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Caging enzyme function: α-chymotrypsin in reverse micelle

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Abstract

We report studies of the enzymatic activity of α-chymotrypsin (CHT) in aqueous buffer and AOT reverse micelle with various degrees of hydration using the substrate Ala–Ala–Phe–7-amido-4-methylcoumarin (AMC). From Michaelis–Menten kinetics, we determined equilibrium and rate constants for catalytic activity in aqueous buffer. In the reverse micelle we found that the activity of CHT to be *retarded* by two orders of magnitude compared to that in aqueous buffer. The activity is also found to be nearly *insensitive* to the degree of hydration of reverse micelle. From these studies, we attempt to elucidate the influence of hydration on enzyme activity.

1. Introduction

The role of protein hydration in enzyme function (catalysis) is well recognized and has recently been reviewed by several authors [1–4]. One of these studies has shown that the dehydration of a protein, which makes it more rigid and increases its denaturation temperature, is correlated with the loss of its physiological function [1]. There are many techniques by which one can control the degree of hydration of a protein molecule. As reported in [1], the hydration level was controlled by maintaining a particular humidity around a protein sample. Reverse micelle (RM) is another system that is commonly used to control the degree of hydration.

Reverse micelle are tiny aqueous droplets (Fig. 1), surrounded and stabilized by a monolayer of surfactant molecules, and dispersed in a water immiscible organic solvent (for reviews, see [5,6]). In some cases it is possible to solubilize enzymes in RM without the loss of the native structure of those enzymes. Enzymes in micellar systems usually exhibit maximal activity when the size of the internal cavity of the RM corresponds to or is close to the size of the solubilized proteins [7–9]. A direct relationship exists between the radius of RM of AOT (aerosol OT) and their hydration degree (w_0), which is described by the following empirical equation [6]:

$$r_m\ (\text{Å}) = 2w_0, \tag{1}$$

where r_m is the radius of the water pool in Å and $w_0 = [\text{water}]/[\text{AOT}]$. For proteins with nearly spherical shape, the empirical relationship between the hydration degree of RM and molecular mass (M) of a solubilized protein is described by the following relation [9]:

$$w_0 = (0.083 \pm 0.008)\sqrt{M}. \tag{2}$$

In this Letter, we measured the rate of the reaction between the enzyme α-chymotrypsin (CHT) and the substrate Ala–Ala–Phe–7-amido-4-methylcoumarin (AMC). Fig. 1 depicts the chemical structure of the substrate and X-ray structure of the enzyme. The enzyme, isolated from bovine pancreas belongs to a class of digestive serine protease, and has biological function of hydrolyzing polypeptide chains. It is a globular protein with molecular weight of 24,800 and dimensions $40\times40\times51$ Å [7]. It can therefore be represented as a sphere with a radius of approximately 22 Å. Eqs. (1) and (2) then indicate that the maximum degree of hydration for this protein is expected [7–9] to occur at $w_0 \approx 10$–12, where the radius of the water pool of the RM is of the same order as that of the CHT. Recently, Zewail's group [10] found a correlation between the

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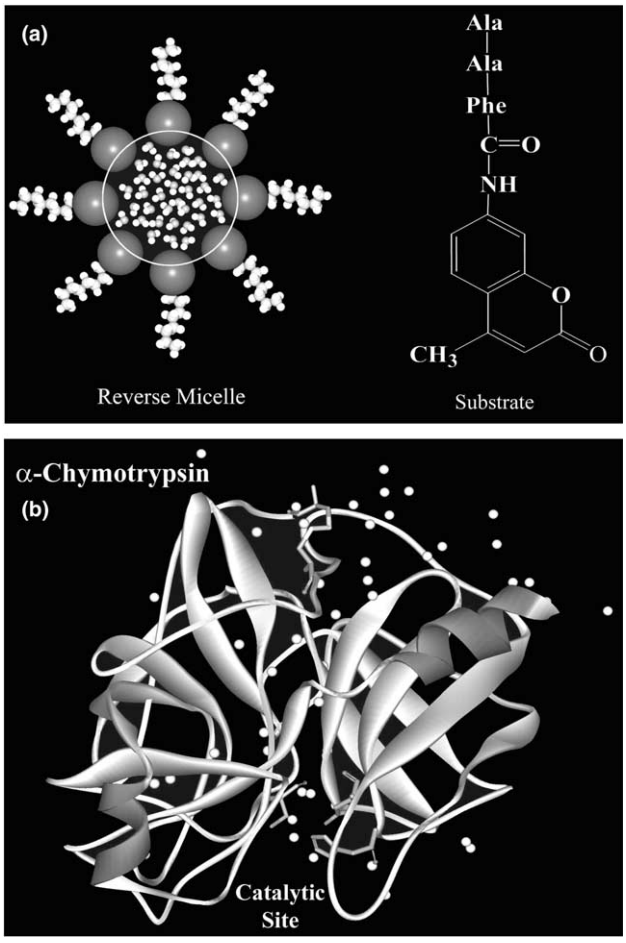

Fig. 1. A schematic structure of AOT reverse micelle ((a), left). The substrate ((a), right) Ala–Ala–Phe–7-amido-4-methylcoumarin (AMC) and the enzyme (b) α-chymotrypsin. The enzyme structure was downloaded from the protein data bank (ID code 2CHA) and handled with program WEBLAB VIEWERLITE.

ultrafast surface hydration and the functionality of the enzyme CHT. Time-dependent studies carried out by this group using fluorescence up-conversion technique with femtosecond time-resolution indicate that in the physiological active state of the enzyme, water molecules at the surface possess relatively a high degree of mobility, whereas in the inactive state the water is more rigidly structured.

Here, by using Michaelis–Menten kinetics we obtained the equilibrium and rate constants for the enzymatic activity in buffer at pH 7.0. Then we measured the catalytic rates of CHT in RM with various degrees of hydration (w_0) and compared them with those observed in the bulk buffer (physiological condition). We followed this procedure to examine the influence of degree of hydration on reactivity in the aqueous and confined (RM) environments. Note here that the reactions were studied at those values of w_0 where the native structure of CHT was preserved. Circular dichroism (CD), electron paramagnetic resonance (EPR) and active-site titration studies made previously have indicated that encapsulation of CHT with $w_0 > 10$ brings out negligible changes in the global structure of the protein. Interestingly, the perturbation is also negligible even when $w_0 = 10$ [7]. We are therefore studying the enzymatic reaction in the limit of the structural integrity of CHT.

2. Experimental

Lyophilized CHT powder was purchased from Sigma (highest grade). *n*-Heptane (Spectrochem, 99.5%), bis(2-ethylhexyl) sulfosuccinate sodium salt (AOT; Fluka 99%), di-sodium hydrogen phosphate dihydrate (Merck, 99%), sodium dihydrogen phosphate dihydrate (Merck, 99%) and Ala–Ala–Phe–7-amido-4-methylcoumarin (AMC; Sigma, 99%) were used as received. Aqueous stock solutions of the protein were prepared in a phosphate buffer (0.01 M) at pH 7.0 using double distilled water. Concentration of the enzyme samples in aqueous solution was determined using the supplied (by the vendor) extinction coefficient value at 280 nm, $\varepsilon = 51\ \mathrm{mM^{-1}\ cm^{-1}}$. AOT solutions were prepared by dissolving solid AOT in *n*-heptane. The final AOT concentration was 0.1 M.

Activity measurements were performed using AMC as the substrate. Concentration of the substrate in aqueous buffer was estimated on taking extinction coefficient value at 325 nm to be $15.9\ \mathrm{mM^{-1}\ cm^{-1}}$. The enzyme cleaves the substrate and produces a free coumarin derivative. The absorbance of this product (coumarin derivative) was monitored in Shimadzu Model UV-2450 spectrophotometer. A cell of 1 cm path length was used for measurements both in aqueous buffer (pH 7.0) and RM. The enzyme concentration was 0.668 μM. Enzyme–substrate reactions in the aqueous buffer were started by the addition of an aliquot (20 μl) of the stock aqueous buffered enzyme solution to the pre-equilibrated desired AMC buffer solution at 25 °C. The final enzyme concentration was 0.668 μM. The initial concentration of AMC was maintained in excess to that of the enzyme and was varied over a wide range. The increase in absorption at 370 nm due to the release of 7-amido-4-methyl-coumarin ($\varepsilon_{370} = 7.9\ \mathrm{mM^{-1}\ cm^{-1}}$) was followed (Fig. 2) as time progressed. Note here that the substrate does not absorb at this monitoring wavelength. Initial rates were measured in the regime where the absorbance varies *linearly* with time. The reaction followed the Michaelis–Menten kinetics [11]. The apparent K_M and k_{cat} values were derived by least squares fitting of the double reciprocal Lineweaver–Burk plot (Fig. 3).

To measure the rates of enzyme–substrate reactions in RM at various w_0, the stock substrate was first injected into the AOT solution and stirred until the solu-

tion became clear. Subsequently, the stock enzyme solution (10 μl) was added to the AOT solution. The cuvette was inverted several times and the absorbance was measured for 1 h at 370 nm. The final enzyme and substrate concentrations in the RM were 1 and 14 μM, respectively. For comparison, enzyme–substrate reaction in aqueous buffer with similar enzyme and substrate concentrations were performed (Fig. 4). No reactions were observed in aqueous buffer and RM in the absence of the enzyme.

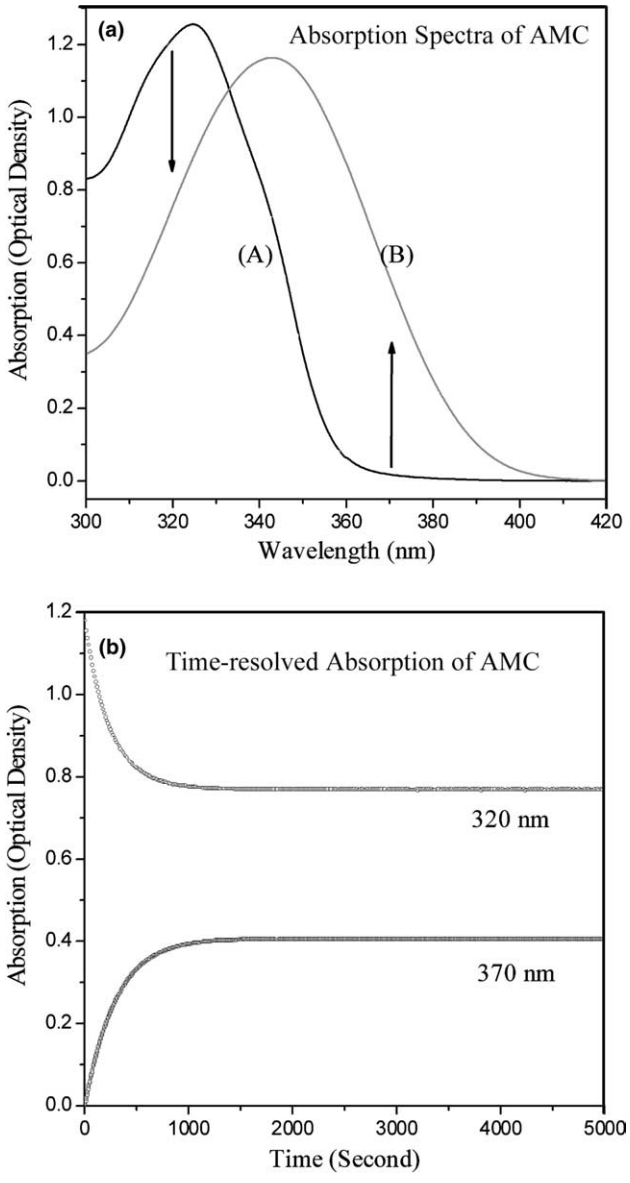

Fig. 2. (a) Absorption spectra of the substrate (A) and the released coumarin derivative product (B). Arrows indicate the wavelengths (320 and 370 nm), which were used to monitor the time-resolved absorbances as shown in (b). (b) The absorbances at 320 nm (top) and 370 nm (bottom) as a function of time. Decay in the short wavelength (320 nm) and rise in the long wavelength (370 nm) indicate a spectral shift due to catalytic activity on the substrate.

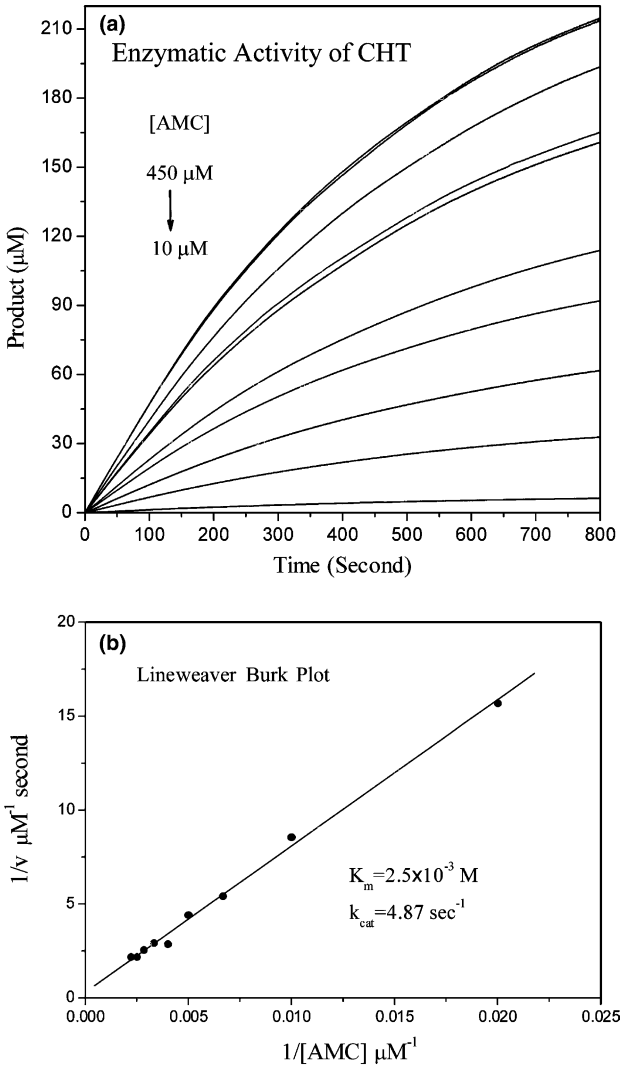

Fig. 3. (a) The concentrations of the product as a function of time at various substrate concentration. The enzyme concentration was kept constant at 668 nM. (b) Lineweaver–Burk plot of the enzyme CHT showing its catalytic activity with substrate AMC in aqueous buffer pH 7.0. The solid line is a linear fit following Michaelis–Menten kinetics.

3. Results and discussion

Fig. 3b depicts the Lineweaver–Burk plot, where the reciprocal of the reaction velocity (v) is plotted as a function of the reciprocal of the initial concentration of AMC in the aqueous buffer solution. The linear behavior is appropriate because the kinetics is in the Michaelis–Menten regime where the concentration of the substrate is much higher than that of the enzyme CHT ([E]). This allows one to obtain the dissociation and rate constants, (K_m) and k_{cat}, respectively. From the slope (K_m/v_{max}; v_{max} is the maximum velocity) and intercept ($1/v_{max}$) of the numerical fitting, the values of K_m and k_{cat} ($v_{max}/[E]$) were found to be 2.53 mM and 4.87 s^{-1}, respectively. We would like to mention here that one of the early studies [12] of enzyme–substrate

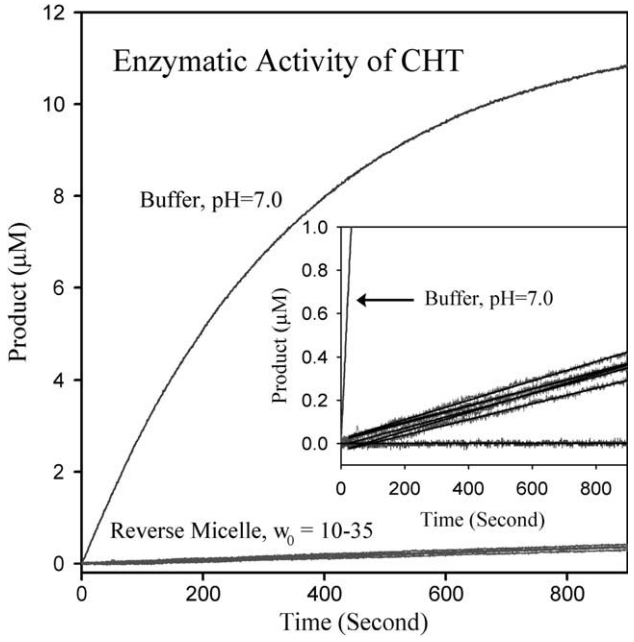

Fig. 4. The concentrations of the product as a function of time in *aqueous buffer* and in *reverse micelle* at various degrees of hydration. Inset shows that the catalytic rate of the enzyme CHT in reverse micelle is nearly *insensitive* to the degree of hydration. The data points *approximately* horizontal to the time axis indicate that no catalytic reaction has taken place in the absence of the enzyme. The data obtained for buffer at pH 7 is also shown (indicated by an arrow) in the inset for comparison.

kinetics involving same substrate (AMC) reported that the values of K_m and k_{cat} are 0.5 mM and 0.83 s^{-1}, respectively.

However, information reported in [12] is rather limiting. The source of the chymotrypsin was not mentioned clearly; being obtained from Worthington Corporation. The enzyme assays were conducted at 24 °C (in our case 25 °C) utilizing 50 mM TES buffer, pH 8.0, containing 10 mM calcium chloride and 1% dimethyl sulfoxide (DMSO) in a final assay volume of 1 ml. The substrate was initially dissolved in 100% DMSO. No kinetic plot or additional information was provided in this article except values for the K_m and k_{cat}. The variation in the experimental conditions is most likely to be the reason for the observed differences between the results reported in this Letter and those in [12].

In Fig. 4, we show the concentration of the product (measured from the absorbance at 370 nm) as a function of time for different environmental conditions of the enzyme–substrate complex. It is clear from the above figure that the changes in product concentration (obtained from the measured absorbance at 370 nm) in RM are relatively small (inset of Fig. 4). However, the variations in slopes are distinguishable even when the absorbance is plotted as a function of time for all the cases studied. In order to obtain the rate of hydrolysis of the substrate AMC by CHT in RM, the extinction coefficients of the free product coumarin were measured at various values of w_0. The extinction coefficients varied approximately linearly in AOT-RM from 11.19 mM^{-1} cm^{-1} at $w_0 = 10$ to 12.3 mM^{-1} cm^{-1} at $w_0 = 35$. The velocities of enzymatic reactions estimated from the changes in product concentrations with time, in the aqueous buffer and in RM are tabulated (Table 1). From Table 1, it is clear that in reverse micelle the velocity of enzymatic reaction is about two orders of magnitude slower than that found in the aqueous medium. However, the velocity of the reaction is independent of the degree of hydration (w_0) of the RM.

Given that the global structure of CHT remain almost the same in all the w_0 values studied here [7], the present observation clearly indicates that the retardation of the enzymatic activity in the RM is *not* due to the structural perturbation of the enzyme in the water pool of the RM. Reaction may also be slowed down through inhibition of active sites in enzyme when it is encapsulated in RM. The active-site titration study mentioned in [7] indicated that at higher water contents, the number of active sites of CHT in AOT-RM is approximately constant at 80% of the value in aqueous solution. The active site concentration of CHT in aqueous solution was measured to be 63% of the total existing catalytic sites [7]. The number of active sites is therefore *not* equal to the total number of catalytic sites (equal to the number of protein molecules) in aqueous solution. Note that approximately 80% (as mentioned in [7]) of the active sites existing in aqueous solution is available for enzymatic reaction in RM. It is therefore expected that the 20% reduction in available catalytic sites compared to that in bulk may not account for the observed retardation of reaction in RM. The cause of the retardation of enzymatic activity in RM is yet to be conclusively determined. Several mechanisms have, however, been conjectured in the literature ([13] and references therein). These include: (i) the lower degree of hydration (compared to aqueous buffer) make the enzyme relatively rigid [4] and decreases its functionality; (ii) the modification of the apparent pK_a at the active site of the en-

Table 1
Velocity (v, μM s^{-1}) of the catalytic reaction of the enzyme CHT in aqueous *buffer* and *reverse micelle*

Medium	Velocity (v, μM s^{-1})
Aqueous buffer, pH 7.0	2.9×10^{-2}
AOT reverse micelle	
$w_0 = 10$	4.0×10^{-4}
$w_0 = 15$	4.4×10^{-4}
$w_0 = 20$	4.0×10^{-4}
$w_0 = 25$	4.5×10^{-4}
$w_0 = 30$	3.6×10^{-4}
$w_0 = 35$	3.8×10^{-4}

The concentrations of the enzyme and the substrate were 1 and 14 μM, respectively.

zyme due to polarity changes in the interior water pool; (iii) immobilization of the enzyme due to electrostatic interactions between the enzyme and the surfactant molecules; (iv) distribution of the substrate in RM (interface versus water-pool) and the partitioning of the population of the substrate in the organic and aqueous RM. This may lead to enzyme–substrate complexation less probable.

The distribution of the substrate (at various w_0) is expected to affect the enzymatic activities of CHT in reverse micelle in a very important manner. For example, a larger distribution in the *n*-heptane compared to that in RM might result in an apparent reduction of the activity. However, the substrate studied here is sparingly soluble in bulk *n*-heptane, but soluble in AOT–*n*-heptane solution. It is therefore unlikely that the majority of the substrate population would reside in the organic phase. Rather, it would like the interface. Our preliminary experiments show a red shift (due to solvatochromism) of about 5 nm only (peak-position) when the environment changes from $w_0 = 0$ to 35 (highest degree of hydration studied here). This observation, however, cannot specifically indicate whether the substrate is located entirely inside the RM or exploring various layers of water-pool. Dynamical studies coupled with computer simulation will be able to bring out microscopic details of such events. It should be noted here that some of the mechanisms mentioned above could contribute to the enzymatic activity in a positive way, i.e. increase the substrate specificity and/or reduce substrate inhibition and hence increase overall catalytic efficiencies of enzymes [13,14].

For other substrates in the RM involving hydrolysis of *N*-glutaryl-L-phenylalanine *p*-nitroaniline, *p*-nitrophenyl acetate and *p*-nitrophenyl caprylate by CHT a bell-shaped dependencies of enzymatic activity on the degree of hydration (w_0) with a maximum around $w_0 = 10$ were reported [7,8]. Our studies do not find any significant dependency (Table 1) of the catalytic activity on w_0. Overall enzymatic activity is the convoluted effect of enzyme flexibility (hydration), which is maximum in the aqueous buffer and the efficiency of enzyme–substrate complexation. The present observation of constant catalytic activity on the variation of w_0 reveals that other mechanisms as detailed below have to be taken into account along with the hydration of the enzyme in the RM. First, from X-ray small-angle scattering experiment it has been demonstrated [8] that after inclusion of CHT into the AOT-RM, the enzyme resides in the water pool and *not* at the interface. The study is in agreement with previous reports on the location of CHT as observed by the kinetic decay measurement of hydrated electrons ($w_0 > 20$) [15] and fluorescent active-site probing [16].

Given the fact that the size of the enzyme-included water pool increases with w_0 [17], any surface affinity of the substrate would expectedly increase enzyme–substrate distance. This will effectively increase the free energy barrier between the enzyme and the substrate. Thus, enhanced flexibility of the enzyme [4] and decreased efficiency of enzyme–substrate complexation with increasing w_0 may make the overall catalytic activity apparently independent of the degree of hydration. Second, in a recent work based on small angle neutron diffraction experiment [18] it has been concluded that there is no variation of protein-filled micellar size except at low hydration level ($w_0 < 5$). The conclusion is further supported by time-resolved fluorescent active-site probing [16]. These experimental findings are in contrast to the model described above and depicted in [17] which predicted an almost linear dependence of the core radius of protein-filled micelle with $w_0 = 5 - 25$. Thus, in first approximation one can expect that the enzyme activity will be unaffected at various water content (w_0), because the size of the enzyme-filled droplet is the same in the regime $10 < w_0 < 30$ [16]. This is consistent with our observation.

4. Conclusion

There is a wealth of information available concerning the protein encapsulated in the AOT reverse micelle, such as structure of a protein at various water contents, possible location of a protein in the water pool and catalytic behavior of a protein inside the RM. From a number of reports involving different experimental techniques it has been confirmed that structure of CHT in AOT RM with higher hydration degree ($w_0 \geqslant 10$) remains unperturbed. In this study, we measured the activity of the protein CHT to a substrate AMC in AOT RM and compared them with that in bulk water (aqueous buffer). We found the rates of catalytic reactions in the RM at a constant enzyme concentration are retarded by, at least, two orders of magnitude compared to that in bulk water (buffer). In this way, we examined the effects of the degree of hydration on reactivity of the protein, since the structure were found to be unperturbed for all these RM [7]. Currently, we are examining the effect of degree of hydration at the different sites of the enzyme; e.g. at the surface, site of the catalytic center and also probing environmental information around the substrate included in the RM. Theoretical studies aided by molecular dynamics simulations will render a better understanding at the microscopic level of this phenomenon.

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