Escherichia coli TEM1 β-lactamase in CTAB reverse micelles: exchange/diffusion-limited catalysis

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Abstract **We report kinetic data of penicillin hydrolysis catalyzed by β-lactamase entrapped in reverse micelles formed with cetyl trimethylammonium bromide (CTAB), *n*-octane, hexanol and aqueous buffer. The K_{cat} of this diffusion-limited reaction can be improved in aqueous buffer by a factor of 1.1–1.2 just by increasing the phosphate buffer concentration from 50 to 100 mM. In reverse micelles, increasing the buffer concentration has little effect on K_{cat} when the size of the empty micelle is below the size of the protein. However, in larger micelles, the effect is enhanced and the K_{cat} improves several fold, changing the form of the curve of K_{cat} versus *Wo* from bell-shaped to almost hyperbolic. The results indicate that micellar exchange and internal diffusion may limit the reaction in reverse micelles and provide further evidence that the form of the curve depends on other factors besides the relationship between the size of the enzyme and that of the empty reverse micelle.**

Key words: Reverse micelle; TEM1 β-lactamase; Diffusion-limited catalysis; Exchange-limited catalysis; Bell-shaped activity

1. Introduction

Reverse micelles are formed when a small amount of water and surfactant are mixed in an apolar organic solvent [1,2]. The activity of many enzymes entrapped in the water space of reverse micelles has been extensively studied (for recent reviews, see [3,4]). Most of the latter work has been done in reverse micelles formed with sodium bis(2-ethylhexyl)sulfosuccinate (aerosol OT or AOT) as surfactant, but other amphiphilic molecules, such as cetyl trimethylammonium bromide (CTAB), have also been employed. The water concentration in reverse micelles is commonly expressed as *Wo*, which is the ratio of water to surfactant molecules, and It has been documented that at a constant concentration of surfactant, the size of the water pool inside reverse micelles increases with the amount of water introduced [5]. Thus, one of the questions regarding the properties of enzymes entrapped in reverse micelles concerns the effect of the water pool size on enzymatic activity. Some reports describe that activity increases with *Wo* (e.g. [6–8]). However, other studies show that enzyme activity has a 'bell-shaped' behavior. These studies also indicate that the maximal activity is attained when the dimensions of the enzyme equal those of the water pool inside the empty reverse micelles [9]. Likewise, it has been reported that some multimeric enzymes exhibit several activity peaks and that each peak corresponds to the point in which the size of the empty reverse micelle interior equals the dimensions of the enzyme at different levels of aggregation (monomer, dimer, tetramer) [10–12]. Attempts have also been made to establish kinetic models that account for the activity that enzymes express in reverse micelles [13]. These models are difficult to elaborate since they must take into account the partition coefficients of the substrates, the location of the enzyme (in the water phase or in the interface of the micelle), the number and size of reverse micelles and the rate of micellar exchange.

In an attempt to gain insight into the factors that control the activity of enzymes in reverse micelles, we determined the kinetics of TEM1 β-lactamase entrapped in a system formed with CTAB, hexanol, octane and aqueous buffer. In pure aqueous buffer, the hydrolysis of penicillin by *Escherichia coli* TEM1 β-lactamase is a diffusion-limited reaction [14]. Structurally, β-lactamase is a monomer [15,16] whose dimensions are within the range of the water pools that can be experimentally attained in the reverse micelle system we choose. The activity of bovine liver catalase, which is also diffusion-limited in aqueous buffer, has been previously studied in reverse micelles formed with AOT. The data show that the activity of this enzyme is controlled by exchange of micellar contents [17]. However, liver catalase is a tetramer. In this work, we determined the activity of TEM1 β-lactamase entrapped in reverse micelles. The purpose was to ascertain if the behavior of a diffusion-limited enzyme exhibited or not a bell-shaped type of curve with the water concentration and whether activity was limited by micellar exchange or diffusion of substrates within the water pool of the micelle at all water concentrations.

2. Materials and methods

CTAB, *n*-octane, hexanol and penicillin were obtained from Sigma. The β-lactamase gene was modified to contain a COOH-terminal six histidine tag and the protein was purified using a Ni-NTA agarose column (Qiagen), using the instructions of the manufacturer for purification under denaturant conditions. The enzyme concentration was calculated from its absorbance at 280 nm (the molar extinction coefficient used was 94 000 M^{-1} cm^{-1}).

2.1. Reverse micelles

The basic micro-emulsion was prepared by mixing *n*-octane/hexanol (8.7:1 v/v), CTAB (200 mM final concentration) and 1.5% (v/v) of aqueous buffer consisting of 50 or 100 mM potassium phosphate buffer, pH 7.0 (for this, the optimum pH of the enzyme). The mixture was vigorously stirred and adjusted to the desired final volume by adding more *n*-octane:hexanol mixture. The penicillin and β-lactamase solutions were prepared using the same phosphate buffer. We adjusted *Wo* by addition of the appropriate amount of buffer and penicillin or enzyme solutions.

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2.2. Kinetics

All reactions were carried out at 25°C. Penicillin hydrolysis was determined with a Beckman DU650 spectrophotometer by recording absorbance changes at 240 nm, a spectrum region where the absorbance of reverse micelles is minimal and the penicillin concentration can still be confidently measured. The difference in molar extinction coefficient for penicillin hydrolysis at this wavelength is 540 M^{-1} cm^{-1}, in pure buffer as well as in reverse micelles. For activity measurements, we prepared separate micelle solutions at the desired water concentration that contained enzyme or penicillin. After recording the absorbance of penicillin in reverse micelles, the enzyme entrapped in reverse micelles at the same *Wo* was added and absorbance changes were recorded throughout time. K_{cat} and K_m parameters were determined from continuous degradation curves fitted to a Michaelis-Menten equation using the Kaleidagraph program (Synergy Software), in a Macintosh computer. Our calculations considered the overall penicillin concentration. The overall enzyme concentrations were kept in the tens of nM range. The starting overall penicillin concentrations ranged between 0.8 and 1.2 mM, which permitted us to follow kinetics at concentrations 4–6 times the apparent K_ms.

3. Results and discussion

3.1. β-Lactamase catalytic efficiency in reverse micelles

Fig. 1 displays the specificity or catalytic efficiency constant (K_{cat}/K_m) of penicillin hydrolysis by β-lactamase in reverse micelles formed with 50 mM potassium phosphate buffer. The highest constants were obtained at *Wo*s between 5.5 and 11 (between 3 and 5×10^6 M^{-1} s^{-1}). As the water concentration was increased, the catalytic efficiency of the enzyme progressively decreased. Since the rate of micellar exchange ranges between 10^6 and 10^8 M^{-1} s^{-1} [18,19], the results suggest that the reaction is limited by exchange of micellar contents or diffusion of substrates within the water pool of the micelles. Table 1 summarizes the K_{cat} and K_m parameters used to calculate the catalytic efficiency constants for Fig. 1.

3.2. β-Lactamase activity in reverse micelles using 50 and 100 mM potassium phosphate buffer

We have observed that although in aqueous buffer, the activity of β-lactamase activity is diffusion-limited, the K_{cat} is between 1.1 and 1.2 times higher in 100 mM than in 50 mM potassium phosphate, the specificity constants being around 5.7×10^7 M^{-1} s^{-1} and 4×10^7 M^{-1} s^{-1}, respectively. Higher buffer concentrations have no further effect. A similar effect can be attained by the addition of 100 mM NaCl to 50 mM phosphate buffer. These observations suggest that the composition of the buffer affects the diffusion of substrates into the active site. Therefore, we determined the activity of β-lactamase entrapped in reverse micelles formed with 50 and 100 mM phosphate buffers in order to explore if under such conditions the composition of the buffer also affects the activity of the enzyme.

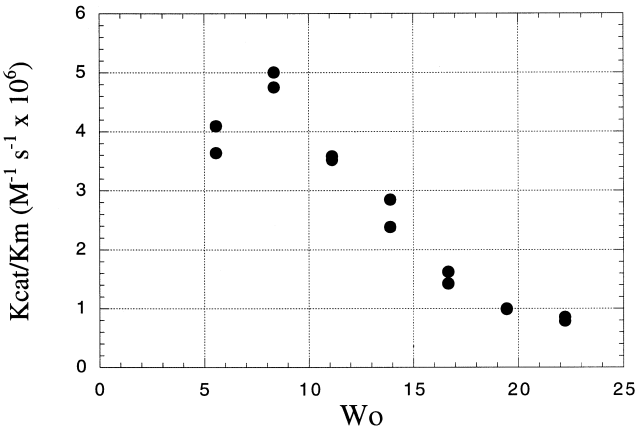

Fig. 1. Catalytic efficiency of penicillin hydrolysis by β-lactamase entrapped in reverse micelles at various *Wo* values. In these experiments, the aqueous phase was 50 mM potassium phosphate buffer, pH 7. We calculated K_m from overall penicillin concentration values. Note that the activity is above 10^6 M^{-1} s^{-1} in most cases.

Up to a *Wo* of 11, the K_{cat} of β-lactamase was almost identical in reverse micelles formed with 50 and 100 mM phosphate buffer (Fig. 2a). At higher water concentrations, an effect of the buffer composition on the enzyme activity became clearly apparent. In 50 mM buffer, there was a sharp decrease in activity as *Wo* was raised from 11 to 23, whereas in reverse micelles formed with 100 mM buffer, the highest activity was observed at a *Wo* of 14 and the activity seems to stabilize at higher water concentrations. The activity versus *Wo* curve we obtained using 50 mM buffer is very similar to that obtained by Chakravarty et al. [20] with a *Bacillus cereus* β-lactamase entrapped in AOT reverse micelles, also hydrolyzing penicillin. They used a phosphate buffer of 20 mM, pH 7.0.

As noted, some reports show that the highest activity of enzymes entrapped in reverse micelles occurs at the point where the size of the water pool of empty reverse micelles equals that of the enzyme [9]. Fig. 2b shows the theoretical calculations of the radii of the water pool of empty reverse micelles at different *Wo* values and the radius of β-lactamase. The former were calculated from simple geometrical considerations leading to the formula: $r = 3v^*Wo/s$. We used 33.6 $Å^2$ as the polar head area of CTAB (s) and 28.0 $Å^3$ as the volume of water molecules (v). The radius of the enzyme was calculated from the volume determined from its three-dimensional

Table 1
Kinetic data of penicillin hydrolysis by β-lactamase in aqueous buffer and in reverse micelles

Wo	K_m (mM)		K_{cat} (s^{-1})	
	First experiment	Second experiment	First experiment	Second experiment
5.5556	0.08900	0.10617	364.60	386.76
8.3333	0.11081	0.11902	554.88	565.61
11.111	0.21236	0.18575	748.48	666.20
13.889	0.20430	0.15462	487.80	440.78
16.667	0.17624	0.13162	251.12	214.39
19.444	0.16320	0.17117	163.51	168.89
22.222	0.18034	0.22081	154.23	175.00
Buffer	0.04640	0.03739	1511.00	1751.20

The aqueous phase was 50 mM sodium phosphate buffer, pH 7. K_m was calculated based on penicillin overall concentrations. Data from duplicate experiments.

structure, using the program 'asc' [21,22]. Note that the radii of the enzyme and the empty micelle are nearly equal when *Wo* is around 10. Therefore, in 50 mM phosphate buffer, the prediction that maximal enzyme activity is attained when the dimensions of the empty micelle coincide with those of the enzyme is fulfilled. Nonetheless, the activity of β-lactamase in reverse micelles formed with 100 mM buffer shows the pattern observed with other enzymes in which activity levels off after a certain water concentration is reached [23–25]. Thus, the overall data of Fig. 2 illustrate that the activity of β-lactamase depends on the dimensions of the micelles, but that this behavior can be affected by the composition of the aqueous phase. Preliminary results show that 50 mM NaCl has a similar though less pronounced effect, i.e. in small reverse micelles ($Wo \sim 8.3$), there is no appreciable effect on K_{cat}, but in large micelles ($Wo \sim 19.4$), K_{cat} increases about 1.5 times (data not shown).

Since the activity of β-lactamase is diffusion-limited, the data imply that the activity of the enzyme entrapped in reverse micelles would be controlled by the rate of micellar exchange or by diffusion of substrates within the water pool of micelles that contain the protein. The reports on micellar exchange rates indicate that it slows down with *Wo* [18,19]. This reinforces the notion that the activities obtained at each *Wo* also depend on successful well-positioned encounters

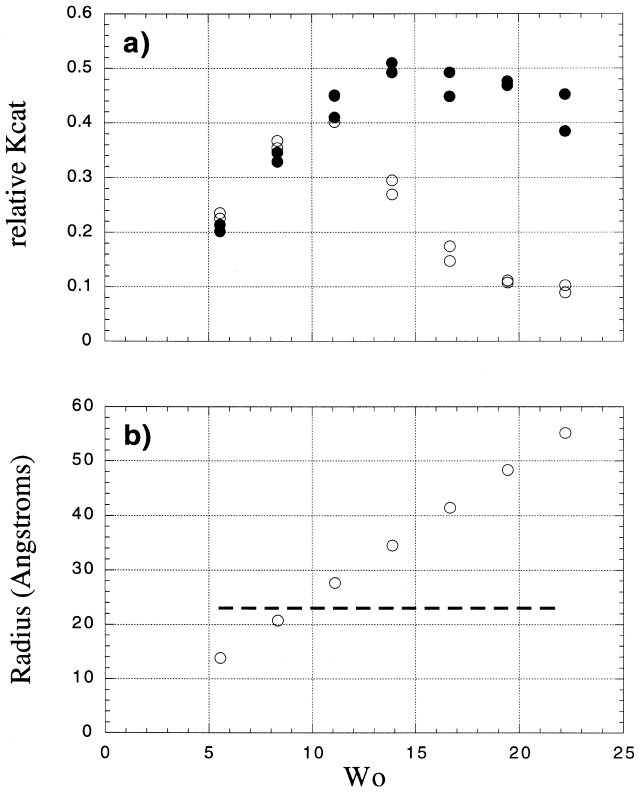

Fig. 2. (a) Plot of the relative K_{cat} of penicillin hydrolysis by β-lactamase entrapped in reverse micelles formed with 50 mM (open circles) and 100 mM (closed circles) potassium phosphate buffer. Plotted K_{cat}s are relative to those in pure 50 mM potassium phosphate buffer. Data from duplicate experiments. (b) Comparison between the radius of β-lactamase (dashed line) and empty reverse micelles internal radii (open circles) at different *Wo* values. The reverse micelles radii were calculated from simple geometrical considerations.

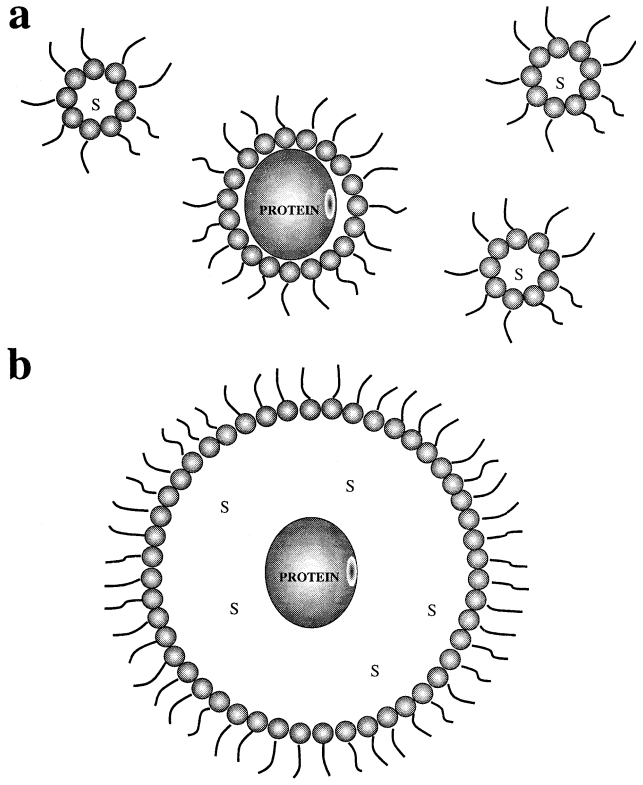

Fig. 3. Different situations for an enzyme entrapped in reverse micelles. (a) When empty reverse micelles are smaller than the enzyme, substrate (S) molecules can only reach the active site (small spot at the right side of the protein) by micellar exchange. (b) In reverse micelles larger than the enzyme, the substrate molecules have to move through water molecules to reach the active site. Thus, the buffer composition might play a more important role than in smaller micelles.

when micelles are smaller than the enzyme and on internal diffusion in larger micelles.

Up to the point in which the micellar size equals that of the enzyme, the enzyme is close or in contact with the micellar wall. Therefore, there would be strong hindrances in the diffusion of substrates to the enzyme active site (Fig. 3). Under these conditions, the composition of the buffer would not exert a significant influence on the enzyme activity. Indeed, the activities observed in 50 and 100 mM buffer were nearly the same. In contrast, at water concentrations in which the dimensions of the micelles are larger than those of the enzyme, the contribution of the buffer in the water phase to the diffusion of substrates becomes the controlling factor in the expression of activity. This is clearly evidenced by the sharp increment in the ratio of activity in 100 and 50 mM phosphate buffer as *Wo* is raised above 13.

Regarding the mechanisms through which the composition of the buffer affects the diffusion of substrates within the water pool of reverse micelles, the studies of Li et al. [26] are relevant. The authors found that in reverse micelles formed with CTAB, *n*-butanol, octane and water, 45 water molecules were bound to the polar head of CTAB, five of these were more tightly bound than the other 40. Additional water molecules were considered as free water. In our experiments, the *Wo* values ranged from 5.5 to 22.2, indicating that all water molecules were bound to CTAB. The particular organization of water in the water pool of the micelle probably

accounts for the hindrances in the diffusion of substrates. Phosphate ions very likely affect the interactions of water with the polar heads of the surfactant and this can account for the different activities observed in 50 and 100 mM phosphate buffer. It is noteworthy that the ratio of the K_{cat} of β-lactamase in 100 and 50 mM phosphate buffer is much higher in reverse micelles than in standard aqueous media. This suggests that the factors that contribute to the diffusion of substrates to the active site of the enzyme, in enzymatic reactions limited by diffusion, may be more easily studied when such enzymes are entrapped in reverse micelles.

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