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Kinetic Studies on Pancreatic Lipase Activity in Micellar Systems. III.[1] Effect of Micellar Size

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Pancreatic lipase activities toward fatty acid vinyl esters solubilized in micelles of five surfactants, sodium taurodeoxycholate (NaTDC), octa-oxyethylene dodecyl ether (ODE), and three polyoxyethylene nonylphenyl ethers (PNE), were kinetically investigated. The kinetic data for all the micellar systems were well interpreted on the basis of fully competitive inhibition mechanisms, where substrate-free micelles act as an inhibitor.

For NaTDC micellar systems, the maximum velocity V, the Michaelis constant K_m, and inhibition constant K_4 were determined with respect to four substrates. The K_m/K_4 values for all the substrates were larger than unity but smaller than those previously obtained for sodium deoxycholate micellar systems.

For the micellar systems of ODE and two PNEs with average numbers of oxyethylene units of 10 and 15, the Michaelis plots at constant surfactant concentrations were linear, indicating that the K_m/K_4 value is close to unity. On the other hand, for PNE with an average number of oxyethylene units of 30, a similar plot was concave, which suggests that the K_m/K_4 value is larger than unity.

The alteration of the K_m/K_4 value with surfactants was closely correlated with the change in stability of the enzyme–micelle solubilizing substrate complex relative to the enzyme–substrate-free micelle complex; the relative stability was shown to depend on the micellar size. The maximum velocity was also affected by micellar size.

Keywords——pancreatic lipase; sodium taurodeoxycholate; octa-oxyethylene dodecyl ether; polyoxyethylene nonylphenyl ether; fatty acid vinyl ester; heterogeneous enzymatic reaction; fully competitive inhibition; binding constant; micellar size

We have studied the pancreatic lipase-catalyzed hydrolysis of a fatty acid vinyl ester solubilized in sodium deoxycholate (NaDC) micelles and have elucidated the mechanism of the enzymatic reaction.[2] The effects of fatty acid chain length of the substrates on the maximum velocity (V) and the Michaelis constant (K_m) have been evaluated.[1] Substrate-free micelles inhibit the enzymatic reaction by competing with micelles solubilizing substrate for the enzyme, so that the reaction depends significantly on the stability of the enzyme–micelle solubilizing substrate complex relative to that of the enzyme–substrate-free micelle complex. The stability of the former complex is less than that of the latter complex and is affected by the fatty acid chain length of the substrates. This is probably because the size of the NaDC micelle is so small that both the enzyme and substrate cannot bind simultaneously to the micelle with their characteristic affinities. Thus it is expected that an increase in the micellar size will reduce the difference between the stabilities of the two complexes mentioned above. Other possible effects of micellar size on the enzymatic reaction should also be considered. Thus, it is of interest to investigate the enzymatic reaction in micellar systems of various surfactants with different aggregation numbers.

In this work, pancreatic lipase activities toward fatty acid vinyl esters solubilized in

micelles of five surfactants, sodium taurodeoxycholate (NaTDC), octa-oxyethylene dodecyl ether (ODE), and three polyoxyethylene nonylphenyl ethers (PNE) with different average lengths of polyoxyethylene chain, were kinetically investigated.

Experimental

Materials——Pancreatic lipase was a product of Sigma Chemical Co., lipase Type VI from porcine pancreas. According to the supplier, the specific activity is 340 unit/mg protein using olive oil at pH 7.7 and 37 °C. Vinyl octanoate (VO), vinyl decanoate (VD), vinyl dodecanoate (VDD), and vinyl tetradecanoate (VTD) were of high purity (>99%) and were used without further purification. NaTDC from Sigma Chemical Co. was recrystallized twice from methanol–ethanol and then dried *in vacuo* at 110 °C. ODE was a homogeneous product from Nikko Chemicals Co. and was used without further purification. PNE (10), PNE (15), and PNE (30), which are heterogeneous PNEs with average numbers of oxyethylene units of 10, 15, and 30, respectively. were obtained from Tokyo Kasei Kogyo Co. and Nippon Oils & Fat Co. Rhodamine 6G was obtained from Katayama Chemical Co. Water was purified as described previously.[2] All other chemicals were of reagent grade.

Determination of Critical Micellar Concentration——The critical micellar concentration (cmc) of NaTDC was determined by the spectral shift method with Rhodamine 6G.[3] The cmc's of nonionic surfactants (ODE and PNEs) were not determined because all the values are expected to be less than *ca.* 0.2 mM,[4] *i.e.*, negligible compared to the total surfactant concentration (see Eq. 5).

Kinetic Measurement——In all the surfactant micellar systems, the total substrate concentrations were chosen so as not to exceed the total micelle concentration, and thus the solubilization of more than two substrate molecules per micelle can be neglected. Kinetic measurements were carried out by essentially the same method as described previously.[1,2] However, to obtain an appropriate initial rate, three concentrations of the enzyme stock solutions were used for the different surfactants: 0.005% for PNE (30), 0.0075% for NaTDC, and 0.05% for ODE, PNE (10), and PNE (15). The initial rate (v) was calculated from the liner absorption–time curves in the presence and in the absence of lipase.[2] Thus, v does not include any contribution from non-enzymatic reaction.

Results

NaTDC Micellar Systems

The enzyme activities towards VDD as a substrate were measured for various NaTDC concentrations at several constant substrate concentrations. When the initial rates were plotted against NaTDC concentration, hyperbolic curves were obtained as in the case of the NaDC micellar system reported previously[1] (Fig. 1).

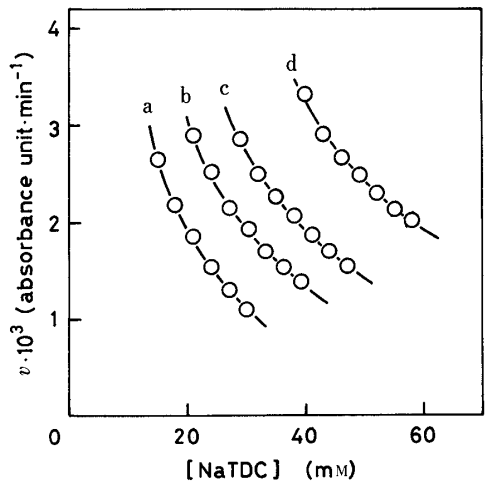
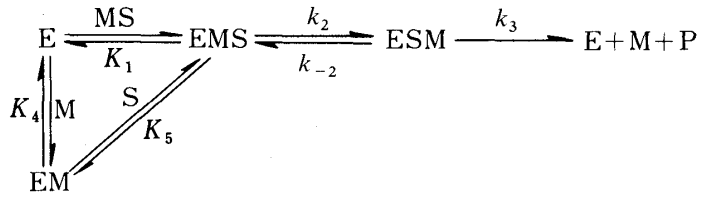


Fig. 1. Plots of Initial Rate *vs.* NaTDC Concentration at Several VDD Concentrations

Concentrations of VDD: a, 0.50 mM; b, 0.75 mM; c, 1.00 mM; d, 1.50 mM.
Conditions: Tris-HCl buffer 40 mM, NaCl 0.18 M, pH 8.0, 25 °C.

The pancreatic lipase-catalyzed hydrolysis of a fatty acid vinyl ester in NaDC micellar systems follows the fully competitive inhibition mechanism shown in Chart 1, and the initial rate is given by Eqs. 1, 2, or 3.[1,2]



E:	lipase	MS:	micelle solubilizing substrate
S:	substrate	EM:	lipase–micelle complex
M:	micelle	EMS:	lipase–micelle solubilizing substrate complex
P:	products	ESM:	lipase–substrate complex at micelle interface

Chart 1

$$v=\frac{V[\mathrm{MS}]}{K_m(1+[\mathrm{M}]/K_4)+[\mathrm{MS}]} \tag{1}$$

$$\frac{1}{v}=\frac{K_m}{K_4V[\mathrm{MS}]}\cdot[\mathrm{M}]+\frac{1}{v}(1+K_m/[\mathrm{MS}]) \tag{2}$$

$$\frac{1}{v}=\frac{K_m(K_4+[\mathrm{M}])}{K_4V}\cdot\frac{1}{[\mathrm{MS}]}+\frac{1}{V} \tag{3}$$

where V and K_m denote the maximum velocity and the Michaelis constant, respectively. In NaDC micellar systems, when $[\mathrm{M}]_t \geq [\mathrm{S}]_t$, the concentrations of MS and M can be approximated by Eqs. 4 and 5, respectively;[1,2)]

$$[\mathrm{MS}]=[\mathrm{S}]_t \tag{4}$$

$$[\mathrm{M}]=[\mathrm{M}]_t-[\mathrm{MS}]=\frac{[\mathrm{D}]_t-\mathrm{cmc}}{N}-[\mathrm{MS}] \tag{5}$$

where $[\mathrm{S}]_t$, $[\mathrm{M}]_t$, and $[\mathrm{D}]_t$ represent total concentrations of substrate, micelles, and surfactant, respectively, and N is the aggregation number. That is, it was considered that the solubilization of the substrate was quantitative and did not significantly modify the cmc or the aggregation number.

A similar analysis was carried out for NaTDC micellar systems. By the spectral shift method,[3)] the cmc of NaTDC under the present experimental conditions (Tris-HCl buffer 40 mM, NaCl 0.18 M, pH 8.0, and 25 °C) was found to be 1.20 mM. The aggregation number of NaTDC micelles under the present conditions is expected to be 23 to 27.[5)] When the initial rates were plotted against [M] calculated from Eqs. 4 and 5 with respect to each expected N value, lines intersecting in the left upper quadrant were obtained for each N value as in the case of NaDC micellar systems;[2)] the plot for $N=26$ is shown in Fig. 2. This result suggests that the solubilization of VDD by NaTDC is also quantitative and does not significantly modify the cmc or the aggregation number, since, in vinyl hexanoate–NaDC systems where the solubilization was considered not to be quantitative, the plots of $1/v$ *vs.* [M] did not give straight lines intersecting in the left upper quadrant.[1)] As described previously,[2)] Lineweaver–Burk plots for each N value (23 to 27) were prepared, using estimates for $(1/v)_{[\mathrm{M}]=\mathrm{const}}$ obtained by regression analysis of $1/v$ *vs.* [M] plots. As expected from Eq. 3, straight lines intersecting on the $1/v$ axis were obtained in the case of $N=26$ (Fig. 3), suggesting that the enzymatic reaction in NaTDC micellar systems follows the same mechanism as shown in Chart 1, as in NaDC micellar systems, and that the aggregation number of NaTDC micelles under the present conditions is 26. When the slopes and the intercepts of $1/v$ *vs.* [M] plots for $N=26$ (Fig. 2) were replotted against 1/[MS], based on Eq. 2, both the replots gave linear relationships as shown in Fig. 4. From the slopes and the intercepts of these replots, the values of the kinetic parameters, V, K_m, and K_4, were calculated (Table I).

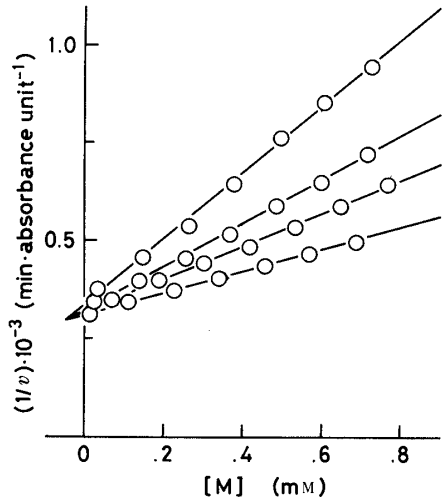


Fig. 2. Plots of $1/v$ *vs.* [M] for VDD–NaTDC Systems Based on $N=26$

[MS] values are 0.50, 0.75, 1.00, and 1.50 mM from top to bottom.
Solid lines are regression lines.

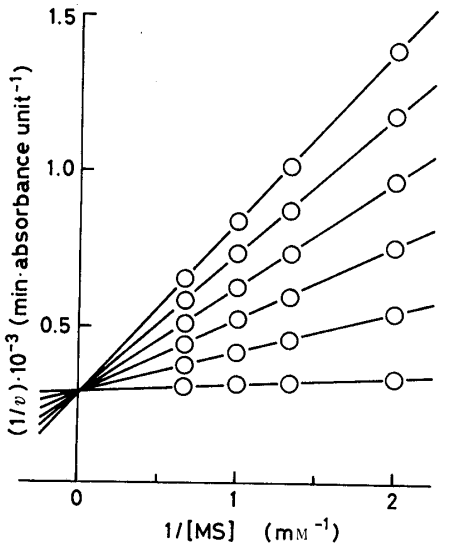


Fig. 3. Lineweaver–Burk Plots for VDD–NaTDC Systems Based on $N=26$

[M] values are 1.25, 1.00, 0.75, 0.50, 0.25, and 0 mM from top to bottom.
Solid lines are regression lines.

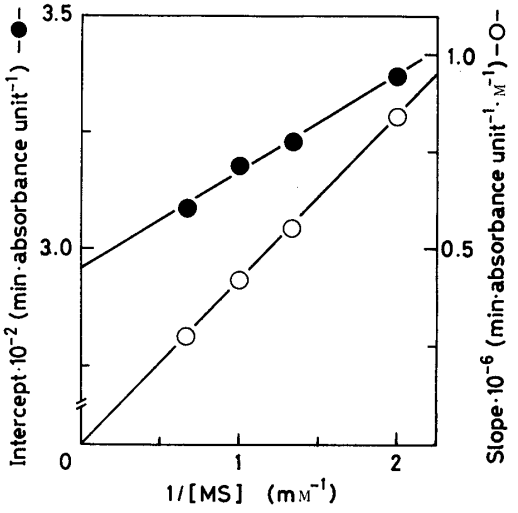


Fig. 4. Replots of Slopes and Intercepts Obtained from Fig. 2 against 1/[MS]

○, slope; ●, intercept.

TABLE I. Kinetic Parameters Obtained for Several Vinyl Esters in NaTDC Micellar Systems

Vinyl ester	$V\times10^3$ (au[a]·min^{-1})	$K_m\times10^5$ (M)	$K_4\times10^5$ (M)	K_m/K_4[b]
Octanoate	4.18	9.49	—	1.95 (17.0)
Decanoate	3.59	8.55	—	1.76 (3.65)
Dodecanoate	3.38	6.93	4.87	1.42 (1.88)
Tetradecanoate	2.94	6.44	—	1.32 (2.28)

a) Absorbance unit.
b) The figures in parentheses are the values obtained in NaDC micellar systems.

Similar measurements at a constant substrate concentration (1.00 mM) were carried out with other substrates, VO, VD, and VTD. The $1/v$ *vs.* [M] plots for these substrates also showed good linearity. In these cases, the values of V and K_m for each substrate were

calculated directly from the slope ($K_m/K_4V[MS]$) and the intercept ($(1+K_m/[MS])/V$) of each line by using the K_4 value obtained for VDD, since K_4, which is the dissociation constant of EM complex, should be independent of the substrate as verified in NaDC micellar systems.[1] The results are summarized in Table I.

The V and K_m values increased with decreasing fatty acid chain length in accordance with the previous results obtained for NaDC micellar systems.[1] Interestingly, the K_m/K_4 values for all substrates were smaller than those obtained for the corresponding substrates in NaDC micellar systems. Since the estimated aggregation number of NaTDC micelles (26) is larger than that of NaDC micelles (17),[2] these results suggest that an increase in micellar size results in a decrease in the K_m/K_4 values. This was indeed found to be the case (see below).

ODE Micellar Systems

As a micellar system with larger size, ODE micelles were selected; the aggregation number has been reported to be 120 at 25 °C.[6] The Michaelis plot for VDD–ODE micellar systems at a constant surfactant concentration was linear, as shown in Fig. 5, in contrast to VDD–NaDC micellar systems, where the corresponding plot was concave.[2] Eq. 1, which satisfactorily explained the initial rate in NaDC and NaTDC micellar systems, can be simplified to Eq. 6, provided that $K_m/K_4=1$.

$$V=\frac{V[MS]}{K_m+[M]+[MS]}=\frac{V[MS]}{K_m+[M]_t} \tag{6}$$

Equation 6 indicates that the Michaelis plot at a constant surfactant concentration should yield a straight line through the origin, with a slope of $V/(K_m+[M]_t)$. Thus, the linear plot (Fig. 5) suggests that the enzymatic reaction in these ODE micellar systems also follows the mechanism shown in Chart 1 and that the K_m/K_4 value is close to unity. According to Eq. 1, the Michaelis plot will be concave when the K_m/K_4 value is larger than unity, an will be convex when the value is smaller than unity (*cf.* Fig. 7 and reference 2).

The Michaelis plots at several constant ODE concentrations also gave straight lines. Similar results were obtained for another substrate, VO. Equation 6 predicts that the reciprocal of the slope of the linear Michaelis plot is given by Eq. 7;

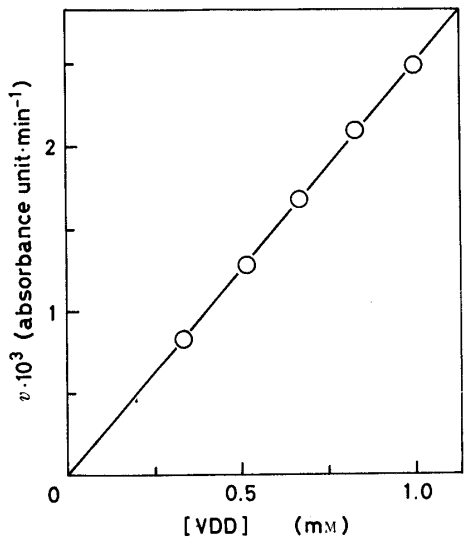


Fig. 5. Michaelis Plot at a Constant ODE Concentration

Conditions: ODE 135 mM, Tris-HCl buffer 40 mM, NaCl 0.18 M, pH 8.0, 25 °C.

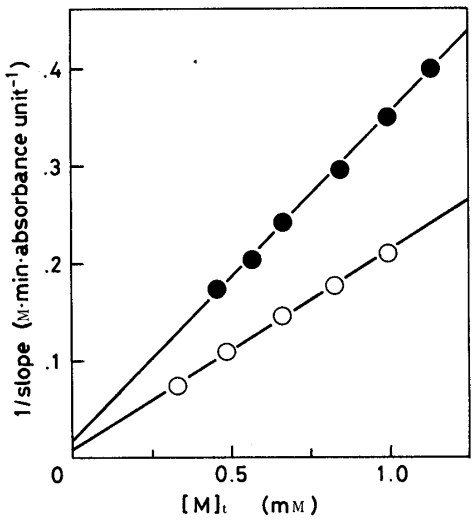


Fig. 6. Replots of Reciprocals of Slopes Obtained from Michaelis Plots against Total Micelle Concentration

●, VDD; ○, VO.

$$1/\text{slope} = \frac{1}{V} \cdot [M]_t + \frac{K_m}{V} \quad (7)$$

that is, a straight line with a slope of $1/V$ and with an intercept of K_m/V should be obtained when the reciprocals of the slopes of the Michaelis plots at various surfactant concentrations are replotted against $[M]_t$. In practice, the replots for VDD and VO gave linear relationships ($r = 0.999$ in both cases), as shown in Fig. 6. From the slopes and the intercepts of these linear replots, the V and the K_m ($= K_4$) values were calculated (Table II).

VO has a larger V value than VDD as observed in NaDC and NaTDC micellar systems. In the ODE micellar systems, however, the K_m values obtained for the two substrates were in agreement with each other within the limits of experimental error, in contrast to the cases of NaDC and NaTDC micellar systems, again supporting the validity of the analysis described above, because the K_m value is considered to be equal to the K_4 value independently of the substrate.

PNE Micellar Systems

To further elucidate the above-mentioned micellar size effect on the K_m/K_4 value, similar measurements were performed for three PNEs with different average lengths of polyoxyethylene chain. When the initial rates were plotted against the substrate (VDD) concentration (Fig. 7), the plots for PNE (10) and PNE (15) were linear, suggesting that the K_m/K_4 value can be regarded as unity in these nonionic surfactant systems, too. On the other hand, the plot for PNE (30) was concave, indicating that $K_m/K_4 > 1$ as in the cases of NaDC and NaTDC. Becher reported that the aggregation numbers of PNE (10), PNE (15) and PNE (30)

TABLE II. Kinetic Parameters Obtained for Two Vinyl Esters in ODE Micellar Systems

Vinyl ester	$V \times 10^3$ (au[a] · min^{-1})	K_m[b] $\times 10^5$ (M)
Dodecanoate	2.99 ± 0.07	5.46 ± 1.88
Octanoate	4.91 ± 0.10	4.28 ± 1.42

a) Absorbance unit.
b) Analyses were made based on the assumption that the values of K_m are equal to that of K_4 (see the text).

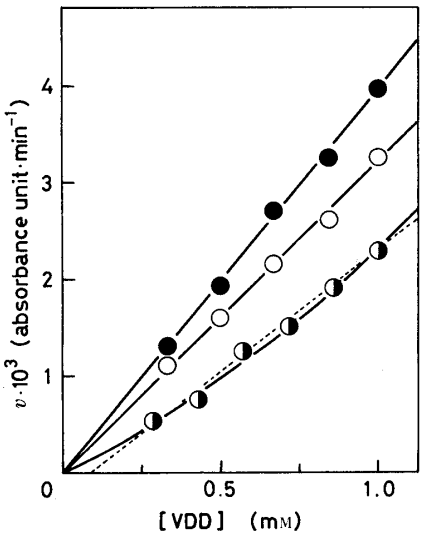


Fig. 7. Michaelis Plots for Three PNEs at Constant Surfactant Concentrations

○, PNE(10) 114 mM; ●, PNE(15) 70.3 mM; ◑, PNE(30) 26.6 mM.
Conditions: Tris-HCl buffer 40 mM, NaCl 0.18 M, pH 8.0, 25 °C.
The dotted line is a regression line. The positive abscissa intercept indicates that the Michaelis plot for PNE(30) is concave.

TABLE III. Comparison of Parameters for Three Surfactant Micelles

Surfactant	N	K_m/K_4	$(1/K_4)^{a)} \times 10^{-4}$ (M^{-1})	$V^{b)} \times 10^3$ ($au^{c)} \cdot min^{-1}$)
NaDC	17	1.88	1.18	3.91
NaTDC	26	1.42	2.05	3.38
ODE	120	1	1.83	0.45

a) This means the binding constant of pancreatic lipase to substrate-free micelles.
b) The substrate is VDD. The values are corrected based on enzyme concentration.
c) Absorbance unit.

are 100, 52, and 19, respectively.[7] Since the PNEs used by Becher and us are not homogeneous but are polydisperse compounds, these values cannot be applied to the present systems as they are. However, it is well known that the aggregation number of PNEs decreases with increase in average length of the polyoxyethylene chain.[4] Therefore, the results shown in Fig. 7 also suggest that an increase in micellar size brings about a decrease in the K_m/K_4 value.

Discussion

We reported previously that the pancreatic lipase-catalyzed hydrolysis of a fatty acid vinyl ester in NaDC micellar systems follows the mechanism shown in Chart 1.[1,2] The present results indicate that the enzymatic reactions in NaTDC, ODE, and PNE micellar systems can also be explained in terms of the same mechanism.

Since pancreatic lipase binds not only to micelles solubilizing substrate but also substrate-free micelles, the presence of substrate-free micelles results in fully competitive inhibition of the enzymatic reaction. However, it should be noted that the competition between the substrate-free and substrate-solubilizing micelles is not for an active site as observed in an ordinary enzymatic reaction, but for the binding site to the hydrophobic interface, namely the hydrophobic head.[8] As pointed out previously,[2,9] the binding of the enzyme to substrate-free micelles may also be responsible for the inhibitory effect of a surfactant on the enzyme activity in an emulsion system. Therefore, it is of interest to estimate the binding constant of the enzyme to substrate-free micelles. As far as we are aware, such an estimation has not been done before.

The reciprocal of K_4 estimated in this work corresponds to the binding constant under consideration; those to bile salt micelles are particularly interesting, because bile salts are the surfactants participating physiologically in lipolysis.[5c,10] The values of $1/K_4$ for NaDC, NaTDC, and ODE are summarized in Table III. Bile salt micelles are negatively charged, but ODE micelles are not charged. Nevertheless, the binding constant to ODE micelles is of the same magnitude as those to NaDC and NaTDC micelles. Therefore, the binding of the enzyme to micelles appears to be primarily attributable to a hydrophobic interaction, in contrast to the case of phospholipase A_2, where an electrostatic interaction plays an important role.[11,12]

The rate of the enzymatic reaction depends on the stability of the EMS complex relative to that of the EM complex, as can be seen from Chart 1. The relative stability may be represented by the K_1/K_4 value. Although the K_1 value cannot be estimated at present, K_m is correlated with K_1 for three possible limiting cases as follows.[2]

Case 1: rapid equilibrium in steps 1, 4, and 5; steady state in EMS.

$$K_m = K_1 \cdot \frac{k_{-2}+k_3}{k_2+k_{-2}+k_3} \tag{8}$$

Case 2: rapid equilibrium in steps 1, 2, 4, and 5; step 3 rate-determining.

$$K_m = K_1 \cdot \frac{k_{-2}}{k_2 + k_{-2}} \quad (9)$$

Case 3: rapid equilibrium in steps 1, 4, and 5; step 2 rate-determining.

$$K_m = K_1 \quad (10)$$

Therefore, the K_1 value should be equal to or greater than the K_m value.

The K_m/K_4 values for NaDC,[1] NaTDC, and PNE (30) are larger than unity, indicating that the EMS complex is less stable than the EM complex in these micellar systems because $K_1 \geq K_m$. Furthermore, the K_m values for NaDC[1] and NaTDC (Table I) are affected by the fatty acid chain length. These effects may arise from the competition of the enzyme with the substrate for the hydrophobic core of micelles with small size (or negatively-cooperative binding).[1] An increase in micellar size should provide a hydrophobic region large enough for both the enzyme and the substrate to bind. In other words, both can coexist stably in large micelles, which will decrease the K_m/K_4 value. In fact, the K_m/K_4 values for NaTDC micelles are smaller than those for NaDC micelles (Table I) and the values for larger micelles, ODE, PNE (10), and PNE (15) micelles, are still smaller than those for NaTDC micelles. In the case of ODE, the K_m value is no longer affected by the fatty acid chain length (Table II). These results support the inference that an increase in micellar size stabilizes the EMS complex.

Micellar size may also affect the maximum velocity V. The value obtained for ODE micelles was smaller by one order of magnitude than those for NaDC and NaTDC micelles (Table III). This may be interpreted as follows: with increasing aggregation number, the relative concentration of substrate in the micelles decreases (surface dilution[15] or substrate dilution phenomenon[16]), so that apparantly k_2 will decrease and/or K_2 will increase, both of which reduce V.

In our previous report, it was not clear which of the three limiting cases mentioned above is the best approximation. As can be seen from Eqs. 8, 9, and 10, the limiting value of K_m/K_4, which is realized at $K_1/K_4 = 1$, is less than unity in Cases 1 and 2 but is equal to unity in Case 3. The results that ODE, PNE (10), and PNE (15) micelles gave the same K_m/K_4 value of unity in spite of a considerable difference in their aggregation numbers tend to imply that Case 3 is most likely.

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