
Fernando Moyano, Evangelina Setien, Juana J. Silber, and N. Mariano Correa*
Departamento de Química, Universidad Nacional de Río Cuarto, Agencia Postal # 3. C.P. X5804BYA Río Cuarto, Argentina

ABSTRACT: The reverse micelle (RM) media are very good as nanoreactors because they can create a unique microenvironment for carrying out a variety of chemical and biochemical reactions. The aim of the present work is to determine the influence of different water-dimethyl sulfoxide (DMSO) mixtures encapsulated in 1,4-bis-2-ethylhexylsulfosuccinate (AOT)/n-heptane RMs on the enzymatic hydrolysis of N-benzoyl-L-tyrosine p-nitroanilide (Bz-Try-pNA) by α-chymotrypsin (α-CT). The reaction was first studied in homogeneous media at different DMSO-water mixture compositions and in DMSO-water/AOT/n-heptane RMs. The hydrolysis rates of Bz-Try-pNA catalyzed by α-CT were determined by UV−vis spectroscopy. The reaction follows the Michaelis-Menten mechanism and the kinetic parameters: $k_{cat}$, $K_M$, and $k_{cat}/K_M$ were evaluated under different conditions. In this homogeneous media, DMSO plays an important role in the solubilization process of the peptide which is almost insoluble in water, but it has a tremendous impact on the inactivation of α-CT. It is shown that the enzyme dissolved in a 20% molar ratio of the DMSO-water mixture does not present enzymatic activity. Dynamic light scattering has been used to assess the formation of DMSO-water/AOT/heptane RMs at different DMSO compositions. The results also show that there is preferential solvation of the AOT RM interface by water molecules. To test the use of these RMs as nanoreactors, the kinetic parameters for the enzymatic reaction in these systems have been evaluated. The parameters were determined at fixed $W_S$ ($W_S = ([\text{water}] + [\text{DMSO}])/[\text{AOT}] = 20$) at different DMSO-water compositions. The results show that the Michaelis–Menten mechanism is valid for α-CT in all the RM systems studied and that the reaction takes place at the RM interface. Surprisingly, it was observed that the enzyme encapsulated by the RMs show catalytic effects with similar $k_{cat}/K_M$ values at any DMSO composition investigated, which evidence that DMSO molecules are localized far from the RM interface.

INTRODUCTION
Reverse micelles (RMs) are aggregates of surfactants formed in nonpolar solvents. The polar head groups of the surfactants point inward and the hydrocarbon chains point toward the nonpolar medium.$^{1–3}$ A common surfactant used to form RMs is sodium 1,4-bis-2-ethylhexylsulfosuccinate (AOT). AOT has a well-known V-shaped molecular geometry, giving rise to stable RMs without cosurfactants.$^{1–3}$ In addition, it has the remarkable ability to solubilize a large amount of water with values of $W_o$ ($W_o = [\text{water}] / [\text{AOT}]$), as large as 40 to 60, depending on the surrounding nonpolar medium, the solute, and the temperature. Besides water, some polar organic solvents, having high dielectric constants and very low solubility in hydrocarbon solvents, can also be encapsulated in RMs.$^{4,6}$ The polar organic solvents that can be sequestrated inside the RM media are dimethyl sulfoxide (DMSO), formamide (FA), dimethylformamide (DMF), dimethylacetamide (DMA), ethylene glycol (EG), propylene glycol (PG), and glycerol (GY)$^{4,6–13}$

Different studies have shown that these polar solvents are confined to the nanometer scale core of the RMs, where they behave differently from the bulk solvents as a result of specific interactions and confined geometries.$^{4,14}$ In others words, when a polar solvent is sequestered inside the RM, there is a competition between polar solvent−polar solvent interactions with polar solvent−surfactant interactions. Falcone et al.$^6$ have
shown that GY, water, EG, DMF, DMA, and FA/AOT/n-heptane RM droplets depend on different polar solvent–AOT interactions and not on their $n_m$ as discussed above. That is, it was shown that the key for the RM droplet size is the polar solvent–surfactant interaction and not the encapsulated polar solvent molar volume. Consequently, if the polar solvent is encapsulated inside the RMs, the droplet sizes should increase with $W_0 = [\text{polar solvent}]/[\text{AOT}]$.6

Very recently, Durantini et al.15 have shown how the confined environment affects dramatically the interaction between GY and DMF; in homogeneous media, GY and DMF interact strongly through hydrogen-bond interactions, while the opposite is found when the mixture is encapsulated inside AOT RMs. Upon confinement of the GY–DMF mixture and because of the strong GY–AOT interaction, GY binds through the hydrogen bond to the AOT $\text{SO}_4^−$ group at the interface and DMF makes complexes with the Na+ counterions in the polar core of the aggregates, diminishing significantly the bulk GY–DMF interaction. Therefore, each solvent (in the mixture) behaves as noninteracting solvents inside RM media.

Several biological phenomena occur at interfaces rather than in homogeneous solution. In particular, interface/protein interactions play a key role in the reactions involving membrane proteins. In this sense, even when reverse micelles (RMs) constitute an oversimplified model, the very large interfacial region provided by these systems can be expected to enhance some effects such as hydrogen bond interactions between peptide bonds, due to the fact that in these media the amphiphilic essence of a biological membrane is preserved.5,13,16,17

Many studies of enzyme kinetics inside RMs have been reported.1,4,5,17–21 One of the enzymes that has been mostly studied in RMs is $\alpha$-chymotrypsin (a-CT), which is a hydrophilic and globular protein and, it exists exclusively inside the micelles.5 In most of the studies, the enzyme is reported totally associated to the micelles, while the substrate is partitioned between the micelles and the external solvent pseudophases.5,17,18,22 For these cases, it was found that the catalytic constant ($k_{\text{cat}}$) of a-CT is very similar in RM media than in bulk water. However, the Michaelis constant ($K_M$) decreases significantly when corrected by the partition of the substrate between the organic solvent and the RM pseudophases, and this leads to an increase on the catalytic efficiency ($k_{\text{cat}}/K_M$) values inside the RMs in comparison with homogeneous media.5,17,18 This enhancement of the catalytic efficiency found upon enzyme encapsulation inside RMs is called superactivity,17 and it has been explained in terms of the peculiar state of water in the RMs, which mimics the status of intracellular water.23–26

The aim of the present contribution is to study the hydrolysis of the substrate N-benzoyl-L-tyrosine $p$-nitroanilide (Bz-Try-pNA) catalyzed by $\alpha$-chymotrypsin (a-CT) in homogeneous media and in DMSO-water/AOT/n-heptane RMs, at different DMSO-water molar ratios. The choice of this water mixture and RM media is because the substrate is completely insoluble in water, but it is soluble in DMSO. Thus, it is valuable to investigate the effect that the DMSO addition has on the hydrolysis reaction. In the present work, dynamic light scattering (DLS) was used to confirm the presence of DMSO-water/AOT/heptane RMs at different DMSO-water molar ratios and to determine the droplets sizes values.

The hydrolysis rates of Bz-Try-pNA in both systems have been determined by means of absorption spectroscopy, following the appearance of the product absorption band in every media. In homogeneous media, a dramatic decrease of the $\alpha$-CT efficiency was found when the DMSO amount increases. Moreover at around a 20% molar ratio of DMSO-water, the hydrolysis reaction is completely inhibited, presumably because the enzyme is denatured. In the RM media, the effect of AOT concentration on the kinetic parameters was determined. The partitioning of the substrate between the organic solvent and the micellar pseudophase in RMs was also obtained using absorption spectroscopy. The best mechanism found that explains the experimental data in RM systems is the Michaelis–Menten. Then, $k_{\text{cat}}$ and $K_M$ were determined by fitting the experimental data according to the mechanism, and the catalytic efficiency ($k_{\text{cat}}/K_M$) was calculated. The value obtained is independent of the DMSO composition used; the results highlight that each polar solvent (in the mixture) exists in different regions within the RM polar cores, and DMSO does not interact with the enzyme as the emission spectroscopy performed to the enzyme in different media also show.

### EXPERIMENTAL SECTION

**Materials and Methods.** General. Sodium 1,4-bis (2-ethylhexyl) sulfoxuccinate (AOT) (Sigma >99% purity) was used as received. AOT was kept under vacuum over $\text{P}_2\text{O}_5$ to minimize water absorption. The absence of acidic impurities was confirmed through the 1-methyl-8-oxynolvinolinium betain $(\text{QB})$ absorption bands.27 $\alpha$-Chymotrypsin, (a-CT), MW 24800, from bovine pancreas (Sigma) and N-benzoyl-L-tyrosine $p$-nitroanilide, (Bz-Try-pNA), (Sigma) were used as received. Dimethyl sulfoxide (DMSO) and n-heptane (Merck HPLC quality) were used as received, and ultrapure water was obtained from a Labonco equipment model 90901-01.

The pH of the bulk water solution has been maintained at 8.7 using a 20 mM phosphate buffer. This pH value was found to be optimal for this enzymatic reaction in homogeneous media. In the RM media, it is known that the pH cannot be measured inside the polar core of the aggregate.28 A meaningful approximation to the pH within the aqueous RM pseudophases can be made using pure AOT and to have sufficient buffering capacity in the bulk solutions. In this sense, the value of the pH inside the polar core is referred to the homogeneous buffer solution and it is called $pH_{\text{ext}}$.

In this work, as all the dynamic light scattering (DLS) experiments were carried out at a fixed surfactant concentration of 0.1 M, consequently, the RM solutions are not at infinite dilution. Thus, it is appropriate to introduce an apparent hydrodynamic diameter (dapp), in order to make the comparison with the system herein studied. A similar approach was used previously.29 The dapp values of the different DMSO-water/AOT/n-heptane RMs were determined by DLS, in a Malvern 4700 with a goniometer and an argon ion laser operating at 488 nm. Cleanliness of the cuvettes used for measurements was of crucial importance for obtaining reliable and reproducible data.30 Cuvettes were washed with ethanol, and then with doubly distilled water, and dried with acetone. Prior to use, the samples were filtered three times to avoid dust or particles present in the original solution, using an Acrodisc with a 0.2 μm PTFE membrane (Sigma) for the RM samples. Previous data acquisition, the samples were equilibrated in the DLS instrument for 10 min at 25 °C. To obtain valid results from DLS measurements, a knowledge of the system’s refractive index and viscosity in addition to well-defined conditions is required. The refractive indices and viscosities for the AOT RMs were assumed to be the same as the neat n-heptane. Multiple samples at each size were made, and thirty independent size measurements were made for each individual sample at the scattering angle of 90°. The instrument was calibrated before and during the course of experiments, using several different size standards. Thus, the magnitudes obtained by DLS measurements can be taken as statistically meaningful for all the systems investigated. The algorithm

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used was CONTIN and the DLS experiments showed that the polidispersity of the AOT RMs were less than 5%.

**METHODS**

Reactions in Homogeneous Media. The reactions were followed by measurement of the increase at the maximum absorption band of the product p-nitroaniline (pNA) at 386 nm ($\lambda = 12500 \text{ M}^{-1} \text{ cm}^{-1}$) and 25.0 ± 0.1 °C. Absorbances were recorded in a Hewlett-Packard, UV-visible 8455 spectrophotometer equipped with a thermostatted cell (3 mL of volume and 1 cm path length).

The UV–visible spectroscopic analysis show that the hydrolysis reaction of Bz-Try-pNA catalyzed by $\alpha$-CT in DMSO-water produces pNA (eq 1) in quantitative yields.

$$\text{Bz-Try-pNA} \rightarrow \text{pNA}$$

The different DMSO-water mixtures were prepared by weight to obtain the desired % molar ratio. Also, a 0.01 M stock solution of Bz-Try-pNA in DMSO was prepared.

To start a kinetic run in homogeneous media, 3 mL of the $\alpha$-CT in the DMSO-water solution (20 mM phosphate buffer at pH 8.7 and $[\alpha$-CT] = $1 \times 10^{-5}$ M) of the molar ratio % investigated was introduced in a thermostatted cell. Then, the enzymatic reaction was initiated by addition of different microliters of the stock DMSO solution of Bz-Try-pNA, in order to have 3 mL of solution with the desired [Bz-Try-pNA]. For example for $[\alpha$-CT] = $1 \times 10^{-5}$ M and for $W_{\alpha}$ = 20, it is necessary to prepare the solution with $W_{\text{DMSO}}$ = 16 and $W_{\text{DMSO}}$ = 4. For a substrate concentration of $[\text{Bz-Try-pNA}] = 7.5 \times 10^{-5}$ M, the following experimental conditions are required to run the kinetic: 86 μL of the buffer solution ([$\alpha$-CT] = 3.5 × 10^{-5} M) and 86 μL of the Bz-Try-pNA DMSO stock solution ($[\text{Bz-Try-pNA}] = 2.6 \times 10^{-3}$ M).

**Bz-Try-pNA Partition Constant Measurements in RMs.** The partitioning of Bz-Try-pNA between the micelles and the organic solvent pseudophases can be treated within the framework of the pseudophase model.32-35 This model considers the RMs as distinct pseudophases whose properties are independent of the AOT concentration and are only determined by the value of the characteristic parameter $W_s$. In this model, only two solubilization sites are considered, that is, the external nonpolar solvent and the reverse micelle (i.e., all the surfactant molecules). In this way, the distribution of Bz-Try-pNA between the micelles and the external solvent pseudophase defined in eq 4 can be expressed in terms of the partition constant $K_p$ shown in eq 5:

$$[\text{Bz-Try-pNA}]_f = [\text{Bz-Try-pNA}]_b^q$$

$$K_p = \frac{[\text{Bz-Try-pNA}]_b^q}{[\text{Bz-Try-pNA}]_f}$$

The terms in brackets represent free (f) and bound (b) Bz-Try-pNA, in terms of local concentrations, that is, the concentration expressed considering the RM volume.36 If $[\text{Bz-Try-pNA}]_b[AOT]$ is the analytical concentration of the micelle bound substrate, eq 6 holds.

$$[\text{Bz-Try-pNA}]_b^q = \frac{[\text{Bz-Try-pNA}]_b[AOT]}{[\text{AOT}]}$$

and, hence, $K_p$ can be expressed as in eq 7

$$K_p = \frac{[\text{Bz-Try-pNA}]_b[AOT]}{[\text{Bz-Try-pNA}]_f}$$

where $[\text{Bz-Try-pNA}]_f$ is the concentration of the substrate in the n-heptane, and $[\text{AOT}]$ is the surfactant concentration. This equation applies at a fixed value of $W_s$ when $[\text{Bz-Try-pNA}]_b \ll [\text{AOT}]$, where $[\text{Bz-Try-pNA}]_f$ is the probe analytical concentration.

The values of $K_p$ can be determined from the changes with the surfactant concentration at a given $W_s$ in the Bz-Try-pNA absorption spectra measured at a given wavelength (319 nm). The values of $K_p$ were calculated with a least-squares fit to eq 8:

$$A' = \left(1 + K_p[AOT]\right)^{-1}$$

where $A'$ is the absorbance at different AOT concentrations and $A'$ and $A''$ are the molar extinction coefficients for the Bz-Try-pNA in n-heptane and at the RM interfaces, respectively. [AOT] is the surfactant concentration and $[\text{Bz-Try-pNA}]_f$ is the total Bz-Try-pNA concentration. It should be noted that $A'$ was determined experimentally from
the spectra of Bz-Try-pNA in n-heptane at λ = 319 nm (ε = 121.62 M⁻¹ cm⁻¹), and ε₉ was obtained from the fitting of the experimental data using eq 8. One should bear in mind that precise spectroscopic estimation of the constants is ruled out by the impossibility of having all the Bz-Try-pNA at the interface, and therefore, the value of ε₉ can only be estimated. The spectroscopic estimation of Kᵢ was, therefore, based on the analysis of the spectroscopic data at 319 nm, using a fitting procedure with the ε₉ as an adjustable parameter.

Mechanism of the Hydrolysis of Bz-Try-pNA Catalyzed by α-CT in Homogeneous and in Reversed Micellar Media. In both media (homogeneous and DMSO-water/AOT/n-heptane RMs), the enzymatic hydrolysis reaction shown in eq 1 follows the classical Michaelis–Menten mechanism depicted in eq 9, and α-CT is totally incorporated into the RM pseudophase, while the substrate partitions between the external and the RM pseudophase.

\[ E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E-S \overset{k_{cat}}{\longrightarrow} E + P \]  (9)

With application of the steady-state approximation to (E-S), the rate law given in eq 10 is obtained:

\[ v_0 = \frac{k_{cat}[E][S]}{(K_M + [S])} \]  (10)

where v₀ is the initial reaction rate (M s⁻¹), [E] and [S] are the analytical enzyme and substrate concentration, respectively, k_cat is the catalytic rate constant, and K_M is the Michaelis constant defined by eq 11:

\[ K_M = \frac{k_{-1} + k_{cat}}{k_1} \]  (11)

Equation 10 can be rearranged into a form that is amenable to data analysis by linear regression, which is known as the Lineweaver–Burk equation (eq 12):

\[ \frac{[E]}{v_0} = \left(\frac{1}{k_{cat}}\right) + \left(\frac{K_M}{k_{cat}}\right)\frac{1}{[S]} \]  (12)

Equation 12 directly provides k_cat from the reciprocal of the intercept and the catalytic efficiency k_cat/K_M from the reciprocal of the slope. The K_M value is obtained from the slope/intercept ratio.

The absorbance at 386 nm was recorded as a function of time, and the v₀ was obtained from the slope of [pNA] versus time profiles. The formation of pNA was linearly dependent on the reaction time during the first 25 min of the reaction. The pooled standard deviation of the kinetic data, using different samples, was less than 5%.

### RESULTS AND DISCUSSIONS

**Reaction in Homogeneous Media. DMSO-Water Mixtures.** As the Bz-Try-pNA solubility in water is very low (and no kinetic data can be obtained), but it is high in DMSO, it is of interest to investigate the reaction in the DMSO-water mixtures. Thus, it is important to study which is the effect of increasing the polar organic solvent content in the enzymatic reactions. In this way, the reaction in different DMSO-water mixtures at the following molar ratios: 7, 15, and 20% was investigated.

Figure 1 shows the typical absorption spectra for the Bz-Try-pNA hydrolysis at different reaction times in the DMSO-water mixture at a 20% molar ratio. The absorption spectra taken at different times of reaction show an increase in the absorbance at λ_max = 386 nm (Figure 1, inset) and a clear isosbestic point at λ = 345 nm. This provides evidence of the lack of intermediates and/or product decomposition.

Figure 2 shows typical results obtained by treating the data in accordance with the Lineweaver–Burk plot (eq 12). The linearity of the plot indicates that, under the condition employed, the Michaelis–Menten mechanism applies for 7 and 15% molar ratio of DMSO-water mixtures. From the slope and the intercept of the line in Figure 2, values of the experimental kinetics parameters, k_cat and (K_M), were obtained and gathered in Table 1. From Table 1, it can be seen that the catalytic efficiency values dramatically decrease with the DMSO content. Furthermore, at a 20% molar ratio of DMSO-water mixture, the reaction was completely inhibited. That is, the addition of DMSO into aqueous solution of α-CT resulted in an increase in the substrate solubility and a notorious loss of the enzymatic activity probably because the enzyme is denatured. In view of these facts, it seems that DMSO acts as a denaturant of the protein. Other authors showed that the enzymatic activity depends on the amount of water in contact with the enzyme and not on the total amount of bulk water in the system. They suggested that the increase in the amount of organic solvent can produce a progressive disruption of the hydrogen bond between the water and protein molecule,
lowering its conformational mobility and consequently reducing the enzymatic activity. It is known that DMSO acts as a strong hydrogen bond acceptor and interacts strongly with water breaking its hydrogen bond structure.39–42

Also, it is known that DMSO is a bad solvent for the protein backbone but a good solvent for the relatively polar tryptophan residues, which form part of the active site of the α-CT.39,43 Therefore, it can be suggested that in the DMSO-water mixtures, the organic solvent preferentially solvates the tryptophan residues in the protein, making less accessible the active site and denaturing the enzyme.

Reactions in the DMSO-Water/AOT/n-Heptane Reverse Micelle. To evaluate the formation of the new RMs, the systems formed by DMSO-water/AOT/n-heptane were studied using the DLS technique at different DMSO composition.

The first question that has to be answered when new reversed micelle systems are explored is if the mixture of DMSO-water is effectively entrapped by the surfactant, creating a true RM in n-heptane.4 Thus, DLS is used to assess this matter because if the DMSO-water mixture is really encapsulated to form RMs and interacting with the interface, the size of the droplet must increase as the Ws value increases with a linear tendency (swelling law of RMs), as is well-established for water and other polar solvents/surfactant RMs.3,6,44,45

Figure 3 reports the dapp values obtained for DMSO-water/AOT/n-heptane at a different DMSO-water composition, namely 7 and 20%. As can be seen, there is an increase in the droplet size values when the DMSO-water content increases in both systems; these results show that the DMSO-water mixture is effectively entrapped by the surfactant layer, yielding RMs. Also, the linear tendency observed in the whole Ws range for both systems suggest that the droplets are spherical and noninteracting.44 An interesting result is observed in Figure 3, when comparing the droplet size values at the same Ws but increasing the DMSO content. There is an increase in the dapp values, as the DMSO content increases. For example, for the 7% DMSO-water composition, the dapp value at Ws = 20 is 9.5 nm, and the value is 14.7 nm for the 20% DMSO-water composition, the value is 14.7 nm at the same Ws. In comparison with water/AOT/n-heptane RMs at W = 20 where the dapp = 9.1,45 the dapp value obtained in the DMSO containing RMs are larger. Elles et al. have reported droplet sizes for DMSO-water/AOT/cyclohexane RMs but at a Ws not larger than 7.47 They also showed a dramatic increase in the dapp values with the DMSO content; for example, they found a dapp value of 9.4 nm for Ws = 5 and Ws = 2, while the dapp value is 15.8 nm for the same amount of water and Ws = 3.47 Nevertheless, there were no attempts to explain the differences in the RM sizes.

To interpret the results, it is important to consider that the RM droplet sizes depend on, among many other variables, the different polar solvent–AOT interactions. It is known that when water is encapsulated in n-heptane/AOT RMs, the hydrogen-bond interaction with the AOT polar headgroup increases the surfactant’s headgroup area (a) values with the consequent decrease in the surfactant packing parameter and the increase in the RM droplet size.4 Also, it was shown that solvents like dimethylformamide (DMF) that do not interact through the hydrogen bond with the AOT interface do not change the RM sizes because they interact with each other in the polar core and not with the AOT interface.4 DMSO is a kind of solvent similar to DMF in the way that it does not interact through the hydrogen bond with the AOT RM interface; thus, when the DMSO-water mixture is encapsulated by the AOT RMs, water goes to the interface to interact with the surfactant molecules, while DMSO moves to the RM polar cores and interacts with the other DMSO molecules. Even more, the results seems to show that as the DMSO content increases, the hydrogen bond interaction between water and AOT increases due to a greater availability of hydration water with the consequent changes in the AOT a value increasing the RM droplet sizes.

In summary, the results show the presence of RMs, that the DMSO-water molecules are effectively entrapped in the AOT/n-heptane at different DMSO-water compositions, and explain the effect in the RM dapp values when the DMSO content increases.

Once it was shown that the RM media exists under our experimental conditions, the next step is to evaluate the enzymatic hydrolysis reaction inside RM systems and to
investigate different variables, such as the surfactant concentration and the polar solvents content.

Figure 4 shows a typical absorption spectrum of the hydrolysis of Bz-Try-pNA, at different reaction times, for the 20% molar ratios of the DMSO-water mixture, in DMSO-water/AOT/n-heptane RMs at $W_S = 20$. The absorption spectra taken at different reaction times show a clear isosbestic point ($\lambda = 334$ nm), evidencing the lack of intermediates and/or product decomposition. As was previously found in the homogeneous media, the band corresponds to the product, pNA.\(^{19−21}\) It is necessary to remark that in the DMSO-water/AOT/n-heptane RMs, the enzymatic reaction was accomplished at the different DMSO-water compositions used, even for the 20% molar ratios of the DMSO-water mixture which was a composition that inhibited the enzymatic reaction in homogeneous media as was discussed before.

Figures 5 and 6 show the surfactant concentration variation for DMSO-water/AOT/n-heptane RMs at different DMSO-water composition and $W_S = 20$. The linearity of the plot in both systems, at every [AOT] considered, indicates that the classical Michaelis–Menten mechanism is valid for $\alpha$-CT in DMSO-water/AOT/n-heptane RMs, as has been observed in water/AOT/n-heptane, for other related reactions.\(^{18,36,48}\)

The dependence with AOT concentration found in Figures 5 and 6, at different DMSO-water compositions in RMs could be due to (i) Bz-Try-pNA partitioning, owing to its solubility in n-heptane, diminishing local substrate concentration within the zone in which the reaction takes place, which is the micellar pseudophase. In other words, this is the result expected due to a simple dilution because the local concentration is inversely proportional to the surfactant concentration when the substrate is totally associated to the micellar pseudophase\(^{17,18}\) and/or (ii) progressive inactivation of the enzyme by the surfactant, diminishing $k_{cat}$ value as [AOT] increases.

In Figures 5 and 6, it can also be seen that both plots have a unique intercept independently of the surfactant concentration. This fact argues against possibility (ii) as expected for a totally incorporated enzyme to the RMs. Therefore, the difference in the kinetic parameter obtained from Figures 5 and 6 is due to the partitioning of the substrate between the micellar pseudophase and the external solvent ($K_p$).

Table 2 shows the results of $K_p$ for DMSO-water/AOT/n-heptane at a different DMSO-water composition obtained through eq 8. The value of $K_p$ at 7% molar ratio of the DMSO-water mixture is half of that obtained at the 20% molar ratio. These differences found in the $K_p$ values can be due to a combined effect of the higher Bz-Try-pNA solubility in DMSO (around 2 M) and the lower Bz-Try-pNA solubility in n-heptane (around $1 \times 10^{-4}$ M). Thus, the substrate is more incorporated in the pseudophases of the RMs when the mixture composition has a higher DMSO content.

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**Figure 4.** Representative absorbance spectra at different times for the hydrolysis of Bz-Try-pNA catalyzed by $[\alpha$-CT] in DMSO-water/AOT/n-heptane RMs at a 20% molar ratio DMSO-water composition at $W_S = 20$. [AOT] = 0.01M. $[\alpha$-CT] = $1 \times 10^{-6}$ M. [Bz-Try-pNA] = $7 \times 10^{-5}$ M; pH = 8.7. Inset: absorbance of pNA in RMs at different time. $\lambda = 386$ nm.

**Figure 5.** Effect of AOT concentration on the Lineweaver–Burk plot for the $\alpha$-CT-catalyzed hydrolysis of Bz-Try-pNA in DMSO-water/AOT/n-heptane RMs with RMs at 7% molar ratio DMSO-water composition. $W_S = 20$. [AOT]: (▲) 0.10, (●) 0.05, and (■) 0.01 M. $[\alpha$-CT] = $1 \times 10^{-6}$ M. pH$_{ext}$ = 8.7.

**Figure 6.** Effect of AOT concentration on the Lineweaver–Burk plot for the $\alpha$-CT-catalyzed hydrolysis of Bz-Try-pNA in DMSO-water/AOT/n-heptane RMs at a 20% molar ratio DMSO-water composition. $W_S = 20$. [AOT]: (■) 0.01 and (●) 0.1 M. $[\alpha$-CT] = $1 \times 10^{-6}$ M. pH$_{ext}$ = 8.7.

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Table 2. Bz-Try-pNA Partition Constant between the Organic Solvent and DMSO-Water Mixture ($K_p^{\text{DMSO-water/n-heptane}}$) and between the Micellar Pseudophase and the Organic Solvent ($K_p$)

<table>
<thead>
<tr>
<th>% DMSO-water</th>
<th>$K_p^{\text{DMSO-water/n-heptane}}$</th>
<th>$K_p$ (M$^{-1}$)$^a$</th>
<th>$K_p$ (M$^{-1}$)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>4.8 ± 0.6</td>
<td>10.6 ± 1.0</td>
<td>10.0 ± 1.0</td>
</tr>
<tr>
<td>15</td>
<td>12.5 ± 1.0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>20</td>
<td>–</td>
<td>20.3 ± 1.7</td>
<td>21.2 ± 2</td>
</tr>
</tbody>
</table>

$^a$Value obtained through eq 8. $^b$Value obtained through eq 13.

Also, as was previously described,$^{17}$ it is possible to determine the $K_p$ value from the kinetic data using eq 13:

$$[\text{Bz-Try-pNA}]_T = \frac{n}{K_p} + n[AOT]$$

(13)

where $[\text{Bz-Try-pNA}]_T$ is the total (analytical) substrate concentration and $n$ is the average number of substrate molecules incorporated into RMs per AOT molecule; $n = [\text{Bz-Try-pNA}]_T/[AOT]$. The proposed method is based on the assumption that, at a given $W_5$ value ($W_5 = 20$) and $(\alpha$-CT) = 1 × 10$^{-6}$ M, the value of $v_0/((\alpha$-CT)) is determined only by the concentration of the substrate in the organic solvent. Since equal $[\text{Bz-Try-pNA}]_T$ implies equal $n$, a simple mass balance leads to the above eq 13.$^{15,17}$ For a set of $[\text{Bz-Try-pNA}]_T$ and [AOT] values corresponding to the same $v_0/((\alpha$-CT)) value in Figure 7 A, a plot of the left-hand side of eq 13 against [AOT] allows the evaluation of $n$ from the slope and $K_p$ from the slope/intercept ratio (Figure 7 B), and the results are gathered in Table 2. A good correlation between both methods was obtained.

From Figures 5 and 6, the values of the experimental kinetic parameters ($k_{\text{cat}}$ and $K_M$) at different AOT concentrations in the RMs can be obtained. As these parameters are practically constant at all surfactant concentrations used in RMs at $W_5 = 20$, only shown are the values obtained in DMSO-water/AOT/n-heptane RMs at $[\text{AOT}] = 0.1$ M and different molar ratios of the DMSO-water mixture in Table 3.

Also shown in Table 3 are the values corrected using eq 14,$^{17,18,49}$ by the partitioning of $k_{\text{cat}}$ and $K_M$ for DMSO-water 7% /AOT/n-heptane RMs and $K_p = 20.7$ ± 1.7 M$^{-1}$ for DMSO-water 20% /AOT/n-heptane RMs) between different pseudophases within the RMs.

$$[(K_M)_{\text{corr}}]_{\text{mic}} = \frac{(K_M)_{\text{exp}}}{{(1 + K_p[AOT])}}$$

(14)

The $k_{\text{cat}}/(K_M)_{\text{corr}}$ ratio values obtained for DMSO-water/AOT/n-heptane RMs at different DMSO-water compositions are practically independent (within the experimental error) of the DMSO compositions. Results indicate that the enzyme is located at the AOT RM interfaces, where it is surrounded by the interfacial water and does not detect DMSO molecules at any DMSO composition. It is known that $\alpha$-CT is one hydrolytic enzyme,$^{13}$ and it can be located at the interface of the micelles of AOT increasing the catalytic activity.$^{17,18}$ Therefore, $\alpha$-CT located at the interface RMs could be sensing a microenvironment solvated by interfacial water and showing a good catalytic effect. In this case, it is necessary to remark that, as shown by the DLS measurements, in the DMSO-water mixture, water binds through the hydrogen bond to the AOT $SO_3^-$ group at the interface increasing the droplet size values. The fact that $\alpha$-CT only senses water in these RMs provides other strong evidence that confirms that DMSO is not associated with the AOT RM interfaces and, therefore, does not compete for the protein active sites solvation, in contrast to what was suggested by Moniruzzaman et al. in studying the lipase-catalyzed hydrolysis of olive oil in water/DMSO AOT RMs.$^{50}$

In other words, DLS measurements and the kinetic parameters obtained would indicate that DMSO molecules...
are located on the polar core of the AOT RMs, while the water molecules are at the interface and interact through the hydrogen bond with the surfactant.

In order to corroborate the assumption mentioned above, the emission spectroscopy of α-CT was followed in the different DMSO-water mixtures and in DMSO-water/AOT/n-heptane RMs at $\lambda_{exc} = 280$ nm. The results are shown in Figure 8 (panels A and B). The conclusions that can be obtained from the Figure can be summarized as follows: (i) The data show that the spectroscopic properties of α-CT are similar in homogeneous and RMs at any DMSO-water molar ratio %.

Moreover, as the DMSO content increases in the water mixture, the emission spectra shift to the red, which shows that the enzyme is being denatured by the organic solvent. Note that at 20% molar ratio content, the spectrum is almost identical to the one obtained in pure DMSO. On the other hand, Figure 7 B shows that the emission maxima is around 330 nm at any DMSO-water composition in the RM media, results that show that the enzyme is solvated only by water in the confined media as was discussed above.

To make a significant comparison of the kinetic parameter values obtained in the AOT RMs with the values obtained in homogeneous DMSO-water mixtures in terms of a common thermodynamic substrate activity scale and not in terms of substrate concentrations, some correction has to be applied.\(^{7,18}\) The simplest approach is to compare the rate constants, taking as the reference the external solvent of the RMs (i.e., n-heptane). In other words, the catalytic efficiency of the enzymatic reaction obtained in the bulk solvents, $(k_{cat}/K_p)^{exp/bulk}$, must be corrected by substrate partitioning between the organic solvent and the DMSO-water mixture $(K_p^\text{DMSO-water}/n\text{-heptane})$. This correction is shown in eq 15.\(^{22}\)

$$[(K_M)^{bulk} / (K_{M\text{corr}}^{exp/bulk})] = (K_p^{exp/bulk})^{DMSO-water/n\text{-heptane}}$$

Table 2 shows the partition constant values in homogeneous media for the Bz-Try-pNA distribution between DMSO-water mixture and n-heptane, $(K_p^{DMSO-water/n\text{-heptane}})$. Then, the $[k_{cat}^{exp} / (K_{M\text{corr}}^{exp/bulk})]^{DMSO-water/n\text{-heptane}}$ values are corrected according to eq 15 and are gathered in Table 1. With this correction taken into account, it can be seen that the catalytic efficiency in the homogeneous media depend on the amount of DMSO in the mixture, as was argued previously. The catalytic efficiency, $[k_{cat}^{exp} / (K_{M\text{corr}}^{exp/bulk})]^{DMSO-water/n\text{-heptane}}$, of the enzymatic reaction obtained at 7% is $1.04 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, while for 15% the value is $2908 \text{ M}^{-1} \text{ s}^{-1}$. It must be noted that for the 20% DMSO-water mixture, the reaction was completely inhibited. Now, the catalytic efficiency in DMSO-water mixture can be compared with those obtained in DMSO-water/AOT/n-heptane RMs at different DMSO-water compositions (Table 3). The first comparison is that the catalytic efficiency in homogeneous medium depends more strongly on the DMSO composition than in the RMs. These facts were discussed above through to the solvation of DMSO with enzyme in homogeneous medium and to the different localization of the enzyme and DMSO in RMs. Then, another comparison is possible. It can be noted that in homogeneous medium at a 7% DMSO-water mixture, the catalytic efficiency is higher than in DMSO-water/AOT/n-heptane RMs at the same DMSO-water composition. These data can be explained, considering the different microenvironments that sense the enzyme. The enzyme in RMs is solvated by interfacial water, and therefore, the amount of water is lower compared with the water in homogeneous media. As is known,\(^{37,38}\) the enzymatic activity depends on the amount of water in contact with the enzyme and the catalytic efficiency in DMSO-water/AOT/n-heptane RMs decreases in comparison with homogeneous media at 7% v/v DMSO-water.

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**CONCLUSION**

This work shows that DMSO acts as a denaturant of the protein in homogeneous DMSO-water mixtures. It proves that the catalytic efficiency of the enzyme in homogeneous media diminishes with the increase of DMSO to the total inactivation of α-CT is denatured when dissolved in DMSO.
of the enzyme, probably because it is denatured. It seems that DMSO solvates the residues of tryptophan in the protein or produce a progressive disruption of the hydrogen bond between the water and protein molecule, causing the loss of activities.

On the other hand, in DMSO-water/AOT/n-heptane RM, the enzyme is totally incorporated to the pseudophases of the RM and its location is the AOT interface. The enzyme is solvated by the interfacial water, and therefore, the catalytic activity is practically the same at any DMSO content. Furthermore, in DMSO-water/AOT/heptanes, RM at W\textsubscript{0} = 20 for the DMSO content in the DMSO-water mixture, which was inhibited in a homogeneous medium (20\% DMSO-water); the catalytic efficiency is similar to that obtained in RM at 7\% DMSO-water compositions. Thus, DMSO inside of the RM helps to solubilize the substrate and increases the effective concentrations inside the RM. The results highlight the importance of RM as nanoreactor media for enzymatic reactions, where the enzyme not only retains but increases its catalytic activity in comparison with homogeneous media.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: mcorrea@exa.unrc.edu.ar.

Notes

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