Models for enzyme superactivity in aqueous solutions of surfactants

Paolo VIPARELLI, Francesco ALFANI¹ and Maria CANTARELLA

Dipartimento di Chimica, Ingegneria Chimica e Materiali, Università di L'Aquila, Monteluco di Roio, 67100 L'Aquila, Italia

Theoretical models are developed here for enzymic activity in the presence of direct micellar aggregates. An approach similar to that of Bru et al. [Bru, Sánchez-Ferrer and Garcia-Carmona (1989) *Biochem. J.* **259**, 355–361] for reverse micelles has been adopted. The system is considered to consist of three pseudo-phases: free water, bound water and surfactant tails. The substrate concentration in each pseudo-phase is related to the total substrate concentration in the reaction medium. In the absence of interactions between the enzyme and the micelles, the model predicts either monotonically increasing or monotonically decreasing trends in the calculated reaction rate

as a function of surfactant concentration. With enzyme–micelle interactions included in the formulation (by introducing an equilibrium relation between the enzyme confined in the free water and in the bound water pseudo-phases, and by allowing for different catalytic behaviours for the two forms), the calculated reaction rate can exhibit a bell-shaped dependence on surfactant concentration. The effect of the partition of enzyme and substrate is described, as is that of enzyme efficiency in the various pseudo-phases.

Key words: enzyme catalysis, kinetics models, micelles.

INTRODUCTION

The concept of a micelle as an aggregate of surfactant molecules in an aqueous solution was first introduced at the beginning of the twentieth century [1]. Many investigations have since been performed into the physicochemical properties of micelles and their possible applications. Micellar aggregates have been suggested as catalysts for organic chemical reactions on the basis that the reactants can interact with the micelles, thereby improving the yield [2]. Studies have been reported over the past fifteen years into the use of surfactants in reverse micelle bioprocessing applications: surfactant micro-emulsions [3–6], the extraction of active enzymes [7] and the preparation of media for hosting enzymic reactions [4,8–18]. In contrast, enzymic biocatalysis in aqueous solutions of buffer and surfactant has been largely disregarded.

The kinetic parameters in ternary systems (water/immiscible organic solvent/surfactant) are generally comparable with those in aqueous solutions [17] and are derivable from Michaelis–Menten type kinetic relations. Some enzymes can also show an activity that is higher in such systems than in pure buffer solutions (superactivity) [19]. There is also some experimental evidence for enzyme superactivity in purely water/surfactant media owing to positive interactions between the enzyme and the surfactant. Some soft detergents fail to inactivate many enzymes and can even reactivate enzymes previously inactivated with SDS. Other detergents [17] are able to increase the catalytic activity of enzymes: Brij 35 stimulates cytoplasmic glycerol-3-phosphate dehydrogenase 1.5-fold; alcohol dehydrogenase from rat liver is activated 4–7-fold by deoxycholate and other bile salts; the incorporation of substrates into micelles or the addition of Tween 20 results in a 3–6-fold increase in the activity of mitochondrial carnitine palmitoyltransferase; non-ionic surfactants stimulate fire-fly luciferase up to 7-fold; lysophosphatidylcholine and non-ionic surfactants activate the NAD: arginine ADP-ribosyltransferase of erythrocytes up to 6-fold. We have previously reported superactivity of α -chymotrypsin in surfactant/buffer aqueous systems [20,21]. Cetyltributyl-

ammonium bromide (CTBABr) and cetyltrimethylammonium bromide gave the most significant effects; it was shown that the interaction between the enzyme and the surfactant needs to be taken into account to explain the higher catalytic activity of α -chymotrypsin than that exhibited in a pure buffer.

In spite of this important evidence, little work has been reported on the catalytic behaviour of enzymes in aqueous solutions enriched with surfactants [17]; the kinetics have yet to be modelled. In contrast, much effort has been devoted to describing the mechanism of enzyme-catalysed reactions in reverse micelles; several models of this process have been developed [9–11,22,23]. Published interpretations [9–11] of kinetic data for enzyme reactions in reverse micelles fall into two categories: (1) all the enzyme parameters are expressed with respect to the total volume of the reverse micellar solution, and (2) the enzymic conversion is related only to the volume fraction of the aqueous solution (pseudo-phase model).

The purpose of the present paper is to present models for enzyme superactivity in the presence of micellar aggregates in water. The pseudo-phase approach has been adopted to describe the kinetic behaviour of the enzyme at surfactant concentrations higher than the critical micellar concentration (CMC). The enzyme kinetic parameters are assumed to depend on the local enzyme concentrations in the system.

THEORY

Direct micellar aggregates

Micellar aggregates in an aqueous solution can be depicted as structures with the hydrophilic heads of the surfactant oriented towards the water molecules and the hydrophobic tails oriented towards the inner part of the structure. The water molecules linked to the polar heads of the surfactant form the so-called bound water layer. The aggregates appear as the dominant form above the CMC. The size and shape of these aggregates depend on the surfactant concentration. Free surfactant is also

Abbreviations used: CTBABr, cetyltributylammonium bromide; CTABr, cetyltrimethylammonium bromide; CMC, critical micellar concentration; GpNA, *N*-glutaryl-L-phenylalanine *p*-nitroanilide.

¹ To whom correspondence should be addressed (e-mail alfani@ing.univaq.it).

Figure 1 Schematic structure of a micellar aggregate

present in the system as monomers or small assemblies not organized in micelles. The system is depicted schematically in Figure 1. There are two distinct kinds of micelle, depending on the surfactant concentration: small, with an aggregation number of approx. 100–200 molecules per micelle, and large, with aggregation numbers rising to 1000 molecules per micelle [1].

Generally, no great size increase in micelles is observed with increasing surfactant concentration under dilute solution conditions. A transition to much larger micelles has been observed at very high amphiphile concentrations. Factors that favour size growth produce relatively small size increases as long as the system remains within the small-micelle regime. Marked changes are observed as the upper limit of surfactant concentration for small micelles is approached and the transition to large micelles occurs [24]. The dependence of the aggregation number on the surfactant concentration varies with the nature of the surfactant employed (the magnitude of the repulsive force between amphiphile head groups profoundly influences micelle size and the conditions required for transition from small to large micelles), with temperature and with the presence of other chemicals in the solution.

In the small-micelle regime, a continuous variability in the aggregation number can be assumed. Micelles in equilibrium with the monomeric amphiphile must therefore be heterogeneous with respect to micelle size. The evaluation of a distribution function for small micelle size is not important for the purpose of this study. In fact, because the variation in micelle size is small, the number of micelles can be assumed to be solely an increasing function of the surfactant concentration, the micelle size remaining effectively constant.

A first attempt to describe this phenomenon is reported in the Appendix. A dimerization equilibrium between simple and dimerized micelles has been introduced to take into account the variation in micelle size.

A spherical shape is often assumed for the micelles, even though the instantaneous and dynamic structure of these aggregates is probably anything but spherical [25]. The surface might be only sparsely covered with the hydrophilic groups and extensive contacts might occur between the lipophilic groups and the water. However, the uncertainties in the aggregate geometry imply no limitation to the model developed in this study because it relies only on the assumption of interactions of both substrate and enzyme with the surfactant molecules, present either as micelles or as free molecules at the equilibrium concentration (CMC). If such interactions occur, changes in the kinetic and catalytic behaviour of the system can take place.

The multiphase approach

For reverse micelles, Bru et al. [9] introduced the multiphase model based on the existence of four different microenvironments in the micellar system: the free water, the bound water, the zone of the surfactant tails and the organic solvent. The enzyme and the substrate are regarded as being partitioned into three phases. A difficulty in the application of this model lies in the determination of the four pseudo-phase volume fractions. However, once this problem has been resolved, the partition coefficients for the enzyme and the substrate can be introduced. It follows that the overall enzymic activity is the sum of the three activities: in free water, in bound water and in surfactant tails. Reaction rates are assumed to follow a Michaelis–Menten-type kinetic law, with component concentrations relating to specific pseudo-phases and defined as follows:

$$[X'_{\text{psph}}] = \frac{\text{moles of X in the pseudo-phase}}{\text{volume of the pseudo-phase}} \quad (1)$$

As has been pointed out above, the model proposed here for describing enzyme kinetics in the presence of direct micelles is based on that employed by Bru et al. [9–11] for reverse micelles. In the present application only three pseudo-phases need to be defined (free water, bound water and surfactant aggregates), there being no organic solvents present. The substrate can partition in all three pseudo-phases but the aliquot associated with the micellar aggregates cannot participate in the catalytic process. Enzyme partition is restricted to two pseudo-phases, free and bound water, the dimension of an enzyme molecule being of the same order as that of the micellar aggregates. Accordingly, two different enzyme forms can be considered to be present in the system: one affected by the surfactant aggregates, the other (most probably) by the free surfactant. Both of these interactions can give rise to increases in enzyme activity that can be determined by means of incubation experiments performed at surfactant concentrations that are respectively higher and lower than the CMC, as reported in [20,21].

The volume fractions of the various pseudo-phases, the molar volume of surfactant in the aqueous phase, the dimensions of a surfactant aggregate, the number of surfactant molecules involved in each aggregate and the number of water molecules forming the bound water layer are not easily determined. Consequently, in this study a different definition of component concentration has been adopted and is given by:

$$[X_{\text{psph}}] = \frac{\text{moles of X in the pseudo-phase}}{V_T} \quad (2)$$

where V_T is the total volume of the system. The conversion of parameters used in this study to those of the standard multiphase approach is reported in the Appendix, where the volume fractions of each pseudo-phase are estimated.

Finally, it should be pointed out that the application of the multiphase approach requires that the characteristic time for enzyme reaction be much greater than that for micellar aggregate formation: enzyme reaction is assumed to be the rate-determining step.

Substrate partitioning and enzyme–surfactant interactions

The substrate can partition between the three pseudo-phases (free water, bound water and surfactant aggregate) as shown in Figure 2; its overall mass balance can be written:

$$[S_t] = [S_f] + [S_m] = [S_f] + [S_b] + [S_s] \quad (3)$$

Figure 2 Substrate partition

where the subscripts t, f, m, b and s refer to the total system volume, the free water, the whole micellar aggregate, the bound water and the surfactant molecules respectively.

Thermodynamic equilibrium is assumed for substrate distribution between the free water and the whole micellar aggregate:

$$[S_f] + [D_N] \xrightleftharpoons{K_s} [S_m] \quad (4)$$

where K_s is the association constant and $[D_N]$ is the concentration of surfactant micellar aggregates, which can be determined from the difference between the CMC and the total concentration of surfactant in the system.

A partition equilibrium between the substrate in the bound water, $[S_b]$, and that associated with the surfactant ('micellized'), $[S_s]$, is introduced as follows:

$$[S_b] \xrightleftharpoons{P_{b,s}} [S_s] \quad (5)$$

where $P_{b,s}$ is the partition coefficient. The above conditions lead to the following relationships for the concentrations of substrate able to react in the presence of micelles:

$$[S_f] = \frac{[S_t]}{1 + K_s \cdot [D_N]} \quad (6)$$

$$[S_b] = \frac{K_s \cdot [D_N]}{(1 + K_s \cdot [D_N]) \cdot (1 + P_{b,s})} [S_t] \quad (7)$$

$P_{b,s}$ is the only unknown parameter in eqns. (6) and (7). The surfactant CMC can be easily determined, so it is possible to evaluate $[D_N]$. K_s can also be measured experimentally with the method described in [21]. This limitation in the number of adjustable parameters increases the predictiveness of the model.

The above approach holds if the number of micelles in the system is directly proportional to the concentration of surfactant forming aggregates, $[D_N]$, while the dimension of the aggregates remains unchanged. This occurs within a range of surfactant concentration sufficiently close to the CMC [1,24]. On the basis of this hypothesis the definitions given by eqns. (1) and (2) are basically analogous.

Enzyme superactivity observed above the CMC [17,21] might be the result of modifications of the catalytic properties of the enzyme owing to positive interactions between the protein and the surfactant, whether dissolved in the aqueous system as a monomer, as aggregates containing a few molecules, or as micelles. In fact, experimental results indicate that superactivity is possible at surfactant concentrations below the CMC, as with α -chymotrypsin and cationic surfactants such as cetyltrialkylammonium bromide [20].

Figure 3 Possible interactions between an enzyme and surfactant micellar aggregates

(a) Enzyme linked to the bound water; (b) enzyme interacting with the surfactant's lipophilic tails.

Taking into account the amphiphilic nature of the surfactants, the physicochemical properties of the enzyme, the type of buffer and the pH, at least three different situations can be considered.

(1) The hydrophobicity of the surfactant and the hydrophilicity of the enzyme tend to prevent interactions; all the enzyme is located in the free water and its activity can be modified only by the non-micellized surfactant.

(2) The enzyme interacts with the micelle through the bound water; an important role is taken by the ionic strength of the surfactant polar heads; little or no modification of the surfactant aggregate structure occurs, as shown in Figure 3(a).

(3) The enzyme interacts with the hydrophobic part of the surfactant molecules; in this case the structure of the aggregate must be strongly modified, as shown in Figure 3(b).

The model compares the reaction rate in the presence of aggregates with r_0 , the reaction rate in the same system evaluated at the surfactant concentration equal to the CMC. This implies no increased enzyme activity for case 1 because the only enzyme modifications are those induced by the non-micellized surfactant and these effects are already considered in r_0 . Cases 2 and 3, in contrast, assume that the enzyme conformation and its catalytic properties are affected by interactions with the micellized surfactant.

No assumptions are made regarding the initial rate of reaction at the CMC, which can be either higher or lower than its value in pure aqueous solution, i.e. in the absence of surfactant. All the estimated values refer to the initial reaction rate or differential reaction conditions, because both substrate consumption and enzyme deactivation are not taken into account in the model. The hypothesis of a Michaelis–Menten rate equation is assumed, as with models for enzyme kinetics in reverse micelles.

RESULTS AND DISCUSSION

Model 1: no enzyme–micelle interactions

The model assumes in this case that both the free substrate in aqueous solution, S_f , and that in the micelle-bound water, S_b , can participate in the enzymic reaction. All the enzyme added to the system, E_t , is free and can react with the two forms of substrate. The specificity towards the substrate and the catalytic constant differ for the reaction in the free water (k_{cat}^f and K_m^f) and that in the bound water (k_{cat}^b and K_m^b). The overall rate of reaction is the sum of the two contributions, r_f and r_b , representing the rates of enzymic reaction with the substrate in the free and bound water respectively.

Substitution of eqns. (6) and (7) for $[S_f]$ and $[S_b]$ into the Michaelis–Menten rate equations gives:

$$r_f = \frac{k_{cat}^f [E_t] [S_f]}{(1 + K_s [D_N]) \left[K_m^f + \frac{[S_f]}{(1 + K_s [D_N])} \right]} \quad (8)$$

and

$$r_b = \frac{K_s [D_N] (k_{cat}^b) [E_t] [S_f]}{(1 + P_{b,s}) (1 + K_s [D_N]) \left[K_m^b + \frac{K_s [D_N] [S_f]}{(1 + P_{b,s}) (1 + K_s [D_N])} \right]} \quad (9)$$

To relate the results to the well-known definition of enzyme efficiency, $\eta_x = k_{cat}^x / K_m^x$, where x indicates the pseudo-phase, it should be noted that the kinetic parameters of the reaction catalysed by the enzyme in the free water pseudo-phase are those measured in the absence of micelles at a surfactant concentration equal to the CMC, k_{cat}^0 and K_m^0 , so that $\eta_f = \eta_0$.

The model predictions show that values of (r/r_0) greater than unity are possible only if $\eta_b > \eta_0$. An increase in superactivity can be explained only by assuming that the reaction of the enzyme in the free water is more efficient with the micellized substrate than with the substrate in the free water.

Figures 4 and 5 show plots of (r/r_0) against micellar concentration at a constant η_b of 10 η_0 . Figure 4 refers to different

Figure 4 Effect of K_s on model 1

Numbers to the right of the curves are K_s , in units of M^{-1} . Simulated conditions: $[S_f] = 2.5$ mM, $P_{b,s} = 1$, $\eta_b/\eta_0 = 10$.

Figure 5 Effect of $P_{b,s}$ on model 1

Numbers to the right of the curves are $P_{b,s}$. Simulated conditions: $[S_f] = 2.5$ mM, $K_s = 100$ M^{-1} , $\eta_b/\eta_0 = 10$.

values of K_s , the substrate–micelle binding equilibrium constant, with $P_{b,s} = 1$. Figure 5 refers to different values of the substrate partition coefficient between the bound water pseudo-phase, $P_{b,s}$, and the surfactant, with $K_s = 100$ M^{-1} . Each curve starts at $(r/r_0) = 1$, for $[D_N] = 0$, which is the condition of surfactant concentration equal to the CMC, i.e. present only as a monomer or as small assemblies not organized as micelles. The ratio of reaction rates tends to asymptotic values, either higher or lower than unity, with increasing micelle concentration. Higher K_s and lower $P_{b,s}$ values result in higher (r/r_0) values. The higher K_s becomes, the more rapid is the increase in r . Both of these conditions imply that most of the substrate is associated with the micelles and is present in the bound water pseudo-phase. It is worth noting that the assumption of an improved efficiency of the enzyme in a micelle-rich medium is a necessary but not a sufficient condition for an increase of the overall reaction rate, because substrate partition is also important. An unfavourable partition can annihilate the advantages of better kinetics.

This model can predict only characteristics that are increasing or decreasing monotonically. In contrast, experimental results can reveal a different behaviour pattern for enzyme superactivity in the presence of surfactant micelles: for example, for α -chymotrypsin in the presence of CTBABr [21], the ratio (r/r_0) plotted as a function of $[D_N]$ exhibits a bell-shaped trend.

Model 2: interactions between enzyme and micelles

Consider now the hypothesis of the interactions between the enzyme and the micelles, leading to a strong modification of the catalytic properties of the enzyme. Such a situation has been depicted by introducing an enzyme partition that generates two different forms of enzyme with different kinetic parameters.

The total enzyme concentration $[E_t]$ is expressed as the sum of two contributions: that in the free water, $[E_f]$, and that in the bound water, $[E_b]$. To describe the enzyme partition in the pseudo-phases, we assume the following equilibrium:

Figure 6 Effect of $\eta_{b,f}$ on model 2

Simulated conditions: $[S_f] = 2.5$ mM, $K_S = 50$ M $^{-1}$, $K_E = 100$ M $^{-1}$, $P_{b,s} = 10^5$, $\eta_{b,f}/\eta_0 =$ (a) 1, (b) 2.5, (c) 5, (d) 7.5, (e) 10.

where K_E is the constant of association between the enzyme and surfactant aggregates. Taking into account the enzyme mass balance, we have:

$$[E_f] = \frac{[E_t]}{1 + K_E [D_N]} \quad (11)$$

and

$$[E_b] = \frac{(K_E [D_N]) [E_t]}{1 + K_E [D_N]} \quad (12)$$

As the dimension of a protein molecule is comparable with that of a micelle, the enzyme can react only with the substrate confined in the free water and in the bound water. Therefore the overall rate of reaction can be evaluated as the sum of four different contributions according to the following:

Finally, the overall rate of substrate consumption, r , is given by:

$$r = \frac{k_{cat}^{f,f} \cdot [E_f] \cdot [S_f]}{K_m^{f,f} + [S_f]} + \frac{k_{cat}^{f,b} \cdot [E_f] \cdot [S_b]}{K_m^{f,b} + [S_b]} + \frac{k_{cat}^{b,f} \cdot [E_b] \cdot [S_f]}{K_m^{b,f} + [S_f]} + \frac{k_{cat}^{b,b} \cdot [E_b] \cdot [S_b]}{K_m^{b,b} + [S_b]} \quad (14)$$

The double superscripts refer to the pseudo-phase in which the enzyme is confined (first superscript) and to that in which the substrate is confined (second superscript). It is worth noting that $k_{cat}^{f,f}$ and $K_m^{f,f}$ coincide with the k_{cat}^f and K_m^f introduced in model 1 and can be determined by experiments at the CMC.

On applying eqns. (11) and (12) for $[E_f]$ and $[E_b]$ and eqns. (6) and (7) for $[S_f]$ and $[S_b]$, the overall reaction rate can be obtained in principle from eqn. (14); however, it still depends on a number

Figure 7 Effect of K_E on model 2

Numbers to the right of the curves are K_E in units of M $^{-1}$. Simulated conditions: $[S_f] = 2.5$ mM, $K_S = 50$ M $^{-1}$, $P_{b,s} = 10^5$, $\eta_{b,f}/\eta_0 = 10$.

Figure 8 Effect of K_S on model 2

Numbers to the right of the curves are K_S in units of M $^{-1}$. Simulated conditions: $[S_f] = 2.5$ mM, $K_E = 100$ M $^{-1}$, $P_{b,s} = 10^5$, $\eta_{b,f}/\eta_0 = 10$.

of kinetic and physicochemical parameters whose values can vary considerably, giving rise to plots of (r/r_0) against surfactant concentration with quite different shapes.

To decrease the number of adjustable parameters and to refer to enzyme efficiency, the simulations are reported as functions of $\eta_{x,y} = k_{cat}^{x,y}/k_m^{x,y}$ in each pseudo-phase and at the CMC. The first and second subscripts of η refer to the pseudo-phase for the enzyme and the substrate respectively.

In Figures 6–9, the diagrams of (r/r_0) refer to two key system conditions, cases 1 and 2. In case 1, the concentration of the substrate in the bound phase is negligible in comparison with that of the substrate associated with the surfactant, $[S_b] \ll [S_s]$, i.e. the substrate aggregated in the micelles is all segregated by the surfactant. This condition is attained at $P_{b,s} \gg 1$ and implies

Figure 9 Effect of $\eta_{b,b}$ (solid lines) and $\eta_{t,b}$ (dotted lines) on model 2

Simulated conditions: $[S] = 2.5 \text{ mM}$, $K_s = 10^4 \text{ M}^{-1}$, $K_E = 100 \text{ M}^{-1}$, $P_{b,s} = 10^{-4}$. A key to the curve numbers is given below.

Curve	$\eta_{b,b}/\eta_0$	$\eta_{t,b}/\eta_0$
A1	1	1
A2	1	5
A3	1	10
A4	1	50
B1	5	1
B2	10	1
B3	50	1
B4	100	1

that $[S_m] \approx [S_s]$. In case 2, the micellized substrate is present mainly in the bound water, i.e. $[S_b] \gg [S_s]$. This condition is attained at $P_{b,s} \ll 1$ and implies that $[S_m] \approx [S_b]$. Moreover, for $K_s \gg 1$, no free substrate is present in the system and $[S_b] \approx [S]$.

The assumption of case 1 determines that $[S_b] \approx 0$, independently of the equilibrium constant of the substrate-micelle association, K_s . As a consequence, the second and fourth reactions in eqn. (13) can be neglected.

In the Figures for case 1, $(\eta_{b,t}/\eta_0)$ varies from 1 to 10, $[E_b]/[E]$ ranges between 0 and 0.83, and $[S_m]/[S]$ increases from 0 to 0.72.

The results of this model simulation show clearly that a monotonically decreasing behaviour is still possible at low values of $(\eta_{b,t}/\eta_0)$ or K_E and at high values of K_s . These mathematical conditions refer to the following physical situations: (a) low efficiency of the bound enzyme in the reaction with the free substrate; (b) high efficiency but low concentration of bound enzyme; (c) high concentration of micellized substrate but segregated by the surfactant. In contrast, the model provides bell-shaped curves for different sets of parameters. The two opposite effects that can determine this trend are (a) higher efficiency of the enzyme in the bound water pseudo-phase, $[E_b]$, and (b) partition of the substrate between the free water pseudo-phase and micellar aggregates.

The dotted lines in Figures 7 and 8 are the loci of the maximum values of (r/r_0) for each set of parameters. The abscissa allows the determination of $[D_N]_{\max}$, which represents the optimum theoretical surfactant concentration to be used in the system. In

Figure 7, $(r/r_0)_{\max}$ initially increases with K_E , then decreases and approaches 0 as K_E approaches $+\infty$.

This behaviour of the curve maximum results from the sum of two effects: the bell-shaped trend of $r_{b,f}$ and the decreasing trend of $r_{t,f}$. At very low values of K_E , the maximum is reached for $(r/r_0) = 1$. An increase in K_E modifies the relative importance of $r_{b,f}$ and $r_{t,f}$ and determines an optimum condition at higher values of $[D_N]$. At very high values of K_E , $r_{b,f}$ begins to prevail and the maximum of the (r/r_0) curve shifts towards lower values of $[D_N]$.

The presence of an optimum at some intermediate value of $[D_N]$ cannot be expected in the plots of Figure 8. These curves refer to a constant partition of the enzyme (constant K_E) and to a constant efficiency ratio, $(\eta_{b,t}/\eta_0)$, assumed higher than unity. Therefore an increase in $[D_N]$ is always associated with a higher concentration of the most active enzymic form, $[E_b]$. It is therefore advantageous to operate with the highest possible concentration of a surfactant that has positive interactions with the enzyme and a low tendency to associate with the substrate, i.e. small K_s .

When the assumptions in case 2 apply, only the second and the fourth reactions in eqn. (13) are important. The model predictions now depend on the two enzyme efficiencies $\eta_{b,b}$ and $\eta_{t,b}$, representing bioconversion of the substrate in the bound water with enzyme in the bound water and in the free water respectively.

Figure 9 shows that bell-shaped curves can be obtained at fixed values of K_s and K_E (i.e. constant conditions of substrate and enzyme partition) when the enzyme in the bound water has an efficiency lower than in the free water: $\eta_{b,b} > \eta_{t,b}$.

Values of (r/r_0) greater than unity (increasing superactivity) can arise if at least one of the two efficiencies $\eta_{b,b}$ and $\eta_{t,b}$ is greater than η_0 . For $\eta_{b,b} < \eta_{t,b}$, the larger positive effect of the free enzyme with higher efficiency prevails in the lower range of surfactant concentration, whereas in the upper range most of the enzyme is bound to the micelles and the superactivity tends to disappear. In the reverse case, when $\eta_{b,b} > \eta_{t,b}$, increasing values of $[D_N]$ lead to increasing superactivity because of a higher concentration of the micellized enzyme at higher efficiency. The effect of K_E on (r/r_0) is shown in Figure 10. The curves refer to conditions A3 and B2 in Figure 9 (positive enzyme-micelle interactions). It is clear that the extent of superactivity always grows with the association constant of the enzyme, leading to the possible disappearance of bell-shaped behaviour.

Conclusions

The models presented here show how the presence of direct micelles can lead to superactivity in enzyme reactions. The pseudo-phase approach, previously employed for describing enzyme kinetics in reverse micellar systems, can be applied to the problem, the only modification being the absence of an organic phase. The model enables the initial rate of reaction, r , calculated as the sum of various contributions, to be compared with r_0 , the value relating to surfactant concentration equal to the CMC. A plot of (r/r_0) against the concentration of surfactant in the aggregated form shows monotonically decreasing, monotonically increasing or bell-shaped characteristics, depending on the partition of enzyme and substrate and on the enzyme efficiencies in the various pseudo-phases. From a comparison of the numerical results presented here with published trends for reaction rates in reverse micelle systems [9–11], it seems that the surfactant concentration, $[D_N]$, in direct micelle systems can have the same role as the molar ratio of water to surfactant, w_0 , in reverse micelle systems.

The model can be applied to surfactant systems that at the CMC give rise to enzyme efficiencies that are either higher or

Figure 10 Effect of K_E on model 2

Simulated conditions: $[S_1] = 2.5 \text{ mM}$, $K_S = 10^4 \text{ M}^{-1}$, $P_{b,s} = 10^{-4}$. A key to the curve numbers is given below.

Curve	$\eta_{b,b}/\eta_0$	$\eta_{t,b}/\eta_0$	K_E
A1	1	10	1
A2	1	10	10
A3	1	10	100
A4	1	10	1000
B1	10	1	1
B2	10	1	10
B3	10	1	100
B4	10	1	1000

lower than in a pure buffer solution. By increasing the surfactant concentration beyond the CMC, the presence of direct micelles can cause either a decrease or a further increase in activity, perhaps leading to very high levels of superactivity.

Finally, it should be emphasized that the usual assumption that the enzyme cannot interact with the micelles but only with the surfactant in the monomeric form, or in small assemblies not organized into aggregates, cannot explain the evidence reported [21] for an optimum surfactant concentration in certain cases.

APPENDIX

Volume fraction of the micellar pseudophase

At a surfactant concentration close to the CMC, the average dimensions of micelles, assumed as spheres, are: $R_m = 30 \text{ \AA}$, $\psi = 100 (\pm 15)$, $A_m = 1.13 \times 10^{-12} \text{ cm}^2$ and $V_m = 1.13 \times 10^{-19} \text{ cm}^3$, where R_m is the micellar radius, ψ is the number of surfactant molecules per micelle, A_m is the surface of the micelle and V_m is the volume of the micelle. The number of micelles per unit system volume, $[M]$, is related to the micellized surfactant concentration, $[D_N]$, and to the volume of the single micelle by the following relationship:

$$[M] = \frac{N[D_N]}{\psi} \quad (\text{A1})$$

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REFERENCES

- Ben-Shaul, A. and Gelbart, W. M. (1994) in *Micelles, Membranes, Microemulsions and Monolayers* (Gelbart, W. M., Ben-Shaul, A. and Roux, D., eds.), p. 1, Springer-Verlag, New York
- Bunton, C. A. and Savelli, G. (1986) *Adv. Phys. Org. Chem.* **22**, 213–309
- Fletcher, P. D. I., Freedman, R. B., Mead, J., Oldfield, C. and Robinson, B. H. (1984) *Colloid Surf.* **10**, 193–203
- Mao, Q. and Walde, P. (1991) *Biochem. Biophys. Res. Commun.* **178**, 1105–1112
- Martinek, K., Klyochko, N. L., Kabanov, A. V., Klmelnitsky, Y. L. and Levashov, A. V. (1989) *Biochim. Biophys. Acta* **981**, 161–172
- Shoemaeker, R., Robinson, B. H. and Fletcher, P. D. I. (1988) *J. Chem. Soc. Faraday Trans.* **84**, 4203–4212
- Hatton, T. A. (1989) in *Surfactant-Based Separation Processes* (Scamehorn, J. F. and Harwell, J. H., eds.) (Surfactant Sciences Series, vol. 33), pp. 57–90, Marcel Dekker, New York
- Blanco, R. M., Halling, P. J., Bastida, A., Cuesta, C. and Guisán, J. M. (1992) *Biotechnol. Bioeng.* **39**, 75–84
- Bru, R., Sánchez-Ferrer, A. and García-Carmona, F. (1989) *Biochem. J.* **259**, 355–361
- Bru, R., Sánchez-Ferrer, A. and García-Carmona, F. (1990) *Biochem. J.* **268**, 679–684
- Bru, R., Sánchez-Ferrer, A. and García-Carmona, F. (1995) *Biochem. J.* **310**, 721–739
- Creagh, A. L., Prausnitz, J. M. and Blanch, H. W. (1993) *Biotechnol. Bioeng.* **41**, 156–161
- Ishikawa, H., Noda, K. and Oka, T. (1990) *Ann. N.Y. Acad. Sci.* **613**, 529–533
- Larsson, K. K., Adlercreutz, P. and Mattiasson, B. (1987) *Eur. J. Biochem.* **166**, 157–161
- Levashov, A. V., Klyochko, N. L., Bogdanova, N. G. and Martinek, K. (1990) *FEBS Lett.* **268**, 238–240
- Luisi, P. L., Giomini, M., Pileni, M. P. and Robinson, B. H. (1988) *Biochim. Biophys. Acta* **947**, 209–246
- Martinek, K., Levashov, A. V., Klyochko, N., Klmelnitsky, Y. L. and Berezin, I. V. (1986) *Eur. J. Biochem.* **155**, 453–468
- Menger, F. M. and Yamada, K. (1979) *J. Am. Chem. Soc.* **101**, 6731–6734
- Sarcar, S., Jain, T. K. and Maitra, A. (1992) *Biotechnol. Bioeng.* **39**, 474–478
- Alfani, F., Cantarella, M., Spreti, N., Germani, R. and Savelli, G. (1999) *Appl. Biochem. Biotechnol.*, in the press
- Spreti, N., Alfani, F., Cantarella, M., D'Amico, F., Germani, R. and Savelli, G. (1999) *J. Mol. Catal.* **6**, 99–110
- Sánchez-Ferrer, A., Pérez-Gilabert, M. and García-Carmona, F. (1992) in *Biocatalysis in Non-Conventional Media* (Tramper, J., ed.), pp. 181–188, Elsevier, Amsterdam
- Verhaert, R. M. D., Hilhorst, R., Vermúe, M., Schaafsma, T. J. and Veeger, C. (1990) *Eur. J. Biochem.* **187**, 59–72
- Reiss-Husson, F. and Luzzati, V. (1964) *J. Phys. Chem.* **88**, 3504–3509
- Laughlin, R. G. (1994) *The Aqueous Phase Behaviour of Surfactants*, Harcourt Brace & Company/Academic Press, London

where N is the Avogadro number. Therefore the volume fraction of the micellar pseudo-phase, ϕ_m , is given by:

$$\phi_m = \frac{N[D_N]}{\psi} V_m \quad (\text{A2})$$

and that of the bound water pseudo-phase is given by:

$$\phi_b = \frac{[D_N]}{\psi} \theta \bar{V}_{\text{water}} \quad (\text{A3})$$

where $\bar{V}_{\text{water}}$ is the molar volume of the water and θ is the number of water molecules belonging to the bound water per single micelle.

Figure A1 Effect of the concentration of CTBABr (■) and CTABr (●) micellar aggregates on α -chymotrypsin activity

Experimental conditions: $[\alpha\text{-chymotrypsin}] = 0.2 \text{ mg/ml}$, $[N\text{-glutaryl-L-phenylalanine } p\text{-nitroanilide}] = 2.5 \text{ mM}$, $[\text{Tris/HCl}] = 0.1 \text{ M}$, $T = 25^\circ\text{C}$, $\text{pH} = 7.75$.

Dimerization of spherical micelles

The dimensions of the micellar aggregates increase when the surfactant concentration increases. Variations in the micelle size [1] are considered here. As the distribution function of the micelle size is difficult to determine, we shall simply assume the following dimerization equilibrium of the spherical micelles:

where $[M]$ and $[D]$ are the numbers of single and dimerized micelles per unit volume respectively and K_D is the dimerization constant. The degree of dimerization, ξ , is related to the initial number of single micelles per unit system volume, $[M]_i = N[D_N]/\psi$, by:

$$\xi = \frac{2[D]}{[M]_i} = 1 + \frac{1 - (8 K_D [M]_i + 1)^{\frac{1}{2}}}{4 K_D [M]_i} \quad (\text{A5})$$

A loss of surface occurs on dimerization because the micelles tend to a spherical conformation; it can be assumed that:

$$A_D = f A_m \quad (\text{A6})$$

where A_D and A_m are the surface areas of dimerized and single micelles respectively. The total number of micelles per unit volume is:

$$[M]_T = [M] + [D] = \left(1 - \frac{\xi}{2}\right) \cdot \frac{N[D_N]}{\psi} \quad (\text{A7})$$

but the volume fraction of the micellar pseudo-phase, ϕ_m , is still given by eqn. (A2) if the volume of the dimerized micelle can be assumed to be twice that of the single micelle. The volume fraction of the bound water pseudo-phase can be simply determined using the hypothesis that the number of water molecules

per micelle is directly linked to the surface of the micelle. Consequently, we have $\theta_D = \theta/f$ and:

$$\phi_b = \left[(1 - \xi) + \frac{\xi}{2f} \right] \frac{[D_N]}{\psi} \theta \bar{V}_{\text{water}} \quad (\text{A8})$$

The assumption that the dimerized micelle still maintains a spherical shape implies that $f = 1.33$. If the dimerized micelle had a rod-like geometry (but generally this would imply a transition from small to large micelles), the most plausible shape would be that of an ellipsoid having two of the three semi-axes equal to the radius of the single micelle. Because the volume of the dimerized micelle is still twice that of the single micelle, it is easy to show that the third semi-axis must be twice the radius of the spherical single micelle. In this case the eccentricity of the ellipsoid would be $e = 0.75$ and f would increase to 1.63.

Equivalence of the model parameters

The expressions for the volume fractions of each pseudo-phase can be used to correlate the model parameters with those usually adopted in the standard multi-phase approach to enzyme kinetics in reverse micelles [2].

The substrate mass balance and the partition coefficients can be written as follows:

$$[S] = \sum_i \phi_i [S_i] \quad (\text{A9})$$

$$P'_{t,b} = \frac{[S'_b]}{[S'_t]} \quad \text{and} \quad P'_{b,s} = \frac{[S'_s]}{[S'_b]} \quad (\text{A10})$$

where the partition coefficients $P'_{x,y}$ are related to the coefficients $P_{x,y}$ as follows:

$$P'_{t,b} = P_{t,b} \frac{1 - \phi_m - \phi_b}{\phi_b} \quad \text{and} \quad P'_{b,s} = P_{b,s} \frac{1 - \phi_m - \phi_b}{\phi_m} \quad (\text{A11})$$

The enzyme mass balance is given by:

$$[E]_0 = \sum_i \phi_i [E'_i] \quad (\text{A12})$$

and its partition coefficients $P'_E = [E'_b]/[E'_t]$ must be related to the coefficient K_E introduced previously:

$$P'_E = \frac{K_E (1 - \phi_b) [D_N]}{\phi_b} \quad (\text{A13})$$

Experimental validation of the model

Previous studies [3,4] have shown that α -chymotrypsin activity can be either depressed or increased by cationic surfactants such as cetyltrimethylammonium bromide (CTABr) and CTBABr. The hydrolysis rate of N -glutaryl-L-phenylalanine p -nitroanilide (GpNA) supplied by Sigma (2.5 mM) was measured at 25°C in 0.1 M Tris/HCl buffer, pH 7.75. The reaction was initiated by the addition of sufficient enzyme (crystalline, EC 3.4.21.1, bovine pancreas, type II, molecular mass 24.8 kDa, pI 8.8), also supplied by Sigma, to result in a concentration of 0.2 mg/ml. The experimental procedure is detailed in [3,4]. The specific rate of hydrolysis in pure buffer (r_{buffer}), $0.0303 \mu\text{mol/min per mg}$ of enzyme, was found to be equal (within the limits of the experimental error) to r_0 in the presence of both CTABr ($0.0310 \mu\text{mol/min per mg}$ of enzyme) and CTBABr ($0.0307 \mu\text{mol/min per mg}$ of enzyme) at the CMC.

The specific rate of hydrolysis above the CMC was measured at surfactant concentrations of up to 100 mM: the plot of (r/r_0) against the concentration of surfactant micellar aggregates is reported in Figure A1. The fitting of data was by means of model 2 with experimentally known values of the surfactant–substrate association constant, $K_s^{\text{CTABr-GpNA}} = 2000 \text{ M}^{-1}$ and $K_s^{\text{CTBABr-GpNA}} = 1500 \text{ M}^{-1}$. For both surfactants $P_{b,s}$ was much greater than unity (case 1 of model 2). The following set of model parameters was determined: for CTABr, $K_E = 0.12 \text{ M}^{-1}$ and $\eta_{b,i}/\eta_0 = 2.14 \times 10^3$, whereas for CTBABr, $K_E = 0.31 \text{ M}^{-1}$ and $\eta_{b,i}/\eta_0 = 1.12 \times 10^4$.

In conclusion, the experimental data confirm that behaviours depicted by the models are not dependent on the relative values of r_{buffer} and r_0 . Bell-shaped curves and monotonic curves can be observed even for the same enzyme and the same family of

surfactants. The model can give indications of enzyme partition between the bulk of the solution and the micellar aggregates as well as information on the efficiency of the two enzyme forms.

REFERENCES

- 1 Ben-Shaul, A. and Gelbart, W. M. (1994) in *Micelles, Membranes, Microemulsions and Monolayers* (Gelbart, W. M., Ben-Shaul, A. and Roux, D., eds.), p. 1, Springer-Verlag, New York
- 2 Fletcher, P. D. I., Freedman, R. B., Mead, J., Oldfield, C. and Robinson, B. H. (1984) *Colloid Surf.* **10**, 193–203
- 3 Alfani, F., Cantarella, M., Spreti, N., Germani, R. and Savelli, G. (1999) *Appl. Biochem. Biotechnol.*, in the press
- 4 Spreti, N., Alfani, F., Cantarella, M., D'Amico, F., Germani, R. and Savelli, G. (1999) *J. Mol. Catal.* **6**, 99–110

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