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Review

Experimental validation of a model for α-chymotrypsin activity in aqueous solutions of surfactant aggregates

Paolo Viparelli*, Francesco Alfani, Maria Cantarella

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

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Abstract

The hydrolysis of *N*-glutaryl-L-phenylalanine *p*-nitroanilide and *N*-succinyl-L-phenylalanine *p*-nitroanilide catalysed by α-chymotrypsin was studied in the presence of cationic (cetyltrimethylammonium bromide and cetyltripropylammonium bromide) and non-ionic (*t*-octylphenoxypolyethoxyethanol and polyoxyethylene-9-lauryl ether) surfactants. The ratio of reaction rates in surfactant rich systems to those in pure buffer solutions was simulated by a previously developed model by Viparelli et al. [Biochem. J. 344 (1999) 765] based on the pseudo-phase approach introduced by Bru et al. [Biochem. J. 259 (1989) 355] for enzymatic reactions in reverse micelles. The system was depicted by three pseudo-phases: free water, bound water and surfactant core. The substrate and enzyme concentration in the pseudo-phase was related to total substrate and enzyme concentrations in the reaction medium.

Superactivity occurs only in the presence of cetyltripropylammonium bromide. Plots of reaction rate ratio versus surfactant concentration were bell-shaped. Model simulation indicated that this behaviour could be attributed to the equilibrium between the enzyme confined in the free water and that in the bound water pseudo-phase, and to partition of the substrate in the pseudo-phases. The two enzyme forms must have different catalytic behaviour. The overall reaction rate depends on the two enzymatic reactions. Reasonable agreement was found between experimental results and model predictions.

Keywords: Enzyme catalysis; Surfactants; Aggregates; Kinetics; Models

1. Introduction

The use of surfactants at concentrations above the critical micellar concentration (CMC) has been suggested for organic chemical reactions, because the yield can be improved by interaction of the reactants with the micelles [1]. During the last decade, surfactants have been employed in a number of reverse-micelle bioprocesses: surfactant micro-emulsifications [2], extraction of active enzymes [3], and preparation of media for hosting enzymatic reactions [4].

There is ample experimental evidence for 'superactivity' in biocatalytic processes: the activity of enzymes confined in reverse micelle surfactants can be higher than in pure buffer solution [5]. In contrast, there are few experimental results which point to enzyme superactivity in solely water-surfactant media, although positive interactions between enzymes and surfactants have been reported to lead to an increase in catalytic activity [6].

* Corresponding author. Tel.: +39-862-434215/434232.
E-mail address: viparell@ing.univaq.it (P. Viparelli).

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Superactivity of α-chymotrypsin (α-CT) has been detected in aqueous solutions of buffer and cationic surfactants [7,8]. An enzyme efficiency higher than in pure buffer can occur at surfactant concentrations either below or above the CMC, and its extent depends on both surfactant and buffer concentration. Interactions between the four components of the system (enzyme, surfactant, substrate and buffer) need to be taken into account in order to explain the differences in maximum reaction velocity and Michaelis–Menten constant for the enzymatic hydrolysis of *N*-glutaryl-L-phenylalanine *p*-nitroanilide (GpNA) when carried out, on the one hand, in pure buffer solution, and on the other in the presence of surfactant.

A model has also been developed for simulating enzymatic reactions in aqueous solutions of surfactant aggregates: this enables the ratio of the reaction rate (r) to its value at the surfactant CMC (r_0) to be predicted [9]. The pseudo-phase approach was adopted to describe the kinetic behaviour of the enzyme at surfactant concentration above the CMC. The assumption of Michaelis–Menten kinetics was made for all the reactions catalysed by an enzyme partitioned in the various pseudo-phases, with kinetic parameters varying according to enzyme location. An exact description of aggregate geometry is not required, however, as the model assumes equilibrium in both substrate and enzyme partition between the free water, the bound water, and the surfactant aggregates. An enzyme activity either higher or lower than in pure buffer may be determined by the combined effects of the effective enzyme and substrate concentrations in each pseudo-phase, and the reaction kinetics in the presence of the surfactant, both as aggregates and free molecules.

This study aims to complete the previous investigation on the activity of α-CT in the presence of surfactant aggregates in order to further validate the model assumptions. New experiments were carried out with GpNA in aqueous solutions of buffer with either cationic or non-ionic surfactants. The hydrolysis of a second substrate, *N*-succinyl-L-phenylalanine *p*-nitroanilide (SpNA) catalysed by α-CT, was also studied. The model was used to simulate experimental conditions, and hence to test the consistency of physical–chemical and kinetic parameters estimated by fitting procedures with the model assumptions.

2. Experimental

α-Chymotrypsin from bovine pancreas (molecular weight 24.8 kDa, isoelectric point pI 8.8) was purchased from Sigma (USA) and used without further purification. α-CT was type II Sigma preparation: three times crystallised, dyalised and lyophilised. Both substrates, GpNA and SpNA, were also supplied by Sigma. Enzyme and substrate solutions were always freshly prepared in the appropriate buffer immediately before their use in experiments. The two chemicals of the buffer, tris-(hydroxymethyl)aminomethane (TRIS) (pK_a 8.3) and hydrochloric acid, were obtained from Aldrich (Germany) and Carlo Erba (Italy), respectively. The commercial grade non-ionic surfactants, Triton X100 (*t*-octylphenoxypolyethoxyethanol) and polyoxyethylene-9-lauryl ether (PO9), were obtained from Sigma, and the cetyltrimethylammonium bromide from Fluka (Germany). Cetyltripropylammonium bromide (CTPABr) was part of a stock kindly supplied by Professor Gianfranco Savelli of the University of Perugia. Details of the laboratory-scale preparation and purification of this synthesised surfactant are given in [10].

2.1. Assay of α-chymotrypsin activity

The α-CT-catalysed hydrolysis of GpNA and SpNA was monitored by following the change in absorbance at 410 nm brought about by the formation of *p*-nitroaniline (pNA). Kinetic determinations were performed at 25°C using a Perkin-Elmer Lambda2 UV–VIS spectrophotometer equipped with cell holders thermostatically controlled to within ±0.1°C. The product extinction coefficient was found to be 10.015 M^{-1} cm^{-1} both in pure buffer and in the presence of surfactants. The absence of autohydrolysis of the substrates was confirmed for all the experimental conditions employed for the enzyme tests: no reaction was observed in the absence of enzymes over the time scale of the experiments (10 min). Enzyme activity was assayed in 0.1 M buffer pH 7.75 at α-CT concentration of 8 μM (0.2 mg ml^{-1}). Regular kinetic tests were carried out in 3 ml cuvettes (1 cm path length) filled with surfactant and substrate solutions (both prepared with buffer) to a final volume of 2.94 ml. The enzymatic reaction was started by

the addition of 0.06 ml of the enzyme buffered stock solution (10 mg ml^{-1}). The enzymatic activity was evaluated both in terms of initial reaction rate r, defined as moles of pNA formed per litre per second, and as turnover number, k_{CAT} (s^{-1}), moles of GpNA transformed per second per mole of enzyme. In all the kinetic tests, GpNA consumption during the initial minutes of hydrolysis at saturated substrate concentration was found to be linearly time dependent — as predicted by differential reactor theory. The initial rates were calculated from the gradient of the changes-in-absorbance versus time records. The maximal velocities, V_{max}, and the Michaelis–Menten constants, K_M, in the presence of buffer and/or surfactant were determined from linear regression analysis of the double-reciprocal Lineweaver–Burk plot. The k_{CAT} values were also calculated. Each reported data point represents the average of at least two experimental results obtained under identical operational conditions; discrepancies in repeated evaluations were always less than 5%.

3. System simulation

Surfactant aggregates in aqueous solutions are usually depicted as micro-structures with the hydrophilic heads of the surfactant oriented towards the water molecules and the hydrophobic tails oriented towards the inner part of the assembly. Aggregates appear as the dominant form above the CMC, but free surfactant is also present in the system as monomer or small unorganised assemblies.

Two distinct kinds of aggregates can exist depending on the surfactant concentration: small ones, formed by some 100–200 molecules, and large ones formed by approximately 1000 molecules [11].

In view of the relatively low surfactant concentrations investigated in this work, the system was schematically depicted as small aggregates, with increases in surfactant concentration resulting in increasing numbers of assemblies: the aggregation number and the size of the aggregates were both assumed to remain essentially constant. This conforms to previously reported observations [12]. Based on this hypothesis, one obtains

$$N_M = \beta[D_N] \quad (1)$$

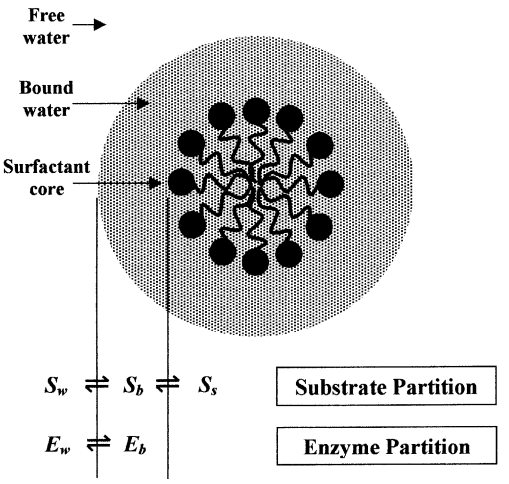

Fig. 1. Schematic representation of the system.

where N_M is the number of aggregates in the system, $[D_N]$ the concentration of the surfactant organised as assemblies, and β a proportionality parameter. $[D_N]$ can be readily calculated as the difference between the total surfactant concentration in the system $[T]$ and the CMC.

Each surfactant aggregate was shaped by three different micro-environments or pseudo-phases: the free water, the bound water (a small region very close to the surfactant heads), and the system core formed by the surfactant molecules (Fig. 1). The substrate partition in the three pseudo-phases is regulated by two constants: K_S and $P_{b,s}$. K_S describes the equilibrium between the substrate in the free water pseudo-phase (S_w) and that in the aggregate ($S_b + S_s$): it can be measured experimentally by a procedure described elsewhere [8]. $P_{b,s}$ describes the equilibrium between the substrate in the bound water pseudo-phase (S_b) and that associated with the surfactant molecules (S_s). This constant cannot be measured experimentally: its value is predicted by model simulation of the (r/r_0) versus $[T]$ curves.

Thus, the substrate available for catalysis is related to its total concentration in the system, $[S_0]$. The derivation of the following relationships is detailed in Appendix A.

$$[S_w] = \frac{[S_0]}{1 + K_S[D_N]} \quad (2)$$

$$[S_b] = \frac{K_S[D_N]}{(1 + K_S[D_N])(1 + P_{b,s})}[S_0] \quad (3)$$

Because the enzyme molecular dimensions are of same order of magnitude as the surfactant aggregates, it was assumed that only two forms of enzyme, E_w and E_b, can be present: confined to the free water and the bound water pseudo-phases, respectively. The partition of the protein is then regulated by the equilibrium constant K_E. Thus, the enzyme concentration in the free and bound water obeys the following equations.

$$[E_w] = \frac{[E_0]}{1 + K_E[D_N]} \quad (4)$$

$$[E_b] = \frac{K_E[D_N][E_0]}{1 + K_E[D_N]} \quad (5)$$

The number of adjustable parameters in the model may be reduced on the basis of the following considerations. In the case of the 'cationic' surfactants, their strong affinity for the substrate allows one to assume that $P_{b,s} \gg 1$ and $[S_b] \approx 0$. Moreover, it would appear reasonable to further assume that the substrate associated with the surfactant S_s, cannot participate in the reaction. In the case of the 'non-ionic' surfactants, experiments showed that $K_S \approx 0$. This result means that $[S_w] \approx [S_0]$.

On the basis that the active site of the enzyme is accessible to the substrate molecules in each pseudo-phase, the reaction rate is the sum of two contributions for both cationic and non-ionic surfactants.

$$r = \frac{k_{CAT}^{w,w}[E_w][S_w]}{K_M^{w,w} + [S_w]} + \frac{k_{CAT}^{b,w}[E_b][S_w]}{K_M^{b,w} + [S_w]} \quad (6)$$

Because of the very short characteristic time of partition equilibrium, together with the assumption of negligible intra- and inter-mass transfer resistances, the enzymatic reaction is always the rate-limiting step (further details of model formulation and simulation are reported in [9]).

4. Results and discussion

α-Chymotrypsin activity was tested initially in pure buffer (0.1 M Tris–HCl, pH 7.75) and in aqueous solutions of buffer plus surfactant at concentration above the CMC. The enzymatic hydrolysis was performed at 25°C, with 8 μM α-CT and surfactant concentration of 5×10^{-3} M. Substrate concentration was 2.5×10^{-3} M, higher than the saturation concentration in pure buffer. Results of a comparison of hydrolysis rates in the presence of surfactant aggregates (r) and in pure buffer (r_{buffer}) are shown in Table 1 and Figs. 2–5. Under the experimental conditions of this study, the rates of GpNA and SpNA hydrolysis at surfactant concentrations approximately equal to the CMC, r_0, were essentially the same as in pure buffer, r_{buffer} (see Table 1). This means that model predictions of (r/r_0) may be compared directly with (r/r_{buffer}) values obtained from the experiments.

Table 1
Effect of various surfactants on α-CT activity[a]

Surfactant	CMC[b] (M)	r_0 (μmol s^{-1} l^{-1})		(r/r_{buffer})	
		SpNA	GpNA	SpNA	GpNA
TX100	2.5×10^{-4}	0.060	0.104	0.92	0.92
PO9	3.0×10^{-4}	0.062	0.107	0.93	0.93
CTABr	8.7×10^{-4}	0.058	0.106	0.76	0.76
CTPABr	5.5×10^{-4}	0.060	0.105	1.83	2.40

[a] $[E_0] = 8$ μM, $[S_0] = 2.5$ mM, [buffer] = 0.1M, $[T] = 5$ mM, $T = 25$°C, pH = 7.75.
[b] In water.

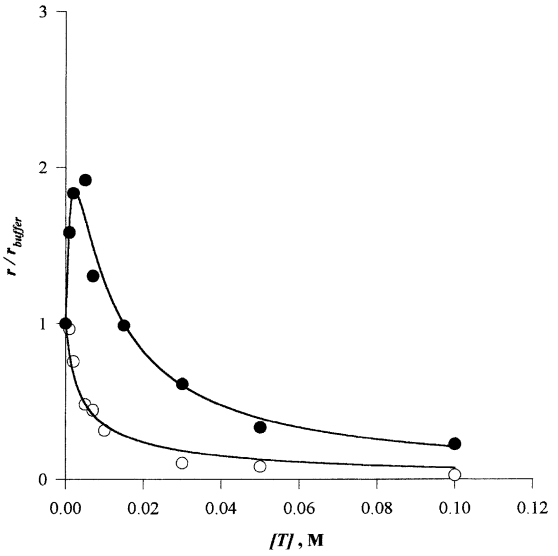

Fig. 2. Hydrolysis of SpNA catalysed by α-CT in the presence of (●) CTPABr and (○) CTABr. $[E_0] = 8$ μM, $[S_0] = 2.5$ mM, [buffer] = 0.1 M, $T = 25$°C, pH = 7.75.

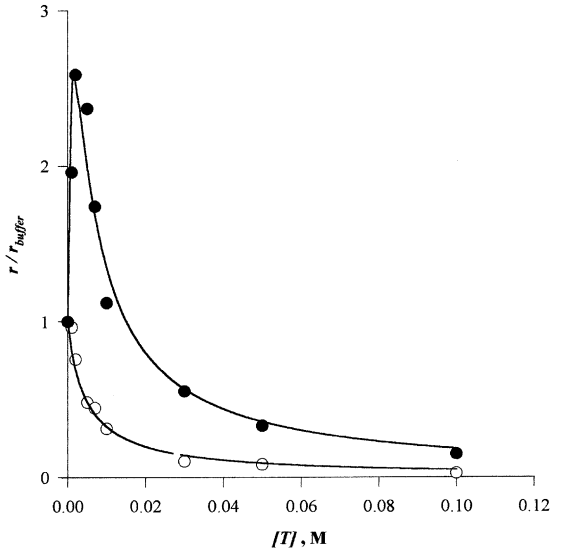

Fig. 3. Hydrolysis of GpNA catalysed by α-CT in the presence of (●) CTPABr and (○) CTABr. $[E_0] = 8\,\mu M$, $[S_0] = 2.5\,mM$, [buffer] = 0.1 M, $T = 25^\circ C$, pH = 7.75.

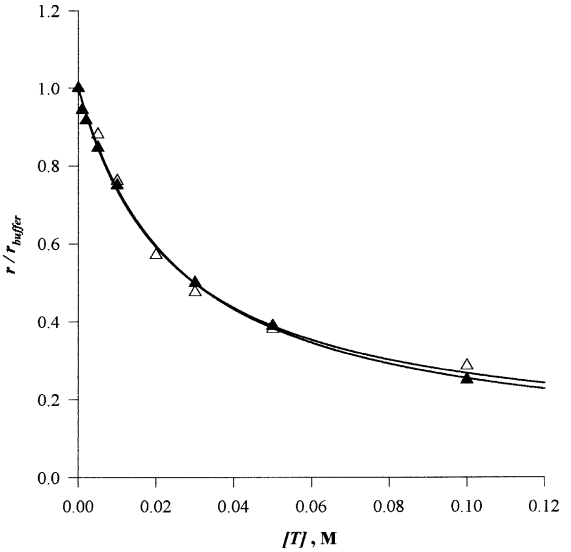

Fig. 5. Hydrolysis of GpNA catalysed by α-CT in the presence of (▲) TX100 and (△) PO9. $[E_0] = 8\,\mu M$, $[S_0] = 2.5\,mM$, [buffer] = 0.1 M, $T = 25^\circ C$, pH = 7.75.

The rates of SpNA and GpNA hydrolysis in pure buffer were found to be 0.060 and 0.101 $\mu mol\,s^{-1}\,l^{-1}$, respectively. Two series of experiments performed with GpNA and SpNA indicate that, with the exception of CTPABr, the ratio of α-CT activity in the two systems with and without surfactant was independent of the substrate employed. α-CT activity, on the other hand, was found to be dependent on surfactant type. Non-ionic surfactants (TX100 and PO9) were found to give rise to a slight loss of activity (7–8%) compared with that observed in pure buffer. More important, however, was the effect on reaction rates (as compared with their values in pure buffer) of cationic surfactant aggregates: these were found to lead to either a considerable reduction in activity (a loss of roughly 25% in CTABr) or a significant increase (83% with SpNA and 140% with GpNA in CTPABr).

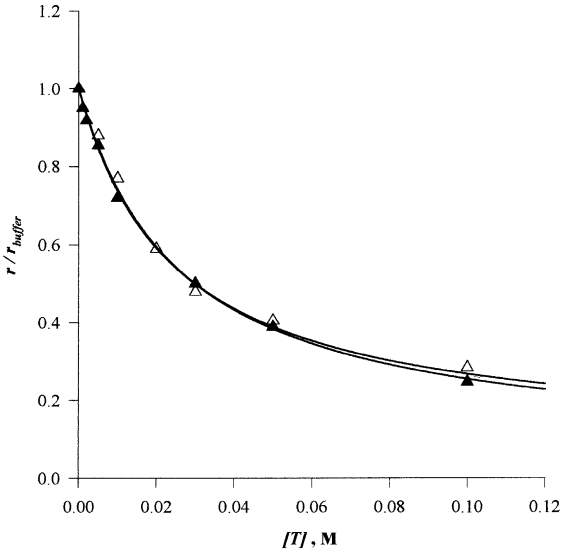

Fig. 4. Hydrolysis of SpNA catalysed by α-CT in the presence of (▲) TX100 and (△) PO9. $[E_0] = 8\,\mu M$, $[S_0] = 2.5\,mM$, [buffer] = 0.1 M, $T = 25^\circ C$, pH = 7.75.

Previous investigations have shown enzyme activity to be dependent on cationic surfactant concentration. This effect was studied by varying the concentration of CTABr, CTPABr, TX100, and PO9 between 0.001 and 0.1 M. The resulting ratios of substrate hydrolysis rate in aqueous solutions of surfactant aggregates to those in buffer (r/r_{buffer}) are shown in Figs. 2–5 as functions of the total surfactant concentration [T].

In the presence of CTPABr aggregates, the α-CT activity was found to depend markedly on surfactant concentration: bell-shaped curves of (r/r_{buffer}) versus [T] were obtained. For SpNA and GpNA hydrolysis,

Table 2
Model results

Surfactant	K_S (M^{-1})	K_E (M^{-1})	$k_{CAT}^{b,w}$ (s^{-1})	$K_M^{b,w}$ (mM)	$\eta^{b,w}$ ($M^{-1}\,s^{-1}$)
GpNA					
None	–	–	–	–	37.4
CTABr	2000	301	0.015	0.29	51.7
CTPABr	2160	413	0.13	0.41	210.5
PO9	–	40.3	0.0012	0.31	3.87
TX100	–	37.5	0.00086	0.48	1.79
SpNA					
None	–	–	–	–	9.5
CTABr	90	350	0.0031	1.50	2.1
CTPABr	404	410	0.053	2.22	23.8
PO9	–	38.8	0.00071	0.94	0.76
TX100	–	35.6	0.00035	0.97	0.36

the highest enzyme activities were obtained at surfactant concentration of 5×10^{-3} and 2×10^{-3} M, respectively, enzyme superactivity reached 92% with SpNA and 160% with GpNA. For all surfactant concentrations, the addition of CTABr and of non-ionic surfactants lead to a monotonic decrease in α-CT activity. The full lines in Figs. 2 and 3 are the results of the model simulations, which show reasonable agreement with the experimental data.

Because kinetic tests at surfactant concentrations below the CMC showed reaction rates to be the same as in pure buffer, it was assumed in the model that enzymatic hydrolysis in the free water pseudo-phase is unaffected by the presence of the surfactant aggregates. This enables values obtained from substrate hydrolysis in pure buffer to be inserted in Eq. (6), yielding: $k_{CAT}^{w,w} = k_{CAT}^{buffer} = 0.0146\,s^{-1}$ and $K_M^{w,w} = K_M^{buffer} = 0.39$ mM.

Model results are listed in Table 2. The substrate partition constant K_S, was also measured experimentally. This leaves K_E, $k_{CAT}^{b,w}$ and $K_M^{b,w}$ as the only estimated parameters. The efficiency of the enzyme in the bound water pseudo-phase, $\eta^{b,w} = k_{CAT}^{b,w}/K_M^{b,w}$, is also reported in Table 2, where it is compared with that in pure buffer.

The constant K_E gives an estimate of the enzyme partition between the free water pseudo-phase and the surfactant aggregates. Results of Table 2 show that K_E was much lower with non-ionic than with cationic surfactants. This implies that the α-CT bound to the PO9 and TX100 aggregates amounted to only 14% of the average of that bound to the CTABr or CTPABr aggregates. This suggests that electrostatic interactions between the enzyme and the surfactant aggregates are stronger than hydrophobic interactions. Their effect depends on the surfactant head groups. However, the fact that non-ionic surfactants give rise to small partition constants seems to suggest that the hydrophobicity of the inner aggregate core should also have a role to play. As enzyme molecular dimensions are of the same order of magnitude as those of surfactant aggregates, α-CT cannot occupy the aggregates, regardless of surfactant presence. Finally, it is noteworthy that K_E is independent of the substrate employed. This result is to be expected and provides further confirmation of the model validity.

Inspection of Table 2 leads to the following conclusions. SpNA exhibits, as expected, a lower binding affinity with the cationic surfactant aggregates than does GpNA. This behaviour is illustrated by the lower measured value of K_S. The addition of non-ionic surfactants (TX100 and PO9) to the buffer does not cause partition of the two substrates between the free water pseudo-phase and the surfactant aggregates: the experiments showed conclusively that all the substrate remained in the free water pseudo-phase ($K_S \approx 0$).

The Michaelis–Menten constant for the enzyme confined in the bound water, $K_M^{b,w}$, depended on both the substrate and the surfactant. However, for all surfactants the affinity of the enzyme for GpNA remained approximately the same as with pure buffer. $K_M^{b,w}$ ranged from −25.6% in CTABr to +23.1% in TX100. Larger variations in the Michaelis–Menten constant were obtained using SpNA as substrate and

cationic surfactants. In CTPABr, $K_M^{b,w}$ reached 231% of its value in pure buffer.

The dependence of turnover number, $k_{CAT}^{b,w}$, on system conditions was even more pronounced: in surfactant solutions its estimated value in the bound water pseudo-phase was as much as an order of magnitude greater than k_{CAT}^{buffer}, its value in pure buffer, for the hydrolysis of GpNA in CTPABr solution; and two orders of magnitude lower than k_{CAT}^{buffer} for the hydrolysis of GpNA in TX100 solution.

Because of the important differences in enzyme activity during the hydrolysis of the two substrates in the presence of the cationic surfactants, the results of Table 2 must be considered with reference to those of Figs. 2 and 3. In the CTABr–GpNA system, the addition of surfactant has little effect on the $k_{CAT}^{b,w}$ value, which remains approximately the same as in pure buffer: $k_{CAT}^{buffer} = 0.0146\,s^{-1}$. The affinity of the enzyme in the bound water for the substrate in the free water is higher than in pure buffer: $K_M^{buffer} = 0.39$ mM. The combined effect is to improve enzyme efficiency. However, the substrate is unfavourably partitioned, as reflected in the high K_S value. Most of the substrate is not available for reaction, which explains the decrease in overall hydrolysis rate with increasing surfactant concentration.

In the CTABr–SpNA system, the interaction of surfactant aggregates with the substrate is weak (low K_S value). Hydrolysis, however, occurred at a lower rate than in pure buffer, in keeping with the results of the model simulation, which indicated a reduction in turnover number, $k_{CAT}^{b,w}$, and an increase in Michaelis–Menten constant, $K_M^{b,w}$.

For both hydrolysis reactions in the presence of CTPABr, the $k_{CAT}^{b,w}$ value was much higher than for the free enzyme in the pure buffer experiments. This increase in enzyme activity indicated positive interactions between the enzyme and the surfactant aggregates. In the presence of surfactant, the affinity for the substrate either remains almost unaffected (in the CTPABr–GpNA system) or else decreases somewhat (in the CTPABr–SpNA system). The resulting enzyme efficiency was significantly greater than in pure buffer. However, the results indicate that strong interactions between the surfactant aggregates and the substrate also occurred. They were very important in the case of GpNA (high K_S). It was therefore possible to attain a maximum α-CT superactivity as a result of the unfavourable substrate partition with increasing surfactant concentration.

An alternative explanation for the higher activity observed with cationic surfactants could be related to the favourable partition of the substrate towards the aggregates. The local concentration of GpNA and SpNA would be higher than in the bulk solution and would lead to an apparent higher activity so long as the reaction rate is not close to V_{max}. This, however, seems unlikely for the following reasons. In the investigated range of surfactant concentration, from 5 to 100 mM, the concentration of the substrate in the local environment of the surfactant aggregates $[S_m]$ can be calculated from the substrate mass balance ($[S_0] = [S_w] + [S_m]$) and Eq. (2). For GpNA, $[S_m]$ varies from 2.27 to 2.5 mM with CTABr aggregates, and from 2.29 to 2.5 mM with CTPABr aggregates. For SpNA, $[S_m]$ varies from 0.77 to 2.5 mM with CTABr, and from 1.67 to 2.5 mM with CTPABr. Therefore, if the phenomenon of superactivity was due only to substrate partition, it should be much more evident in reactions carried out with GpNA than with SpNA, since substrate partition is much more favoured in the former case. This result is not confirmed by the experimental results; nor does it explain the different observed system responses to surfactant concentration changes: the bell-shaped curve with CTPABr aggregates, and the monotonically decreasing curve with CTABr. Finally, enzyme superactivity due to substrate partition should be a continuously increasing function of surfactant concentration — in contradiction with all the experimental results.

5. Conclusions

The results indicate that a model based on the pseudo-phase approach can predict with reasonable accuracy α-CT activity in aqueous solutions of cationic and non-ionic surfactants. The rate of hydrolysis of two model substrates, GpNA and SpNA, can be computed as the sum of two catalytic events related to the enzyme confined in the bound water and free in the aqueous solution; this rate may or may not be affected by interaction with surfactant aggregates. Another important result is that the model

can depict the dependence of (r/r_{buffer}) on surfactant concentration. Both observed behaviours, of a monotonically-decreasing or of a bell-shaped curve, can be described by introducing appropriate parameters for the partition of substrate and enzyme, and for the enzyme efficiency in the various pseudo-phases. Comparisons to rate behaviour of enzyme catalysed reactions in reverse micelles suggest that $[D_N]$ in direct micelle systems can play a similar role to w_0 (water to surfactant molar ratio) in reverse micelle systems.

From the experimental studies, it was deduced that aggregates of non-ionic surfactants lead to reductions in enzyme activity, as compared with the situation in pure buffer. The analysis based on the model simulation showed that this was largely determined by the resulting lower values of the kinetic constant. A reduction of 96.2% was reached with TX100 in the SpNA hydrolysis. The enzyme superactivity observed with the cationic surfactant CTPABr might be the result of both a better enzyme activity and a favourable partition of enzyme and substrate.

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Appendix A. Derivation of model equations

Thermodynamic equilibrium is assumed for both substrate and enzyme distribution between the free water (subscript w) and the whole aggregates (m). The substrate can partition into three pseudo-phases, free water, bound water (b) and surfactant core (s). In contrast, the enzyme partition occurs only between the free water and the bound water.

$$[S_w] + [D_N] \overset{K_S}{\rightleftharpoons} [S_m] \tag{A.1}$$

$$[E_w] + [D_N] \overset{K_E}{\rightleftharpoons} [E_b] \tag{A.2}$$

A further equilibrium between the substrate in the bound water and that associated with surfactant is introduced.

$$[S_b] \overset{P_{b,s}}{\rightleftharpoons} [S_s] \tag{A.3}$$

Taking into account the overall mass balance for substrate and enzyme

$$[S_0] = [S_w] + [S_b] + [S_s] \tag{A.4}$$

$$[E_0] = [E_w] + [E_b] \tag{A.5}$$

and the thermodynamic equilibrium relationships, Eqs. (2)–(5) can be easily derived.

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