the oil-continues phase, which results in a reduced concentration in the aqueous pseudophase [85]. This enzyme can also catalyse the hydrolysis of longer chain alkanoates (up to C_{16}) in RM and w/o microemulsion. It was inferred from the kinetics that substrate partition to the interface, where the lipase must also be active, influences the enzymatic process.

The thermodynamic stability of the substrate in the external solvent influences the K_M values. This effect can be taken into consideration by measuring the partition constant of the substrate between the dispersion (organic) solvent and water. In other words, K_M values measured in aqueous solutions, $(K_M)_W$, can be compared with $(K_M)_{RM}/K_{S/W}$, where $K_{S/W}$ is the partition constant of the substrate between the bulk organic solvent and water. This correction accounts for the large difference in K_M values measured for lipase VII from *Candida Rugosa* in CTAB/water/pentanol/hexane RM and in bulk aqueous solution [141].

Avramiotis et al. [44] have measured the rate of lauric acid esterification by C_3 to C_7 alkanols catalyzed by *P. Cepacia* lipase. Results obtained in lecithin/isooctane and AOT/isooctane microemulsions indicate that the enzyme shows a preference for propanol in both systems. However, the dependence with the alcohol chain length is different. It is concluded that the enzyme behavior is affected by partitioning of the sustrate betweem the dispersed aqueous pseudophase and the organic solvent. This distribution depends upon the alkanol chain length [140].

Kinetic studies of polyphenol oxidase [92] acting on several phenols and catechols in AOT RM have shown that K_M increases, and the catalytic efficiency of the enzyme decreases, as the hydrophobicity of the substrate increases. This type of results also suggests the relevance of the substrate partition among the solvent, the inner core and the micellar interface.

The influence of the electronic and hydrophobic properties of sustrates on kinetics parameters has been tested in the oxidation of substituted benzaldehydes, catalysed by xanthine oxidase, in different RM [45]. Differences observed were explained in terms of differences in sustrate partitioning. Similarly, oxidation of phenols by tyrosinase readily takes place in reverse micelles, being the rate of the process determined by the substrate partitioning [92]. Rodakiewicz-Nowak et al. [95] assumed that the process takes place only in the water pool and applied a three pseudophase model to the substrate (4-t-butylcathechol) distribution.

Khmelnitsky et al. [142] used a three pseudophase model of substrate distribution between the organic phase, the water pool,

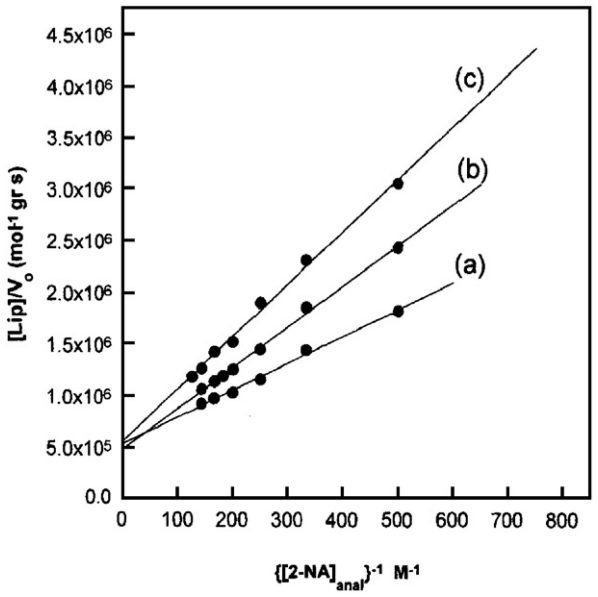


Fig. 2. Dependence of the Lineweaver-Burk plot with AOT concentration for the lipase catalyzed hydrolisis of 2-NA in RM, at W=10. [Lip]/[AOT]=1.25. (a) [AOT]=0.025 M, (b) [AOT]=0.2 M, (c) [AOT]=0.4 M.

and the interfacial layer to describe the activity of horse liver alcohol dehydrogenase in AOT in octane RM. Assuming that the enzyme resides exclusively in the aqueous phase, they obtained a relationship between the apparent Michaelis constant of the substrate and the volume fraction for all considered microphases. Using the experimental values of the partition coefficient for the series of short chain alkanols, they were able to explain the shift in the substrate specificity in reverse micelles, respect to the aqueous system.

Lissi and Abuin [19] have proposed and alternative approach based only in a two pseudophase model that does not require assigning values to the volume fraction of the microphases or to assume a given intramicellar distribution of the substrate. It is proposed that any comparison must be carried out employing the activity of the substrate in bulk water solution as a thermodynamic concentration scale. The kinetic results obtained in RM employing the analytical substrate concentration must be corrected by the solute distribution between the micellar pseudophase and the external solvent, and by the partitioning of the substrate between the external solvent and an aqueous solution. Corrected values of the catalytic efficiency can be obtained employing Eq. (5)

$$(k_{cat}/K_M)_{cor} = \left\{\left(k_{cat}/(K_M)_{exp}\right)\left(1 + K_p[\text{Surf}]\right)\right\}K_{w/s} \quad (5)$$

where K_P is the partition constant of the substrate between the organic solvent and the micelles, and $K_{w/s}$ is the partition constant of the substrate (S) between the organic solvent and an aqueous solution ($K_{w/s}=[S]_{org}/[S]_{water}$). This last factor takes into account that, if the substrate is highly stabilized in the organic solvent ($K_{w/s}$ large) it can be expected a high value of K_M associated to its low chemical potential. After applying these corrections to the data obtained in the oxidation of aliphatic alkanols by alcohol dehydrogenase in RM [86,143], both the dependence of k_{cat}/K_M values on the length of the alkyl chain and their absolute values are very similar to those measured in bulk water.

3.2.6. Determination of the substrate partitioning from enzyme catalyzed reaction rate measurements

Partitioning of the substrate between the micelles and the organic solvent can be treated within the framework of a two pseudophase model [140]. According with this model the partitioning of the substrate, S, is expressed in terms of a pseudo partition constant K_p defined by,

$$K_p = [S]_{mic}/\left\{[S]_{org}[D]\right\} \quad (6)$$

where $[S]_{mic}$ and $[S]_{org}$ are the analytical concentrations of the substrate incorporated to the micelles and remaining in the organic solvent, respectively, and $[D]$ is the concentration of micellized surfactant. Since $[S]_{mic}/[D]$ is the average number of substrate molecules n incorporated to the micelles per surfactant, K_p can be also expressed as

$$K_p = n/[S]_{org} \quad (7)$$

The value of K_p can be independently [85] determined by curve fitting of the kinetic data to multiphasic models [15] or by measuring the kinetic of the process at different surfactant concentrations. Aguilar et al. [17] proposed a method that, by this last procedure, allows a joint evaluation of substrate partitioning and kinetic parameters in a simple two-pseudophase model, for reactions catalyzed by enzymes entrapped in RM, irrespective of the involved kinetic law. The method was applied to the hydrolysis of 2-NA catalyzed by lipase in AOT/buffer/heptane RM. In the presence of RM, the relationship between the initial rate and the analytical concentration of 2-NA was dependent on AOT concentration at a constant W_0 value The dependence of the initial reaction rate profiles with [AOT] was analyzed according with the method proposed to obtain the partition constant of 2-NA between the micelles and the external solvent, K_p. A value of $K_p=2.7$ L mol^{-1} was obtained irrespective of the water content of the RM (W_0 from 5 to 20). However, this value decreases to 0.33 M^{-1} when 2 M urea is added to the water employed in RM preparation ($W_0=10$). This result would suggest a significant incorporation of urea to the micellar interface, decreasing so substrate incorporation. This competition could contribute to the inhibitory effect of urea on the catalytic activity of the enzyme in the RM [28,17].

In 2-NA catalyzed hydrolysis by lipase, the catalytic rate constant k_{cat} in the micellar solution was independent of [AOT] but slightly decreased with an increase in W_0 from 2×10^{-6} mol g^{-1} s^{-1} at $W_0=5$ to 1.2×10^{-6} mol g^{-1} s^{-1} at $W_0=20$. The apparent Michaelis constant, determined in terms of the analytic concentration of 2-NA, increased with [AOT] (at a fixed W_0), and with W_0 (at a fixed [AOT]). The increase with [AOT] is accounted for by considering the partitioning of the substrate. After correction for the partitioning of 2-NA values of $(K_M)_{corr}$ were obtained as 3.9×10^{-3} mol L^{-1} ($W_0=5$), 4.6×10^{-3} mol L^{-1} ($W_0=10$), 2.3×10^{-3} mol L^{-1} ($W_0=15$), and 1.7×10^{-3} mol L^{-1} ($W_0=20$). The rate parameters in the aqueous phase in the absence of RM, were obtained as $(k_{cat})_{aq}=7.9\times10^{-6}$ mol g^{-1} s^{-1} and $(K_M)_{aq}=2.5\times10^{-3}$ mol L^{-1}. In order to compare the efficiency of the enzyme in the RM with that in aqueous phase, values of $(K_m)_{corr}$ were in turn corrected to take into account differences in the substrate activity, obtaining so a set of $(K_M)^*_{corr}$ values. The catalytic efficiency of the enzyme in the RM, defined as the ratio, $k_{cat}/(K_M)^*_{corr}$ was found to be higher than in the aqueous phase, even at high water contents ($W_0=20$). This higher efficiency is due to a significant decrease in $(K_M)^*_{corr}$ values.

3.2.7. Effect of water content

The effect of water content of the reverse micellar system, W_0, upon the enzyme activity is one of the aspects that has been most extensively studied in recent years. It has been found that changing W_0 value from the minimal required to obtain a stable system to higher values leads to three different activity profiles, namely, (i) saturation curves, that have been interpreted in terms of the need of the enzyme for free water in order to reach the maximal activity; (ii) bell shaped curves, usually with an optimum activity taking place at W_0 values between 5 and 15, and (iii) curves where the enzyme activity decreases when the water content increases.

For most of the enzymes a "bell shaped" dependence of activity on W_0 has been observed [23,54,138,144–147] and the optimal values of W_0 appears as linearly correlated with the size of the protein. To explain these facts, it has been proposed that the optimum activity takes place around a value of W_0 at which the size of the reverse micelle is similar to the size of the enzyme. For example, for a hydrogenase from *Desulfovibrio gigas* bacterium [145] in AOT RM, the maximum catalytic activity (k_{cat}) was found at $W_0=18$, condition in which the micelle size theoretically fits the size of the heterodimer enzyme. However, the discussion is open in the literature as to whether the W_0 dependence of the enzyme activity is related or not to the size of the enzyme.

There are reports on enzymatic activity varying monotonously with the radius of micellar aggregates, beyond the predicted optimal values of W_0 [148,103]. A representative example of the lack of a relationship between the size of the enzyme and the optimal W_0 value is given by the catalytic activity of lipases [21] from *Penicillium simplicissimum, Rhizopus delemar, Rhizopus arrhizus and Pseudomonas cepacia.* These enzymes have molecular weights of 56,000, 43,000, 36,000 and 33,000 Da, respectively. In AOT-based reverse micellar solutions, the optimal value of W_0 for the three lipases with lower sizes are higher than the optimum W_0 value for the lipase from *Penicillium simplicissimum*. This effect has been explained in terms of the solubilization site of the different lipases within the reverse micelles [12]. Lipases work at oil/water interfases and since microemulsions and RM present enormous interfacial area, all the enzymes are activated and located at the interface [149]. For this reason, the enzymatic activity are less influenced by W_0 and consequently by the size of the water pool [85,150].

A bound water model has been developed [151] for the interpretation of kinetics data of β-galactosidase (*E. Coli)* in RM and the kinetic parameter of *p*-nitrophenyl-β-D-galactopyranoside hydrolysis reveals that the major effect on the hydrolitic rate is the amount of free water and not structural changes in the enzyme. Also, for the same enzyme in AOT RM and *o*-nitrophenyl-β-D-galactopyranoside as substrate, a kinetic model proposes that the process occur in two steps. It is assumed that the second step involves the presence of free water considered as the total water minus the "bounded water" which does not take part in the biocatalytic reaction, but it is needed to maintain the structure of the RM [152].

The catalytic rate constant of trypsin (a hydrophilic enzyme) compartmentalised within the water-pool of a CTAB/isooctane/*n*-hexanol/water microemulsion has been observed [18] to increases by about 4-fold (from 3.91 s^{-1} to18.1 s^{-1}) as W_0 increases from 12 to 44. This has been interpreted as due to changes in the local concentration of water and bromide counterions at the locus of the enzyme localization. On the other hand, the lipase catalyzed hydrolysis of *p*-nitrophenylcaproate in the same microemulsion was found [150] to be essentially unchanged across a wide range of W_0 values, from 12 to 44. It was suggested that the interfacial regions of cationic RM are densely populated by counterions and cosurfactant head groups and that their interfacial concentrations remain essentially unchanged with W_0 in the presence of catalytic amount of interfacially solubilized lipase. This unchanged interfacial composition in cationic RM seems to be the origin of the unchanged catalytic activities of surface bound enzymes. It was then proposed [18,150] that the local molar concentrations of the various species present inside the water-pool, as well the local activities of water at the site of reaction, need to be taken in consideration in explaining the observed "activity versus W_0" profiles in reversed micellar enzymology. For example, in the triolein hydrolysis catalyzed by wheat germ lipase in AOT/cyclohexane/water [96], the profile of enzyme activity *vs.* size of the water pool was found to be dependent on which enzyme concentration: i) the overall concentration in the RM or ii) the concentration in the water pool, was kept constant thus, when the overall concentration was constant, the profile showed a bell-shaped curve. It was also found that the optimum value of $W_0=9$ is not only for maximum enzyme activity but also for the stability of this lipase in the RM [224].

The proteolitic activity of lysine-*p*-nitroanilide was studied [146] in RM of lecihin/1-propanol/iooctane/water and it was found that the enzyme activity followed a bell-shaped pattern with a maximun at $W_0=20$. The ratio k_{cat}/K_M in the RM was higher than that observed in aqueous solution.

Oxidative degradation of phenolic environmental pollutants in organic media was investigated [8] using a laccase complexed with surfactants. The catalytic activity of these complexes (surfactant: non-ionic dioleyl *N*-D-glucono-L-glutamate) in isooctane was markedly enhanced by appropriately adjusting the water content of the reaction medium using AOT RM. Also, in the presence of AOT RM, the peroxidase activity of a surfactant-manganese peroxidase complex in toluene was increased in 10-fold by controlling the water content in the reaction system [122], with the highest catalytic activity at $W_0=5$.

The catalytic constant of prostatic acid phosphatase (PAP) entrapped in AOT RM shows a bell shaped dependence with an optimal value that is pH dependent: $W_0=10$ at pH 5.6 and $W_0=23$ at pH 3.8. This result was interpreted as due to optimal hosting of the monomer and dimer [69].

3.2.8. Effect of pH

The presence of the micelles can modify the optimum pH of the process, the range of pH in which the enzyme is stable, and/or the magnitude of the pH dependence. pH definition is not straightforward in RM. Usually, the referred pH corresponds to that of the bulk water solution added to RM aggregates. Local intramicellar pH would depend upon the charge of the surfactant head groups [153]. Enhanced proton concentrations (lower pHs) can be expected near the interface for negatively charged surfactants, while the opposite will take place if the surfactant bears a positively charged head. In agreement with these considerations, lower intramicellar pHs have been reported in reverse micelles formed by AOT [21,154,155]. This effect would shift the optimum pH to higher values, an effect observed in several systems, for example, lacasse/environmental relevant phenols [120] and α-CT/GPNA [84].

The pH-dependence of the hydrolytic activity of trypsin has been studied [125] in cationic CTAB RM in (50% v/v) chloroform/isooctane using a positively charged substrate. The pH was varied from 4.0 to 8.5 with citrate-phosphate buffer and it was found that the optimum pH, for maximum enzyme activity is similar to that observed in bulk aqueous solution, 8.0–8.5. However, the changes observed in the catalityc rate constant (k_{cat}) with W_0, are found to be pH dependent. At low pH (4.0) and low water content ($W_0=5$), the enzyme is more active in RM than in aqueous solution by a factor of 2.

Results from log k_{cat} versus pH plot, for octopus glutatione transferase in RM of AOT, have suggested [67] that amino acid residues with pK(a) values of 6.56 ± 0.07 and 8.81 ± 0.17 should be deprotonated to give optimum catalytic function. It was proposed that the pK(a_1) 6.56 is that of a group that, in the bound enzyme, is 1.40–1.54 pH units lower than in the free enzyme. The observed pK(a_2) 8.81 in RM had been assigned to Tyr(7) of the octopus glutatione transferase, being 0.88 pH units lower than that in aqueous solution.

The effect of pH on the activity of α-CT using GPNA as substrate was studied by Barbaric and Luisi [84] at different W_0 values. These authors analysed the results in terms of the "local pH", called pH_{wp}.The observed effect was to shift the optimum pH (which for GPNA in aqueus solution is 7.8) towards more alkaline pH values, depending on W_0. The smaller the W_0, the higher was the optimum pH. Also, the optimum pH in the micellar solution changed more markedly in the region of low W_0 values and tend to level off at higher W_0's. One explanation of this observation is that the water content in the micelles affects the physical state of the enzyme, bringing about an increase of the pK of certain groups in the active site with more importance at lower water contents. This explanation is in line with results found by Menger and Yamada [83] for the α-CT catalyzed hydrolysis of *N*-acetyl-L-tryptophan methyl ester in reverse micelles. They found a marked shift of the optimum pH for the enzyme turnover number. The interpretation of the changed pH profile was that the enzyme's activity arises from a shift of the pK of the hystidine group located in the active site of the enzyme.

The hydrolysis of triolein catalyzed by *Thermomyces lanuginosa* lipase was studied as a function of pH in the range 4.0–10.0 in AOT/isooctane reverse micellar solutions [157].The behaviour of the enzyme was different from that reported for aqueous media (30 °C, tributyrin as substrate) where it maintained high activity from pH 7.0 to 11.0. In the micellar system, using triolein as the substrate, it was observed a much sharper activity peak, with a maximum at pH 8.0. This different stability was associated to the lower intramicellar water activity, a factor that would reduce its capacity to minimize pH dependent conformational changes. This effect should not be general since in other systems, such as yeast alcohol deghydrogenase [87] or lipase [85] entrapped in AOT RM pH profiles in aqueous solutions and in the micellar solutions are very similar.

The pH of maximum activity depends upon the surfactant properties. This difference can be due to differences in intramicellar pHs and/or to several other factors. This is stressed by data obtained on the enzymatic hydrolysis of cicrocrystalline cellulose. In this system, the optimum pH is 6.0 en Triton X-100 RM and 6.5 in CTAB RM [156]. This shift is the opposite to that expected from simple intramicellar pHs considerations.

3.2.9. Effect of temperature

The effect of temperature in the catalytic activity of enzymes in reverse micelles is complex, due to the number of factors that condition the rate of the process. Usually it is observed a bell shape behaviour, with an increase in rate with temperature at low temperatures and the opposite trend at higher temperatures, attributable to enzyme denaturation. Arrehnius type plots in the low temperature range usually renders activation energies between 20 and 100 kJ mol^{-1}. For example, for fungal lipases in AOT RM systems the values range from 29.3 to 82.2 kJ mol^{-1} [157].

The optimum temperature for the oxidation of 4-methylcatechol to 4-methylquinone by polyphenol oxidase, was observed [158] to shift from 30 °C in buffer to 45 °C in RM of AOT/cyclohexane, a result that could be due to a higher enzyme stability in the RM. Furthermore, the small difference in the activation energies in both media indicates that there is not a significant extra energy barrier for the catalysis in RM. On the other hand, below 30 °C the activation energy of yeast alcohol deghydrogenase entrapped in AOT reverse micelles is considerably higher than in aqueous solution [87].

Chen et al. [156] studied the effect of temperature (in the range 30 °C–60 °C) on the activity of cellulase in three types of reverse micelles, using microcrystalline cellulose as substrate. It was found that, for Triton X100 ($W_0=1.0$) and AOT ($W_0=7.4$), at pH=6.0, the enzymatic hydrolysis of cellulose reached a maximum at 50 °C with reaction rates 35-fold (for Triton × 100) and 5 fold (for AOT) larger than in buffer. In CTAB reverse micelles ($W_0=16.7$) the maximum reaction rate was found at 45 °C (pH=6.5) with an activity 7-fold parger than in aqueous solution in the absence of the surfactant. These results emphasize the importance of the experimental conditions on the temperature effect observed in reverse micellar catalysis.

In spite of the increase in optimum reaction temperature, polyphenol oxidase losses stability when is pre-incubated in the reverse micelles in the absence of substrate, regardless of the temperature, although the effect is more pronounced at higher temperatures [158]. The thermostability was higher when was injected in buffer containing reverse micelles than when injected in empty micelles. Moreover, the thermostability is strongly dependent on the size of the micelles, the bigger the micelle the greater the stability. The inactivation of the enzyme promoted by temperature takes place with a first order kinetics, characteristic of conformational changes. The stability of the protein increases in presence of ligands. So, *p*-Nitrophenol, a competitive inhibitor, and acetyl tyrosine ethyl ester, an alternate substrate, increase the half-life of polyphenol oxidase by a factor 2.5 and 4, respectively. The authors propose that this increased stability may allow the use of the enzyme at higher temperatures [158].

An increased stability in reverse micelles is not a general phenomena. The effect of temperature on the activity of yeast

alcohol dehydrogenase has been measured in water/AOT/isooctane reverse micelles at $W_0=28$ and pH=8.1 [87]. In this system, denaturation is evidenced near 30 °C, both in aqueous solution and in the reverse micelles.

The hydrolysis of triolein catalyzed by *Thermomyces lanuginose* lipase in AOT/isooctane/water has been evaluated in the 23 to 58 °C temperature range. Maximum activity was observed at 37 °C, a behavior typical for fungal lipases in RM [96,157]. At higher temperatures the interface is more fluid and disordered, which could favour inactivation, rendering enzymes with low or negligible activity. The same behaviour was obtained for the hydrolysis of several nitrophenylalkanoate esters catalyzed by *Chromobacterium viscosum lipase* [85].

The catalytic activity of laccase in AOT/isooctane reverse micelles has been also studied as a function of temperature in the range 40 °C–75 °C, using *o*-chlorophenol as substrate [120]. The micelles decrease the temperature of highest catalytic activity (from 70 °C in aqueous solution to 60 °C). Nevertheless, these results have to be considered with care since at temperatures higher than ca. 65 °C the reverse micellar solution separates into two phases.

3.2.10. Effect of the external solvent

Changes in the organic (external) solvent can produce dramatic changes in the properties of the RM, such as the micropolarity of the interface [159,160], the maximum W_0 [160], etc. The external solvent can then modify k_{cat} and K_M, even for enzymes totally incorporated to the micellar interfase or water pools. Furthermore, changes in the external solvent can modify the partitioning of the substrate between the solvent and the micelles, changing so K_M values, particularly when analytical concentrations are employed. In spite of this, very few works have been devoted to describe the influence of the external medium upon the intramicelar catalysis in these systems.

There are very few systematic studies regarding the effect of the external solvent upon the activity of enzymes incorporated to reverse micelles. Naoe et al. [161] have measured the rate of triolein hydrolysis catalyzed by *Rhizopus delemar* lipase in AOT reverse micellar systems formulated in straight-chain alkanes (from C_6 to C_{10}), isooctane and cyclohexane. The initial rate of the process, at $W_0=15$ and fixed substrate concentration, increase by a factor near to six when cyclohexane is changed to isooctane. This difference is due both to changes in k_{cat} and K_M, whose values are influenced by the size and shape of the external solvent molecule. However, these changes affect the rate of the process in a way that is extremely dependent upon the enzyme and substrate considered. In fact, while in triolein hydrolysis catalyzed by *Rhizopus delemar* lipase in AOT the initial rate of the process follows the order: Isooctane > *n*-hexane > cyclohexane; the opposite tendency is observed in the lipase catalysed hydrolysis of *p*-nitro phenyl acetate (PNPA).

When the substrate is distributed among the external solvent and the micelles, and analytical concentrations are employed, changes in the thermodynamic stability of the substrate in the external solvent should be reflected in K_M values. This effect can be taken into account by multiplying the apparent K_M value by the partition constant of the substrate between the external solvent and a reference solvent, such as water [19]. This amounts to express the rate of the process in terms of the concentration of the solute in the reference (water) solvent. We have applied this correction to data obtained in the lipase catalyzed hydrolysis of PNPA in AOT RM prepared in hydrocarbons, aromatic solvents and chlorinated compounds. In order to be able to use several external solvents, experiments were carried out at $W_0=6$. Some of the data obtained [162] are collected in Table 4. These data show that there are significant differences between the rough data and that corrected by the substrate stability in the external solvent. For example, enzyme activity appears as higher in cyclohexane than in cumene, but this result is totally due to the higher chemical potential of the substrate in the former solvent. In fact, after taking into account this difference, the catalytic efficiency is considerably higher in the aromatic solvent.

The fact that k_{cat}, $K_M.K_r$, and V_0/K_r values depend upon the external solvent implies that the enzyme and/or the enzyme/substrate adduct in the water pool (or the water/surfactant interface) are influenced by the external solvent. This is in agreement with noticeable differences observed in the kinetics of the fluorescence decay of tryptophan moieties of a lipase incorporated to the micelles. In particular, the contribution of the long-lived component is considerably higher (c.a. 40%) in aliphatic than in aromatic solvents (c.a. 15%), implying significant differences in the protein conformation [162].

One interesting type of external solvent is represented by supercritical fluids and highly compressed gases. The pioneering works by Zaks and Klibanov [163], Hammond [164] and Randolph [165] have shown that enzymes remain actives in supercritical fluids. Several reviews on the performance of enzymes in these fluids have been published [166–168]. Supercritical fluids have properties that can be tuned by changing temperature and/or pressure. Moreover, supercritical carbon dioxide ($scCO_2$) is regarded as a 'green' solvent, which makes it a promising alternative for traditional solvents used in biocatalysis. In fact, it is the most frequently used do to the fact that its critical point of 73.8 bar and 31.1 °C makes equipment design and reaction set-up relatively simple [169]. Supercritical fluids, which represent a state between the gaseous and liquid phases of the compound, exhibit properties similar to those of hydrophobic solvents such as hexane, so it is likely that the activities and stabilities of enzymes in these systems will be similar to those presented in hydrophobic solvents. Although the use of supercritical fluids is not restricted to hydrolases, studies with this class of enzymes, especially lipases, dominates in the literature [170–180].

The number of works in which it has been measured enzymatic activity in RM formed in compressed or supercritical solvents is scarce. Holmes et al. [181] measured the lipase-catalyzed hydrolysis of *p*-nitrophenol butyrate and lipogenase-catalyzed peroxidation of linoleic acid in a water in CO_2 microemulsion stabilized by a fluorinated dichained sulfosuccinate surfactant. The activity of the enzymes was equivalent to that in water-in-heptane microemulsions stabilized by AOT. Hakoda et al. [182] studied the activity of a lipase in supercritical ethane. Micelles of diameter between 4 and 10 μM were readily formed at W_0 ratios below six. Triolein conversion increased

Table 4
Kinetic and thermodynamic parameters associated to PNPA hydrolysis catalysed by lipase in AOT reverse micelles, W_0=6, phosphate buffer pH 7.0

External solvent	$V_0 \times 10^6$ M s^{-1}	$K_r \times 10^2$	$k_{cat} \times 10^5$ Mol $g^{-1}s^{-1}$	$K_M \times 10^3$ M	$V_0/_r 10^{-4}$ M s^{-1}	K_M K_r 10^{-3} M
Benzene	3.5	0.41	–	–	8.5	–
Toluene	5.8	0.54	–	–	11	–
Cumene	4.6	1.0	–	–	4.6	–
Tetrachloroethylene	19	2.0	1.2	50	9.4	1.0
Cyclohexane	32	12	0.6	10	2.7	1.2
Isooctane	6.8	19	0.34	38	3.6	7.2
n-Hexane	9.3	14	0.36	24	6.6	3.4

with the increase in size of the RM and CO_2 pressure, reaching a maximum rate near critical conditions. Mishima et al. [183] prepared surfactant-coated α-CT complexes. These complexes, in presence of surfactants formed, in supercritical and liquid CO_2 RM-like structures able to incorporate significant amounts of water. The enzyme activity in these structures, employing AOT as surfactant, was higher than that observed in ethyl acetate.

The presence of high pressures of CO_2 can modify the catalytic activity of enzymes incorporated to conventional RM. Chen et al. [184] have evaluated the effect of compressed CO_2 on the chloroperoxidase catalyzed halogenation of 1,3-dihydroxybenzene in RM (CTAC-water-octane-pentanol). The results show that enzyme activity can be enhanced significantly, and that it can be tuned continuously by changing the pressure. This enhancement was related to the changes in sample viscosity elicited by the incorporation of CO_2 to the micellar solution.

3.2.11. Effect of additives

Co-surfactants have been added to improve the stability of RM. Their effects on microencapsulated enzymes are variable, as some may be detrimental for the activity/stability while others enhance the enzymatic performance and the retention of activity [21]. Furthermore, other additives such as urea or salts, can also change the activity of the enzyme.

Addition of a water-miscible organic cosolvent solubilized in the inner cavity of RM, such as glycerol, leads to a pronounced increase of α-CT stability at all tested Wo values [23]. The dependence on the enzyme residual enzyme activity on W_0, in presence of 20 and 30% (v/v) glycerol, had the same bell shaped form as in the absence of co-solvent. It was considered that the addition of glycerol results in a decrease of conformational mobility of the enzyme as a whole due to the micellar matrix becoming more "rigid".

For *Chromobacterium viscosum* lipase in AOT/isooctane RM it has been found [22,185] that their activity and stability is increased in the presence of low molecular weight polyethylene glycol (PEG400). PEG 400 molecules are highly polar and poorly soluble in organic solvents, remaining in the water pool. They can then modify both the properies of the pool and the water/surfactant interface [185]. By increasing PEG concentration, the lipase activity increases to a maximum value and then began to decrease. The maximum value in AOT/PEG reverse micelles was about 200% [22] higher than that in unmodified AOT RM, an effect attributed to the decreased mobility. This reduces fluctuations of enzyme structure that can affect the catalytic activity. FTIR analysis suggests that PEG 400 strongly interact with SO_3^- head groups of AOT molecules, leading to a redistribution of water molecules inside the micelles. This effect would increase the activity and stability of the lipase [185]. Similarly, cutinase stability in AOT RM increases in the presence of 1-hexanol, which acts as a co-surfactant that delays, or even prevents, unfolding of the enzyme [186].

The effect of urea, a well-known protein denaturant, on the properties of reverse micelles has been extensively investigated [187,188]. Urea can affect the enzyme structure by a direct interaction with the macromolecule or by an indirect action through effects on the structure and properties of the water pool and the micellar interface, or by a combination of these effects [189,190]. The effect of urea has been studied [28] in the hydrolysis of 2-NA catalysed by lipase in AOT-heptane-water reverse micelles. As previously mentioned, urea interacts with the inner micellar interface altering the distribution of the sustrate and the protein-interface interaction. Addition of urea to AOT RM provokes a decrease in the activity of lipase, from *Rhizopus arrhizus,* incorporated to the micelles, although the enzyme is more resitant to urea denaturation in the micellar assamply than in bulk water solution. At intermediate urea concentrations (2 M in the added water) the decrease in activity totally results from an increase in K_M value [28].

The effect of salt on enzyme activity has been study [191] and discussed [192] for halopihilic extremozyme entrapped in the microaggregates of a CTAB/1-butanol/cyclohexane microemulsion. The enzymatic catalysis, at high salt concentration (0.85 M NaCl in the added water), follows a Michaelis–Menten kinetics, but K_M and V_{max} values depend on the sample preparation method. However, the extremozyme showed the same dependence on the buffer ionic strength in w/o microemulsion as in aqueous medium. The dependence of the maximum reaction rate (V_{max}) on W_0 showed a bell-shaped curve in presence of NaCl or KCl.

The influence of NaCl concentration on W_0-activity profile of halophylic malate dehydrogenase (hMDH) was studied [193] in CTAB/cyclohexane microemulsion, with 1-butanol as cosurfactant. The W_0-activity profiles, at 1.0 and 0.5 M NaCl in phosphate buffer, were similar and twice more active than without salt added at every W_0. Nevertheless, at low salt (0.05 M) showed a very steep increase reaching a plateau at $W_0 \sim 13$. The enzymatic activity at W_0 higher than 10, followed the order: ([NaCl]=) 0.05 M ≫ 0.5 M > 1 M. It was proposed that the presence of salt may have several effects: i) it can modify water properties, either favouring or limiting its dissolution by the micelles; ii) it can shield electrostatic interactions

of the charged surfactant molecules. This could change the natural curvature of the interface, changing the size (and hence the number) or RM.

The presence of salts can reduce the inner surface potential, affecting so the reactivy and/or stability of RM incorporated enzymes. It has been reported that inactivation of YADH in Brij-30 and mixed Brij-30/AOT reverse micelles can be reduced by bile salts [194] or by reducing the surface charge density by addition of cosurfactants and/or non-ionic surfactants [[62] *and references therein*]. Similarly, halophilic glucose dehydrogenase from Hf. Mediterranei shows [195] a good activity in presence of salts, and the enzyme is more stable, at low salt concentration, in reverse micelles than in aqueous solution.

The effect $NaNO_3$ and NaCl addition on the oxidation of β-D-glucose catalysed by glucose oxidase has been studied in AOT/decane RMs [196]. Their effects on the catalytic rate constant (k_{cat}) and Michaelis constant (K_M) allowed to conclude that both additives act as non-competitive inhibitors.

Modification of AOT RM by alkyl glucosides or nonionic surfactants (Spans, Tweens and Tritons) was attempted [197] to improve the activity of lipase using *p*-nitrophenyl alkanoates as sustrates. Spans solubilize into the micellar water pool, while Tweens, Tritons and alkyl glucosides solubilize at the interface of AOT, forming mixed micelles. Addition of Tweens and Tritons, both bearing poly-(oxyethylene) chains, decreased the hydrophobicity of the systems and significantly improved lipase activity. Tween 85, which forms a hybrid reverse micellar system with AOT in isooctane, has been used [63] to avoid denaturation of putidaredoxin reductase.

DNA polymerases are particularly active in nonionic microenvironment in w/o microemulsions, although they are very sensitive to the ionic strength, particularly at low water content. [198]. The increased activity of DNA polymerase at low water content was explained in terms of an improvement of the protein dynamics in low polarity environments [199]. The activity of the enzyme can also be modulated by changing the hydrophilic-lipophilic balance of the surfactants. For example, it has been found [199] that DNA polymerase has a higher activity in a system composed by Triton X-114, SDS, CTAB and Brij 58 (concentrations of 128, 25, 15 and 10 mM, respectively) in hexanol-decane than in a system containing only Brij 58. The behaviour of the enzyme in the former mixture was further improved by introducing the lipophilic Brij30 and changing the organic solvent to hexanol-octane [200].

Carbohydrates and polyols affect the stability of a cationic peroxidase in AOT RM [201]. The effect observed strongly depends upon the employed additive. For example, addition of arabinose and trehalose (20 mM) increase the enzymatic stability by factors of 4.4 and 2.3, respectively, while melezitose had not effect. From the three tested polyols, inositol and sorbitol increased the peroxidase stability by a factor of 3.8 and 1.8, respectively, while mannitol had no effect.

The presence of lecithin increases the rate of esterifications catalyzed by *Rhizopus delemar* lipase in AOT RM [202]. Similarly, addition of cholesterol to AOT/isooctane RM enhanced the catalytic activity of a peroxidase at various temperatures and pHs [203,10].

3.2.12. Effect of the internal solvent

The catalytic activity of enzymes in RM can be regulated by a partial dehydration of the inner cavities, acchieved by a progressive substitution of water by a water-miscible organic solvents [204,205]. The dependences of the catalytic activity of α-CT, peroxidase and laccase with W_0 in RM of AOT/octane/water-glycerol with different concentrations of glycerol (up to 94-vol.%, referred to the volume of water-glycerol mixtured used to solvate RM) has been studied [206]. Also, butanediol and dimethylsulfoxide were used instead of glycerol. Khmelnitsky et al. [206] described that the characteristic features of enzymatic catalysis in RM solvated by water-miscible organic solvents are: (i) a shift of the profile of the catalytic activity on W_0 towards lower values of W_0 and (ii) an increase in the catalytic activity observed under optimal conditions with increasing concentration of the water-miscible organic solvent.

The influence of glycerol (GY) on the hydrolysis of 2-NA catalysed by α-CT has been recently described [24]. In bulk solution and in AOT/heptane RMs, incorporation of GY notably increases the value of k_{cat}^{exp}. This is particularly so in RM, at GY-water 38%v/v and W_M (=[GY+H_2O]/[AOT])= 13.5, when the rate constant is nearly 35 times larger in presence of GY. The experimental and corrected kinetic parameters obtained are gathered in Table 5. Taking into account the corrections described in Section 3.2.6 and Ref. [19], comparing the catalytic efficiencies ($[k_{cat}^{exp}/(K_M)_{corr}]$) obtained in both reverse micellar systems, GY-water/AOT/*n*-heptane and water/AOT/*n*-heptane, it can be seen that the value in the former is 5 times higher. This fact can be explained by considering that the addition of GY results in a high microviscocity around the enzyme, as suggested by steady state fluorescence anisotropy values (see <*r*> values in Table 5). This reduces the conformational mobility of α-CT, leading to an increase of the enzyme stability and activity. An increment of the enzyme stability has been demostrated [23] to occur in presence polyols, which form a net of hydrogen bonds that fix the protein to a more rigid matrix.

On the other hand, the effect of aprotic solvents, ethylene glycol, acetone, formamide, sulfolane, DMSO, acetonitrile on the enzymatic activity of *C. viscosum* lipase in AOT RM, has been studied recently [207]. DMSO was found to be most effective of the solvents to enhance lipase activity (reduces K_M while V_{max} is not altered). DMSO molecules existed at the micellar interface and modified the micellar interface to reduce the surface charge density which creates a better environment for the enzyme.

4. Catalytic properties of enzymes in aqueous solutions of surfactants

The kinetic behavior of enzymes in reverse micellar solutions have been the subject of numerous studies, as has been discussed in the previous sections. Surfactants in aqueous solutions can also act as biomimetic systems, but, in spite of this, little work has been reported on the catalytic behaviour of enzymes in this type of systems.

One of the first studies on the effect of aqueous surfactants on enzyme activity appeared at the beginning of the 1970's. These

were reported by Jones et al. [208,209] who studied the interaction of pancreatic ribonuclease A with surfactants and its effect on the enzyme activity. They found that DTAB does not interact significantly with the enzyme while SDS binds to the protein, inducing conformational changes and denaturation of the enzyme. The main motivation of the first studies on enzymes properties in aqueous solutions was to gather information on the role of electrostatic interactions between the charged residues of the protein with the charge of the surfactant molecules in reverse micelles. It was found a correlation of the inhibiting effect of AOT on alcohol dehydrogenase and α-chymotrypsin in normal and reverse micelles [210], but afterwards other enzymes, such as lipase, catalase and horseradish peroxidase [211] showed different behaviours in the two systems. At present, it is known that the factors responsible for enzyme catalytic properties in the two systems are different and dependent not only on the nature of the surfactant type, but also on the physicochemical properties of the microenvironment in which the enzyme works. More detailed studies on the catalytic properties of enzymes in aqueous surfactant solutions were done in le last fifteen years. Recently, a brief account of the state of art on enzyme activity and stability control by surfactants in aqueous solutions has been reported by Savelli et al. [212]. A summary of representative systems investigated in the last fifteen years and the main findings is given in Table 6, and a more expanded discussion of some of them is given below.

Similarly to the case of reverse micellar solutions, the main aim of the kinetic studies in aqueous solution is to investigate the influence of the surfactant on the Michaelis constant K_M, the catalytic constant, k_{cat} and the inhibition constant k_I for those surfactants that act as enzyme inhibitors. Nevertheless, in aqueous solutions, the surfactant can exist as monomer (below the CMC) or under the form of micelles (above the CMC) and then, the effect promoted by the surfactant on the behaviour of the enzyme could be dependent on its form of organization. This aspect was barely considered in the first studies performed in aqueous solutions, which were indistinctly performed at surfactant concentrations either below or above the CMC. Furthermore, it must be considered that micelle-like aggregates can be formed on the macromolecule even at concentrations below the surfactant CMC.

Table 5
Summary of Kinetic parameters associated to 2-NA hydrolysis catalysed by α-CT in different media (homogeneous and RM of AOT) and steady state anisotropy (<r>) values of the enzyme

Medium	k_{cat}^{exp} (s^{-1})×10^2	(K_M^{exp}) (M)×10^3	((K_M)$_{corr}$) (M)×10^3	[k_c^{exp}/(K_M)$_{corr}$] (M^{-1} s^{-1})	<r>
Water	2.10	0.57[a]	0.16[a,*]	0.1[a]	0.108
GY-water (38%)	8.80	0.31[a]	0.05[a,*]	1.8[a]	0.120
water/AOT/*n*-hep	0.22	2.12[b]	2.1[b,**]	1.1[b]	0.113
GY-water/AOT/ *n*-hep	7.5	20.0[b]	15[b,**]	5[b]	0.161

[a]Bulk, [b]mic.
Corrections according to: (*), $((K_M)_{corr})^{bulk}=(K_M^{exp})^{bulk}/K_{polar\ solvent/hep}^{bulk}$, and (**) $((K_M)_{corr})^{Mic}=(K_M^{exp})^{Mic}/(1+K_p\,[AOT])$.
Data from Ref. [24].

As can be seen in Table 6, the most used enzyme is α-CT. The reason for this is that, it is a widely studied serine protease, its mechanism of action in aqueous media is well known [213], and its behaviour with different substrates in reverse micelles has been well documented [23,24,68,106]. We will then discuss with more detail the studies reported in Table 6 that were performed using this enzyme.

The study performed by Schomaecker et al. [210] with α-CT in the hydrolysis of GPNA was done at a single pH (8.2) for all the surfactants considered. They investigated the effect of the surfactants at concentrations below or very near the CMC's in two type of experiments: with samples freshly prepared and after a pre-incubation of the enzyme with the surfactant for different times up to 15 min. From these type of experiments they were able to differentiate between inhibiting and denaturing effects of the surfactant. It was found that AOT is a competitive inhibitor of α-CT in aqueous solution without affecting the enzyme stability. For hexaethyleneglycolmono-n-dodecylether, $C_{12}E_6$, and SDS, both inhibition and denaturation was found. CTAB does not inhibit the enzyme (in terms of an increased K_M), but it is an effective denaturing agent. However, the denaturation does not takes place in the presence of the substrate.

Spreti et al. [214] evaluated α-CT activity in solutions of cetyltrialkylammoniun bromides in the series trimethyl, (CTAB), triethyl (CTEAB), tripropyl (CTPAB), and tributyl (CTBAB) that differ in the head group size, and also with SDS and SB3-14. They used GPNA as substrate and results were obtained only above the CMC at pH=7.75, condition under which the enzime has a net positive charge (isoelectric point 8.8). They found that, in comparison with the value in TRIS–HCl buffer, the rate of GPNA hydrolysis was decresed in the presence of CTAB and CTEAB micelles, while was two fold higher and 5.9 times higher when using CTPAB and CTBAB, respectively. These results were interpreted in terms of a progressive increase in the micelle net charge [215], since the affinity of the micelles for counterions decreases with increasing the hydroiphobicity of the alkyl head group (the degrre of ionization of CTAB is ca. 0.2 and for CTBAB is ca. 0.5, [216]). In this work, the effect of the buffer type was also investigated with those surfactants that promoted superactivity. Th authors proposed that the surfactant and the buffer can interact differently and alter the pH in the vecinity of the reaction site. Accordingly, buffers with the same pK may have distinct interactions with the micelle interfaces, with hidrophylic buffers being more favourably partitioned towards the water phase than the hydrophobic ones. Both effects are expected to modulate the activity of the enzyme, by controlling the local pH and by changing the properties of the micelle interface. Buffers employed were: TRIS–HCl, HEPES and phosphate. In the presence of micelles, K_M values (evaluated in terms of the analytical concentration of GPNA) were found to increase by one order of magnitud in TRIS–HCL and phosphate, and three times in HEPES, respectively. The values of k_{cat} also increased, being 13.4, 12.9 and 32 times higher when the micelles were prepared in TRIS–HCl, HEPES and phopshate, respectively. These authors were the first to recognize that the results indicated a greater catalytic efficency

Table 6
Summary of some representative enzymatic reactions that have been studied in aqueous solutions of surfactants in the last fifteen years

Enzyme	Substrate	Surfactant	Observed effect	Ref
Several lipases; α-chymotrypsin (α-CT)	*p*-nitrophenyl-butyrate for lipases and *N*-Glutaryl-l-phenylalanine-*p*-nitroanilide (GPNA) for α-CT	SDS, CTAB, AOT	For lipases competitive inhibition was observed with SDS and CTAB and non-competitive inhibition with AOT. For α-CT, AOT is a competitive inhibitor but enzyme stability is unaffected. SDS, show both, inhibition and denatu-ration. CTAB does not inhibit the enzyme but is a denaturing agent.	[210]
Alcohol dehydrogenase	Ethanol	AOT, CTAB and alkyl sulfates and sulfonates	Short chain alkyl sulfona-tes and sulfates do not affect the activity below the CMC. Longer chain and branched alkyl sulfates and sulfonates decrease the activity above and below the CMC.	[212]
Beef liver catalase	H_2O_2	Several anionic, cationic and zwitterionic	All cationic and zwitterionic surfactants have no effect on the initial activity of catalase but several of them allow the enzyme to retain a high residual activity for longer periods of time than those observed in the absence of any additive.	[214]
Bovine lactoperoxidase	5,5′ -dithiobis (2-nitrobenzoic acid)-thiociana-te	SDS, Benzalconium chloride (Bz), chlorhexidine, Triton X-100	SDS provokes a loss of enzyme activity. The non-ionic do not affect it. Bz is efficient in preserving the enzyme activity for longer times than the native specie.	[224]
α-CT	GPNA	Cetyltrialkylammonium bromides with different head group size, SDS, myristyldimethylammo-nium propane sulfonate, (SB3-14)	Kinetics was measured at surfactant concentrations above the CMC. The head group size of the surfactant had a major weight. Enzyme superactivity was found to be dependent on the buffer type.	
α-CT	GPNA	CTAB; CTBAB	Theoretical models were developed for enzyme activity in the presence of micellar aggregates.	[34]
Cutinase	*p*-nitrophenyl-butyrate (p-NPB) and *p*-nitrophenylace-tate (p-NPA)	AOT ; CTAB	Both surfactants activate the hydrolysis of the esters.	[214]
α-CT	GPNA	CTAB, CTEAB, CTPAB, CTBAB.	Superactivity was observed at surfactant concentrations below and above the CMC.	[219]
α-CT	GPNA and *N*-succinyl-L-phenylalanine *p*-nitroanilide (SPNA)	CTAB, CTPAB, t-octylphenoxyl-poliethoxyethanol and polyoxyethylene-9-lauryl ether.	Superactivity occurs only in the presence of CTPAB. Reaction rates versus surfactant concentration were bell-shaped. The results weresimulated by a three pseudophase model.	[220]
Bacterial α-amylases	Amylase	SDS	The catalytic rates show sigmoidal kinetics increasing the surfactant concentration	[225]
α-CT	2-Naphthyl-acetate (2-NA)	DTAB	The presence of the surfactant, at concentrations above the CMC increases the value of K_M, without changes in the catalytic rate constant.	[221]
α-CT	*p*-Nitrophenyl-acetate (PNPA)	CTAB	Bell shaped profile of α-CT activity with increasing surfactant concentration was observed.	[14]
Lipase	*p*-Nitrophenyl oleate (PNPO)	PNPO-Triton X-100 mixed micelles	Enhancement of the rate of hydrolysis	[226]
α-CT	2-NA	DTAB	The effect of the surfactant upon the enzyme activity under the steady –state conditions for the acyl-enzyme intermediate is compared with the behaviour of the enzyme in the transient phase.	[222]
α-CT	GPNA	DTAB	Superactivity was observed at surfactant concentrations below and above the CMC, when the results are treated in terms of the analytical concentration of GPNA. In terms of the local concentration of the substrate the activity of the enzyme tend to remain constant above the CMC.	[223]

(k_{cat}/K_M) in the presence of the aggregates (due mostly to higher k_{cat}). Furthermore, these values must be only considered as apparent, sine they were evaluated in terms of the analytical concentration of the substrate. To take into account the effect of substrate partitionining on K_M they measured the partition constant of GPNA in CTBA micellar soutions under the experimental conditions that give α-CT superactivity. The values of the partition constants determined were strongly dependent on the buffer type, indicating the importance of the buffer role. After correction for free substrate concentration, k_{cat} values remaing unchanged while K_M dropped to values very close to those in buffer. It was then concluded that superactivity should be related to a catalytically more favourable conformation of the enzyme.

Viparelli et al. [217] have developed theoretical models to interpret the enzymatic activity in aqueous solutions of surfactants. They used a pseudo phase approach, similar to that previously developed for the description of enzyme kinetics in reverse micelles [118,218]. The model considers three pseudophases: free water, bound water and surfactant tails. The substrate concentration in each one of the pseudophases is related to its analytical concentration. When no association between the enzyme and the micelles is considered, the model predicts either a monotonically increasing or decreasing trend in the reaction rate as a function of surfactant concentration. Enzyme–icelle interactions are included by introducing an equilibrium between the enzyme residing in the free water and in the bound water pseudophases, and by allowing for diferrent catalytic behaviours for the two forms. Under these conditions the reaction rate can exhibit a bell-haped dependence with surfactant concentration. The model can be applied to surfactant systems that at the CMC give rise to enzyme efficiencies that are either higher or lower than in pure buffer solution. Experimental validation of the model was afforded by using α-CT activity in solutions of CTAB and CTBAB.

In the work by Alfani et al. [219], α-CT activity was tested with GPNA in buffered media using surfactants of the cetyltrialkylammonium bromide series. It was found that the hydrolysis of GPNA was promoted in the presence of the surfactants and that the extent of superactivity increased with the size of the head group. CTBAB provoked large improvements of the enzyme efficiency both below and above the CMC. However, this occurs below the CMC only when the buffer molaroty is low. The values of k_{cat}/K_m, both apparent and after data correction to take into account the partitioning of the substrate, were found to be higher in the presence of micelles. From reaction rate measurements performed at different pH and ionic strengths in TRIS–HCl buffer it was concluded that enzyme superactivity is dependent on protein-surfactant-buffer interactions of hydrophobic and electrostatic types.

Viparelli at al. [220] further validated the model previously proposed [34] to interpret enzyme kinetics in aqueous solutions of surfactants by modelling the results obtained for the rate of hydrolysis of GPNA and SPNA in CTAB, CPAB, t-octylphenoxyl-poliethoxyethanol, and polyoxyethylene-9-lauryl ether solutions. The dependence of the reaction rates with surfactant concentration was bell-shaped. The modulation of the results with the proposed model indicated that this behavior could be attributed to the equilibrium between the enzyme residing in the free water and in the bound water pseudophases, and to partitioning of the substrate in the pseudophases. However, it must be recognized that these models, that are only suitable for concentrations above the CMC, are conceptually different to those implicit in other studies where it is considered that an homogenous enzyme ensemble is modified by the surfactant, and that this modification is univocally determined by the surfactant concentration [194–196]. This approach has the advantage that, in principle, it can be applied over all the surfactant concentration range.

The relevance of the sensitivity of the enzyme behavior to the substrate and surfactant alkyl chain length was pointed out by Abuin et al. [221] in a study on the rate of hydrolysis of 2-NA catalyzed by α-CT in solutions of DTAB. The presence of the surfactant at concentrations above its CMC, increases the values of K_m without significant changes in the catalytic rate constant. The increase in K_m is larger than that expected from the incorporation of the substrate to the micelles. The effect was interpreted in terms of an interaction between the enzyme and the micellar aggregates which leads to an alteration of the formation of the enzyme substrate complex. This was related to a partial unfolding of the enzyme as suggested by the changes observed in its intrinsic fluorescence. These results are different than those reported for the hydrolysis of GPNA in presence of cetyltrialkylammonium bromides [34,219], were either superactivity below the CMC [219] and superactivity (CTAB) or loss of activity (CTMAB) was observed in the presence of micelles [34].

The effect of cationic (CTAB, cetyldimethylethyl ammonium bromide (CDMEAB) and CTPAB and nonionic (Triton X-100 and PO9) surfactants on the hydrolysis of PNA catalyzed by chymotrypsin iso-enzymes has been studied by Viparelli et al. [35] at surfactant concentration above the CMC. For all the iso-enzymes, superactivity was observed only in presence of CTPAB and CDMEAB. The extent of superactivity was found to be dependent on the enzyme used, following the order δ-CT > β-CT > γ-CT > α-CT. The catalytic behaviour of α-CT in he presence of CTAB micelles was also studied by Celej et al. [14] using PNPA as substrate. The kinetic results were similar to those obtained by Viparelli et al [35], ie., bell shaped profile of activity with increasing CTAB concentration. The novelty of the work by Celej et al. [14] resides in the fact that the authors complemented the kinetic results with measurements of the effect of the micelles upon the α-CT conformation. Changes in the tertiary structure were observed from the increase in intensity and red shift in the enzyme fluorescence spectrum, which were interpreted in terms of the annulment of internal quenching and a more polar environment of the tryptophan residues. Near-UV circular dicroism (CD) spectra were also indicative of the transfer of aromatic residues to a more flexible environment. It was also found that the presence of CTAB micelles induces an increase in α-helix content, as indicated by far-UV CD and Fourier-transform infrared (FTIR) spectroscopies. The far-UV CD spectrum of the enzyme shows and increase in the intensity of the positive band at 198 nm and in the negative band at 222 nm, results that indicate an increase in

the α-helical content. This is in agreement with the results obtained from FTIR, which showed an increase in the band at 1655 nm corresponding to the α-helix. The conclusion drawn from this study is that the higher catalytic efficiency of the enzyme in the presence of CTAB micelles is due to important conformational changes.

The distinct effect of DTAB addition on the transient (pre-steady state or "burst") and steady state phases of 2-NA hydrolysis catalyzed by α-CT was pointed out by Abuin et al. [222]. It was found that, in the transient phase, there is not effect of DTAB on the kinetic parameters at concentrations below the surfacant CMC. On the contrary, super-activity was observed under steady state conditions for the acyl-enzyme intermediate. These results were taken as an evidence that the surfactant does not modifies neither the formation nor the decomposition of the acyl-enzyme intermediate (transient phase) while notably increases the rate of decomposition of the acyl-enzyme complex.

Abuin et al. [223] studied the rate of hydrolysis of GPNA catalyzed by α-CT in aqueous solutions of DTAB at concentrations below and above the surfactant CMC. Superactivity was observed under both conditions with a maximum reaction rate taking place at DTAB concentrations near the CMC. The enzyme behavior was found to be similar when it was partially denatured in the presence of 4 M urea. The decrease of the enzyme activity after the surfactant CMC was found to be mainly due to the partitioning of the substrate, ie., after correction to take into account this effect, the activity remain almost constant. This results from a compensation of a decrease in the catalytic rate constant and a decrease in the Michaelis constant. The relevant point of this work was to show that the behaviour of α-CT in the hydrolysis of PNA in DTAB solutions is at variance with that in the hydrolysis of 2-NA [222] in solutions of the same surfactant, a result that can be explained in terms of different rate-limiting steps for the formation of the products.

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