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EFFECT OF MASS TRANSFER LIMITATION ON THE ENZYME REACTION IN REVERSED MICELLE

Hak-Sung KIM*, M.D. LEGOY, and D. THOMAS

* Dept. of Biological Science & Eng., KAIST, Seoul, Korea
Dept. de Genie Biologie, Univ. de Tech. de Compiegne, B.P. 233, Compiegne, France
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Abstract—The theoretical and experimental studies concerning mass transfer effect on enzyme reaction in reverse micelle were carried out. Firstly, the theoretical analysis showed that in the case of Michaelis-Menten kinetics, the apparent Michaelis constant increased with increasing substrate transfer limitation, but the apparent maximum reaction rate was constant. The experimental evidence obtained for the trypsin catalysed hydrolysis in reverse micelle by Martinek et al. [1] supports the results of our theoretical analysis.

Secondly, an experimental result demonstrating that the substrate transfer across the interface was a rate limiting factor in the enzyme reaction was obtained. When the horse liver alcohol dehydrogenase activity was determined by the rate of increase in fluorescence intensity of NADH, two different slopes were observed and this could be due to the rate limitation of substrate transfer across the interface. As the carbon number of primary alcohol is increased, the ratio between two slopes (second slope over first one) which represents the degree of substrate transfer limitation increases, except for heptanol and octanol. This result could be explained by the rate limitation of substrate transfer and substrate specificity of the enzyme: As the substrate specificity of the enzyme is increased, the limitation of substrate transfer becomes more severe.

INTRODUCTION

Enzymes are usually used in aqueous environments, while, in case of apolar substrate, it is desirable to carry out the enzymic reaction in the presence of non-polar solvent to solubilize the apolar compound. The organic solvents, however, denature the enzyme very rapidly. In order to overcome this problem, the micellar solubilization of enzymes in hydrocarbon solvents with the help of surfactants has been studied [2-4].

The micellar enzymology has two main potential applications. They are the enzymic transformation of lipophilic, water-insoluble, substrates [5-6] and its use as a model system where the confinement of enzymes in the water pool of reverse micelle could help the basic research to simulate the enzyme action in living system [7].

Although a great deal of research on the micellar enzymology has been carried out, several important questions remain to be explained, i.e., the conformational change of enzyme in reverse micelle, its relationship with substrate specificity of the enzyme, and mass transfer effect on the kinetics of an enzyme reaction.

The major advantage of using biphasic system, such as reverse micelle (microemulsion) is that the water-insoluble substrate can be solubilized in the organic solvent. In this case, the substrate transport across the interface could be very low and might become a rate limiting factor in enzyme reaction. Until now, mass transfer effect on enzyme kinetics in reverse micelle has not been studied carefully.

In this paper, we analyze and report how the transport of water-insoluble substrate across the micellar interface could affect the kinetics of an enzyme reaction in reverse micelle.

THEORETICAL CONSIDERATION

Here we shall consider a reaction between enzyme and substrate that obeys the Michaelis-Menten kinetics. In our theoretical approach, the followings are assumed; (a) a solution of a surfactant consists of two phases, i.e., of a bulk phase of an organic solvent and a phase of micelles wetted by water, (b) the structure of both micelle and enzyme are ideally spherical, (c) the enzyme molecule exist in water pool of reverse micelle, and (d) the substrate is poorly water-soluble.

The schematic diagram of reverse micelle and con-

*To whom all correspondence should be addressed.

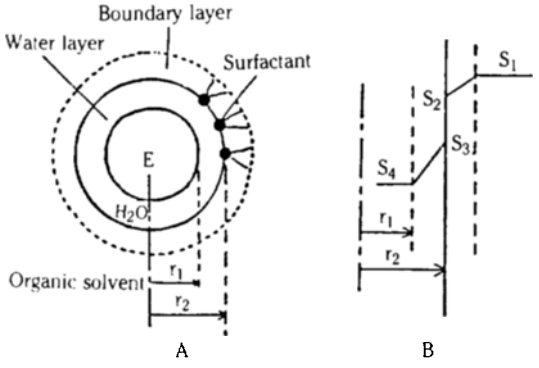

Fig. 1. The schematic diagram of enzyme-containing reverse micelle (A) and the concentration profile of substrate (B).

S_1, S_2, S_3, and S_4 represent the substrate concentrations at each point and r_1 is the inner core radius and r_2 the radius between the center and interface. E represents the enzyme molecule in water pool.

centration profiles of substrate at steady-state are described in Fig. 1.

The substrate transfer flux J from the bulk organic phase to interface will be

$$J = hA(S_1 - S_2) \tag{1}$$

where h; substrate transfer coefficient
A; interfacial area
S_1, S_2; substrate concentrations

The partition of substrate between two phases gives the partition coefficient defined by the following equation.

$$P = S_3/S_2 \tag{2}$$

The diffusional flux through a water layer is written by

$$J = D_s/\Phi \cdot (S_3 - S_4) \tag{3}$$

where D_s; diffusion coefficient of substrate
Φ ; geometric factor defined by $r_1^2(1/r_1 - 1/r_2)$

The reaction rate of enzyme in aqueous phase (water pool) is

$$R = \frac{V_m S_4}{K_m + S_4} \tag{4}$$

where V_m; intrinsic maximum reaction rate
K_m; intrinsic Michaelis constant
S_4 ; substrate concentration in water pool

In order to simplify the above equations, following dimensionless variables are introduced,

$$\sigma_1 = \frac{S_1}{K_m}, \quad \sigma_2 = \frac{S_2}{K_m}, \quad \sigma_3 = \frac{S_3}{K_m}, \quad \sigma_4 = \frac{S_4}{K_m}$$

$$\delta = \frac{\sigma_3}{\sigma_2}, \quad \mu = \frac{V_m}{hAK_m}, \quad \nu = \frac{\Phi V_m}{D_s K_m}, \quad \zeta = \frac{R}{V_m}$$

The term σ represents the dimensionless substrate concentration, the terms μ and ν the dimensionless substrate transfer modulus or modified Thiele modulus and ζ the dimensionless reaction rate. Under steady-state, the substrate transfer flux will be equal to the enzyme reaction rate, i.e., J = R, and the equations (1), (2), (3), and (4) can be rewritten in dimensionless forms.

$$\zeta = \frac{1}{\mu}(\sigma_1 - \sigma_2) \tag{5}$$

$$\delta = \frac{\sigma_3}{\sigma_2} \tag{6}$$

$$\zeta = \frac{1}{\nu}(\sigma_3 - \sigma_4) \tag{7}$$

$$\zeta = \frac{\sigma_4}{1 + \sigma_4} \tag{8}$$

Equation (8) can be rewritten in terms of measurable bulk substrate concentration instead of concentration in aqueous phase, i.e., σ_4 can be replaced by σ_1. From equations (5), (6), and (7), we obtain,

$$\sigma_4 = \delta\sigma_1 - (\delta\mu + \nu)\zeta \tag{9}$$

Here we define the new dimensionless variables $\gamma = \delta\mu + \nu$. This variable represents the dimensionless overall substrate transfer modulus. Equation (9) can be rewritten as follows:

$$\sigma_4 = \delta\sigma_1 - \gamma\zeta \tag{10}$$

The insertion of σ_4 into equation (8) yields

$$\zeta = \frac{\delta\sigma_1 - \gamma\zeta}{1 + \delta\sigma_1 - \gamma\zeta} \tag{11}$$

We now solve the equation (11) to obtain the ζ and

$$\zeta = \frac{1}{2\gamma}[(1 + \gamma - \delta\sigma_1) - \{(1 + \gamma + \delta\sigma_1)^2 - 4\gamma\delta\sigma_1\}^{1/2}] \tag{12a}$$

$$= 2\delta\sigma_1[(1 + \gamma + \delta\sigma_1) + \{(1 + \gamma + \delta\sigma_1)^2 - 4\gamma\delta\sigma_1\}^{1/2}]^{-1} \tag{12b}$$

since $0 \le \zeta \le 1$

In general, for the homogeneous enzyme reaction system, the maximum reaction rate is defined as the reaction rate at a high substrate concentration or $S \to \infty$ and the Michaelis constant is the substrate concentration at which the reaction rate is half the maximal. If these concepts are applied to reverse micellar system, then we can define that

$$\alpha^* = \lim_{\sigma_1 \to \infty} \zeta \tag{13}$$

$$\beta^* = \sigma_1 \mid_{\zeta = 0.5\alpha^*} \tag{14}$$

where α^* represents the apparent maximum reaction rate and β^* apparent Michaelis constant in dimensionless form.

In order to obtain α^* and β^*, the equation (12b) is introduced into above definition, and we have

$$\alpha^* = 1.0$$

$$\beta^* = \frac{1}{\delta}(1 + 0.5\gamma)$$

Based on our theoretical analysis, we found that the apparent maximum reaction rate, α^*, is constant and the apparent Michaelis constant, β^*, can be varied by the partition coefficient, δ, and overall substrate transfer modulus, γ.

EXPERIMENTAL METHODS

1. Materials

Bis (2-ethylhexyl) sodium sulfosuccinate (AOT) was obtained from Fluka (Swiss) and purified as described elsewhere [8]. Isooctane and primary alcohols (butanol-nonanol) purchased from Fluka were used without further purification. Horse liver alcohol dehydrogenase and NAD(H) were obtained from Sigma(U.S.A.)

2. Methods

2-1. Preparation of reverse micelle

A microliter amount of concentrated solution of enzyme with or without NAD was injected into 1 mL of a 50 mM AOT-containing isooctane and this solution was gently stirred in Vortex mixer until the solution became clear. The desired water content was obtained by an additional injection of the same buffer solution into the micellar solution. It was then shaken gently until clear solution (no scattering at 320 nm) was obtained. The water content was expressed in terms of volume percent or molar ratio of water to AOT ($W_0 = [H_2O]/[AOT]$). 0.1 M NaOH-glycine buffer was used for enzyme reaction in reverse micelle.

2-2. Enzyme activity measurement

Horse liver alcohol dehydrogenase activity was determined by measuring the initial rate of increase in fluorescence intensity of NADH in 1 cm pathlength cuvette. Fluorescence intensity was measured by using spectrofluorimeter (Perkin-Elmer, U.K.), excitation at 340 nm, and emission at 460 nm. The 10 nm passing band was used for both excitation and emission. This method is more accurate and reproducible than conventional absorbance measurement. The enzyme reaction was initiated by adding substrate solubilized in AOT-isooctane solution to the micellar solution prepared as mentioned above. Unless otherwise noted, the overall concentration of enzyme, NAD, and substrate were 1 μM, 100 μM, and 40 mM respectively.

2-3. Fluorescence spectra of enzyme

Fluorescence spectra of enzyme either in micellar or in buffer solution was obtained at a excitation of 296 nm, and 5 nm passing band was used for both excitation and emission for the precise resolution. Tris-HCl (0.1 M) at pH 7.2 was used. The maximum wavelength of emission was determined by the fluorimeter equipped with a Prescan.

EXPERIMENTAL RESULTS

1. Test of substrate transfer rate by fluorimetry

When the fluorescence intensity of NADH was followed after initiation of enzyme reaction in reverse micellar solution, two different slopes were observed. Typical raw data for hexanol was shown in Fig. 2. However, ethyl alcohol showed linear behavior in the same time scale due to higher solubility of ethyl alcohol in water (data not shown).

2. Effect of molecular size of primary alcohol on both enzyme activity and substrate transfer rate

Horse liver alcohol dehydrogenase generally has a broad substrate spectrum. The primary alcohols (butanol-nonanol) were tested as substrate, and both en-

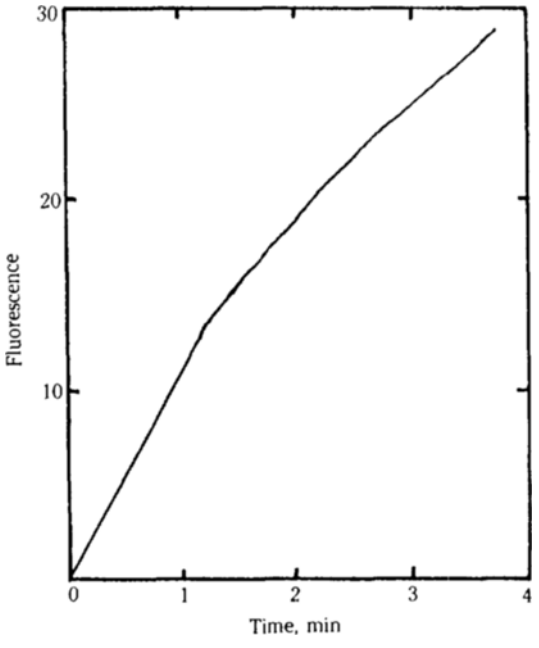

Fig. 2. Variation of fluorescence intensity of NADH as a function of time.
40 mM of hexanol was used as substrate.

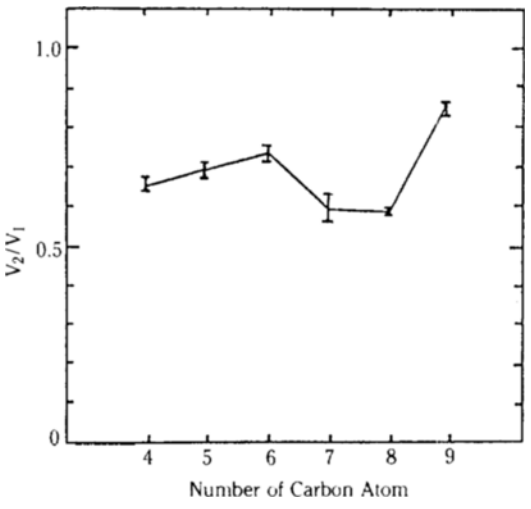

Fig. 3. Effect of carbon number of primary alcohol on the ratio between two slopes obtained from the fluorimetry.
Experimental conditions; W_0 = 16.7, pH = 9.6 and the concentration of substrate is 40 mM.

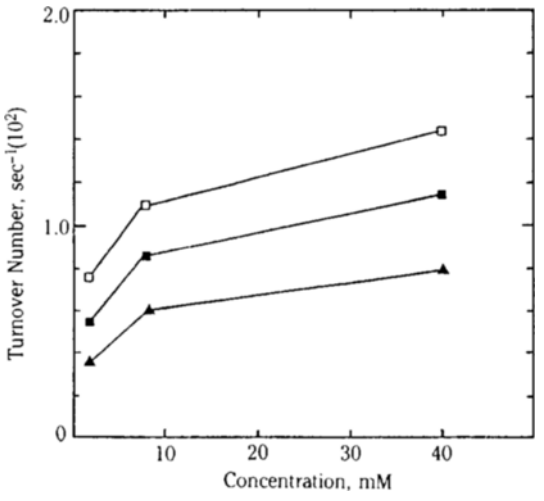

Fig. 5. Effect of substrate concentration on the enzyme activity.
Experimental conditions; W_0 = 16.7, pH = 9.6. The substrates were; pentanol (■), heptanol (□), nonanol (▲).

zyme activity and ratio between the two slopes (second slope over first one) were obtained from the fluorimetry reading.

As shown in Fig. 3 and Fig. 4, the ratio increased with increasing carbon number of primary alcohol except for the heptanol and octanol, but the enzyme activity decreased. The enzyme activity was determined by the first slope of the fluorimetry reading.

The modification of substrate specificity of the enzyme in micellar solution was observed in case of heptanol and octanol: The substrate specificity of enzyme for both heptanol and octanol was enhanced exceptionally.

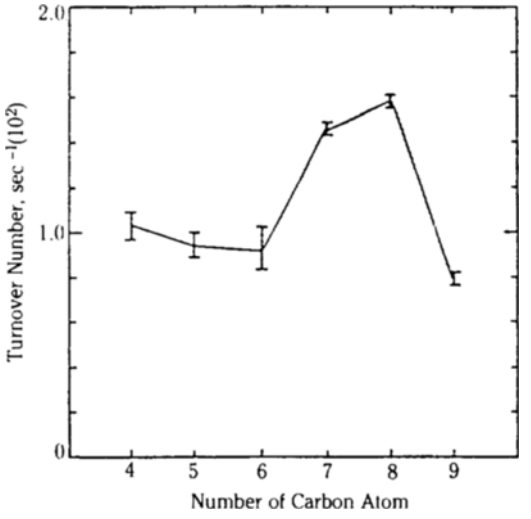

Fig. 4. Effect of carbon number of primary alcohol on the enzyme activity.
The experimental conditions were the same as those used in Fig. 3.

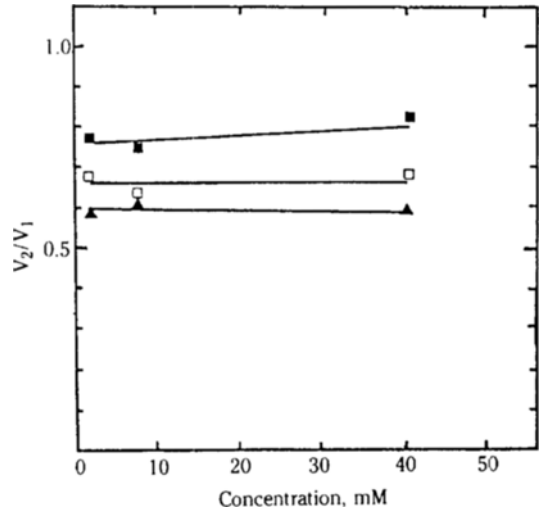

Fig. 6. Effect of substrate concentration on the ratio between two slopes; W_0 = 16.7, pH = 9.6.
The substrates were; pentanol (□), heptanol (▲), nonanol (■).

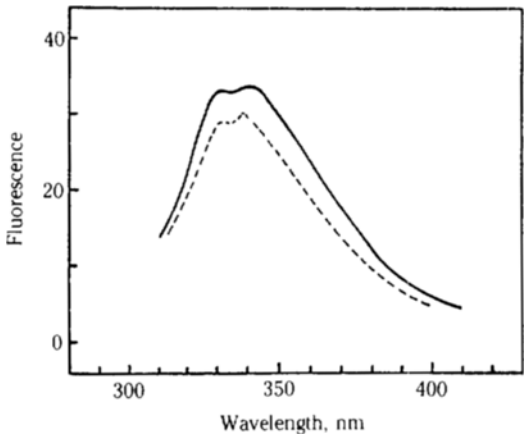

Fig. 7. Fluorescence spectrum of enzyme in buffer solution (—) and in micellar solution (---).
The excitation was at 296 nm.

3. Effect of substrate concentration on both enzyme activity and ratio between the two slopes

The enzyme activity was increased with increasing substrate concentration in organic phase of micellar solution (Fig. 5), but the ratio between two slopes remained practically constant (Fig. 6). Enzyme reaction in micellar solution obeyed Michaelis-Menten kinetics (data not shown).

4. Fluorescence characteristics of enzyme

When enzyme was excited at 296 nm in buffer solution, the maximum wavelength of emission at 329 nm and 340 nm were observed, but in micellar solution, the two maxima were at 329 nm and 335 nm (Fig. 7). A slight reduction in fluorescence intensity in micellar solution was also observed.

DISCUSSION

Based on the theoretical analyses, the apparent maximum reaction rate, α^*, is constant, and the apparent Michaelis constant, β^*, can be varied by the partition coefficient, δ, and overall mass transfer modulus, γ. If the substrate is highly hydrophobic ($\delta \ll 1$), the apparent Michaelis constant could increase greatly at constant γ. In reality, δ can be as low as 10^{-2}–10^{-4} for a poorly water-soluble substrate, so the apparent Michaelis constant could increase as much as 10^2–10^4 times. When we examine the variation of the apparent Michaelis constant at a fixed substrate, i.e., δ is constant, the apparent Michaelis constant increase with increasing mass transfer limitation (i.e., with increasing γ).

When poorly water-soluble substrate is solubilized in organic phase of micellar solution, the substrate transfer rate across the interface is very low in comparison to that of enzyme reaction. Consequently, the substrate transfer across the interface becomes a rate limiting factor in enzyme reaction. Recently, Martinek et al. determined the kinetic parameters as function of water content in reverse micelle by using water soluble substrate [1]. They observed that the apparent maximum reaction rate did not change although the apparent Michaelis constant increased with increasing water content. In this case, the water content in reverse micelle can be related to substrate transfer resistance. The structural model and experimental results for protein-containing reverse micelle obtained by Bonner et al. show that the augmentation of water content increases the micelle size and consequently the thickness of water layer [9]. For example, the inner core radius is 15.5 Å and thickness of water layer is 5 Å at $W_0 = 5$. As water content increase to $W_0 = 20$, the inner core radius is 15.5 Å and the thickness of water layer increases to 22 Å. The mass transfer resistance is directly proportional to the thickness of water layer, and the augmentation of water content is attributable to the increase in mass transfer limitation. Consequently, the apparent Michaelis constant increases with increasing water content.

For the water-soluble substrate, it is not clear whether the mass transfer limitation exist or not in biphasic system. On the other hand, for the immobilized enzymes in homogeneous system, the external diffusion limitation cause the variation of kinetic constant. In the case of Michaelis-Menten kinetics, the apparent Michaelis constant increases with increasing mass transfer limitation, but apparent maximum reaction rate is fairly constant [10].

In view of the analyses presented, it seems possible that a serious mass transfer limitation could exist in reverse micellar system as though the water-soluble substrate is used. The experimental results by Martinek et al. could be explained by our theoretical model and analysis.

Another experimental evidence supporting above results was obtained from the fluorimetric determination of enzyme activity in reverse micelle. When enzyme activity was measured by fluorescence intensity of NADH, two different slopes were observed. This could be explained as follows. As the water-insoluble substrate is added to micellar solution, the substrate is quickly partitioned between the two phases. The initiation of enzyme reaction decreases the substrate concentration in the water-pool of reverse micelle progressively. The substrate transfer rate across the interface is lower than the substrate consumption rate by enzyme reaction, and the second slope with a reduced rate appears. It is possible that this observation is an indication that the substrate transfer across the interface is a rate-limiting factor in enzyme reaction in re-

verse micellar system.

The ratio between the two slopes (second slope over first one) depends upon both the substrate specificity of the enzyme and substrate transfer rate across the interface. The increase of ratio between the two slopes, except for heptanol and octanol, as increasing the carbon number of alcohol could be related to the substrate specificity of the enzyme. If the substrate specificity of the enzyme decrease, the substrate consumption rate by enzymic reaction in aqueous phase would also decrease. Consequently, the ratio between the two slopes increase. It means that the mass transfer limitation becomes less severe as the substrate specificity of the enzyme decrease. In the case of heptanol and octanol, the substrate specificity of the enzyme appears to be significantly higher, consequently, the ratio decreases. This could be due to the fact that as the substrate consumption rate by enzymic reaction increase, the enzymic reaction is more severely limited by substrate transfer rate across the interface. In view of above points, the ratio between the two slopes represent the degree of mass transfer limitation. With increasing the mass transfer limitation, the ratio would become smaller.

It is estimated that the interfacial area in micellar system is about 100 m^2/ml and the effect of diffusion limitation is greatly decreased in comparison to all other systems that have been devised for the enzymic conversion of non-polar compounds [5]. However, when water-insoluble compounds are solubilized in micellar solutions, the substrate transfer rate is very low in comparison to enzymic reaction rate, and it could easily become the rate limiting factor.

The modification of substrate specificity of the enzyme was observed in this work. Martinek et al. also observed the modification of substrate specificity of the enzyme [7]. The micellar effect on the catalytic activity and specificity of the enzyme may be due to; (a) microenvironmental effects in the micelle on the reactivity of amino acid groups of enzyme or substrate molecules, (b) conformational alteration of the enzyme structure, and (c) partition of the substrate or other molecules involved in the reaction between the aqueous and organic phase as well as the surface layer of the micelle. Horse liver alcohol dehydrogenase has two tryptophan residues: Trp-15 is situated on its periphery and trp-314 is buried within the hydrophobic region [11]. In our fluorescence studies, the peak fluorescence emission contributed by trp-15 was shifted by about 5 nm to a shorter wavelength, but that of trp-314 was not shifted. It is likely that trp-15 was exposed to more hydrophobic environment in micellar system so that only a slight conformational change of enzyme occurred, consequently, the substrate specificity of the enzyme was slightly modified. Further study will be required to ascertain the extent of modification of substrate specificity of the enzyme in micellar system.

With decreasing the substrate concentration in organic phase, the enzyme activity was also decreased, but the ratio did not varied. This could be due to fact that when the substrate concentration in organic phase decreased, both enzyme reaction rate and substrate transfer rate decreased, thus, the ratio between the two slopes remained constant.

Our approach could elucidate the mass transfer effect on the kinetics of an enzyme action in reverse micelle and reveal the system such as reverse micelle (micro-emulsion) in which the mass transfer across the interface is a rate limiting factor in enzyme reaction. The analogous examination could be applied to other enzyme reaction system with different kinetics. Based on this kind of analyses presented here, one may be able to determine the advantage and/or disadvantage of using the reverse micelle system for biotransformation by enzymic reactions of known kinetics. For the practical use of micellar system, further careful studies on the stabilization of enzyme in micellar solution as well as the mass transfer problems at the interface of reverse micelle will be essential.

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