Kinetics of α-chymotrypsin catalyzed hydrolysis of 4-nitrophenyl acetate in ethanolamine surfactants

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The kinetics of α-chymotrypsin (α-CT) catalyzed hydrolysis of 4-nitrophenyl acetate has been studied in aqueous solution of alkyldimethylethanolammonium bromide (cetyl, dodecyl, decyl) surfactants at concentrations below and above their critical micelle concentration. From Michaelis-Menten kinetics, the catalytic rate constant $k_{cat}$ and the Michaelis constant $K_M$ have been determined. The bell-shaped profiles of α-CT activity with increasing surfactant concentrations indicate the interaction between the micelle-bound enzyme and substrate.

Keywords: α-Chymotrypsin, 4-Nitrophenyl acetate, Hydrolysis, Micellar enzymology, Alkyldimethyl ethanolammonium bromide, Surfactants

The catalytic rate enhancement by the enzymes in the presence of self-organized aggregates, viz. micelles, reverse micelles and microemulsions etc. has attracted the considerable attention of chemists in recent years. The interactions between the enzyme and surfactant are of crucial significance for the enzymatic hydrolysis. These interactions may involve the free surfactant and or the micelle, leading to conformation changes that could modify the catalytic rate constant and or the enzyme-substrate binding constant. The catalytic activity of a serine protease α-chymotrypsin (α-CT) on the hydrolysis of carboxylic acid derivatives has been studied in the presence and absence of surfactants. α-CT catalyzed hydrolysis of 2-naphthylacetate and N-glutaryl-L-phenylalanine (GPNA) p-nitroanilide has been studied in the presence of alkyltrimethylammonium bromides.

Several studies have been performed on the superactivity of α-CT in aqueous solutions of cationic surfactants. Although hydrolysis of 4-nitrophenyl acetate (PNPA) is rapid and discovered several decades ago, it still attracts the attention of micellar enzymologists. Bianconi et al. studied superactivity and conformational changes on α-CT upon interfacial binding to cetyltrimethylammonium bromide (CTAB) for the PNPA. Recently, pre-steady and steady states kinetics measurements for 4-nitrophenyl esters using several hydrolase enzymes and thermodynamical investigation of hydrolysis of PNPA using α-CT in water-acetonitrile mixtures have also been reported. Very recently, the conformational dynamics at the active site of α-CT has been correlated with its catalytic activity.

The reactivity pattern of ethanolamine surfactants combines the characteristics of both the amine and hydroxy groups, making them an important system for the enzyme kinetics. The use of PNPA as substrate gives the possibility of studying both acylation and deacylation of the enzyme. Moreover, no attempts have been made to study the α-CT catalyzed reaction of PNPA in the presence of novel cationic surfactants based on dimethyl ethanolamines (I). Thus, in the present work, the kinetics of α-chymotrypsin (α-CT) catalyzed hydrolysis of 4-nitrophenyl acetate has been studied in aqueous solution of the novel surfactants i.e., ethanolamine surfactants (Fig. 1).

Fig. 1—Structure of alkyldimethylethanolammonium bromides [R = C$_{10}$, C$_{12}$, C$_{16}$]

Materials and Methods

α-Chymotrypsin (type-II, from bovine pancreas, molecular mass 25 kDa, and isoelectric point $pI$ 8.8) was procured from Sigma (USA) and used without further purification. The substrate 4-nitrophenyl acetate (PNPA) was procured from Sisco, Bombay. Enzyme and substrate solutions were freshly prepared in the appropriate buffer immediately, before their use in experiment. Tris-(hydroxymethyl) aminomethane (Tris) ($pK_a$ 8.3) and HCl were obtained from Qualigens (Bombay). The surfactants i.e., cetyl-dimethylethanolammonium bromides...
bromide (C₁₆DMEAB), dodecyldimethylethanol-ammonium bromide (C₁₂DMEAB) and decyldimethyl ethanol-ammonium bromide (C₁₀DMEAB) were obtained from Prof. R M Palepu, St. Francis Xavier University, Antigonish, Canada as a gift. The critical micelle concentration (CMC) of these surfactants was determined by conductometric method. Absorbances were recorded in Varian Carry 50 UV-visible and Systronics (104) spectrophotometers.

The rate of PNPA hydrolysis, catalyzed by α-chymotrypsin was measured in aqueous and surfactants solution at pH = 7.5 (10 mM Tris/HCl buffer), following the formation of p-nitrophenoxide ion (PNP⁺) at 400 nm (ε = 18000 M⁻¹ cm⁻¹). Because the isoelectric point of the enzyme was 8.8, it had a neat positive charge under the conditions employed in this work as a function of time. The initial reaction rate _V₀_ was determined from the slope of the PNP⁺ ion concentration _versus_ time. The hydrolysis of PNPA could occur both enzymatically and non-enzymatically. The true enzymatic hydrolysis was calculated by subtracting the non-enzymatic rate (in presence of surfactant) from the total reaction rate, instead of measuring it directly. No noticeable variation in absorbance at 400 nm was observed for PNPA hydrolysis in Tris-HCl buffer (no surfactant/enzyme) within the time limits required for an enzymatic assay. The _K_m_ and _k_cat_ of PNPA hydrolysis were determined by measuring the steady-state activity of α-CT with PNPA ranging from 0.04 mM to 0.13 mM in the presence of different ethanolamine surfactants (1-12 mM).

**Results and Discussion**

The hydrolytic activity of α-CT was measured in the presence of three ethanolamine surfactants by determining the rate of enzyme catalyzed reaction of PNPA at 30°C at pH 7.5 (Scheme I). The formation of PNP⁺ ion was linearly time-dependent during the first 5 min of reaction at all the PNPA concentrations considered. The initial rate _V₀_ was calculated from the slope of PNP _versus_ time plots.

Typical plots of substrate saturation curves obtained in the absence of surfactant and presence of different concentrations of C₁₆DMEAB (1-10 mM) are shown in Fig. 2. The results of Fig. 2 showed an increase in reaction rate with surfactant concentration above the CMC. The stability of enzyme is affected by many factors, such as temperature, pH, the organic solvent and the presence of surfactants. The α-CT activity and stability were tested in cationic surfactants by several workers and it was found that enzyme was very stable under the present experimental conditions. The stability of α-CT (in buffer, 30°C) was checked in surfactant for 20 h and was found that the activity was retained.

Fig. 3 shows the effect of surfactant tail group on the initial rate. It is reported that the surfactant head and tail groups strongly affect the catalytic behaviour of the enzyme activity. The C₁₆DMEAB shows...
superactivity, which can be explained in terms of the surfactant hydrophobicity, as it increases in the order $C_{10}$DMEAB$<$C$_{12}$DMEAB$<$C$_{16}$DMEAB. If the effect of surfactant is interpreted in terms of an interaction between the enzyme and the monomeric form of substrate, the increase of chain length hydrophobicity would favour the formation of micelles rather than the association of surfactant with the substrate.

**Effect of surfactant concentration**

In order to see the effect of surfactant concentration, the rate of hydrolysis of PNPA was determined at constant $\alpha$-CT (11 $\mu$M) concentration in the presence of different concentrations of surfactant (below and above the CMC). The hydrolysis of PNPA catalyzed by $\alpha$-CT in the micellar system follows the Michaelis-Menten kinetics shown in Eq. (1):

$$E + S \stackrel{k_1}{\longrightarrow} ES \stackrel{k_{cat}}{\longrightarrow} E + P \quad \ldots \ (1)$$

Applying the steady-state approximation to ES, the rate law given in Eq. (2) was obtained:

$$V_o = k_{cat} [E]_o [S]/K_M [S] \quad \ldots \ (2)$$

where $V_o$ is the initial rate, $[E]_o$ is the initial enzyme concentration, $[S]$ is the concentration of the substrate, $k_{cat}$ is the catalytic rate constant and $K_M$ is the Michaelis-Menten constant defined by Eq. (3).

$$K_M = (k_1 + k_{cat})/k_1 \quad \ldots \ (3)$$

Eq. (2) can be rearranged as Lineweaver-Burk plot for the determination of $k_{cat}$ and $K_M$.

$$[E]_o/V_o = 1/k_{cat} + (K_M/k_{cat})/S \quad \ldots \ (4)$$

From the Lineweaver-Burk linear plot between $[E]_o/V_o$ and $1/[S]$, $k_{cat}$ (reciprocal of the intercept) the catalytic efficiency $k_{cat}/K_M$ (reciprocal of the slope) and $K_M$ (slope/intercept ratio) could be obtained. All the steady-state kinetic parameters are given in Table 1. The catalytic constant increases with surfactant ($C_{16}$DMEAB, $C_{12}$DMEAB, $C_{10}$DMEAB) concentration initially and then decreased with future addition of surfactant. This might be explained on the basis of the decreased thermodynamic activity of the substrate due to its incorporation into the micellar pseudophase$^{13}$ at higher concentration of surfactant.

According to Bianconi et al$^{19}$, if there is an enzyme-micelle interaction, a bell shape curve was observed for the activity of $\alpha$-CT as a function of surfactant concentration. As observed in other systems, a bell-shaped behaviour was observed for all the three surfactants (Fig. 4). Due to an increase in the number of micelles in solution, there was a decrease in the concentration of free substrate, the unfavourable partition led to a decrease in activity when the concentration of surfactant was increased. The dependence of $k_{cat}$ and $K_M$ with surfactant concentration was similar to that of the catalytic efficiency and, being the increase above the CMC higher for the surfactant having longer tail respectively. The superactivity increased in the order of increasing the surfactant chain length, indicating that surfactant hydrophobicity exerted influence on the CMC than upon its association with the enzyme.

**Mechanism**

$\alpha$-Chymotrypsin catalyzes the hydrolysis of PNPA by means of a double displacement mechanism. After non-covalent binding of substrate to the enzyme, the reaction is initiated by nucleophilic attack by the active site serine hydroxyl group on the acyl carbonyl group of the substrate to form initially the tetrahedral intermediate. The nucleophilicity of serine hydroxyl group is enhanced by a proton transfer to a nearby

<table>
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<th>Surfactant</th>
<th>CMC (mM)</th>
<th>$10^5 k_{cat}$ (s$^{-1}$)</th>
<th>$10^5 K_M$ (M)</th>
<th>$k_{cat}/K_M$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
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<tr>
<td>Aqueous</td>
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<tr>
<td>$C_{16}$DMEAB</td>
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<tr>
<td>$C_{12}$DMEAB</td>
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<td>15.4</td>
<td>17.8</td>
<td>86.5</td>
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<tr>
<td>$C_{10}$DMEAB</td>
<td>8.94</td>
<td>7.35</td>
<td>117</td>
<td>117</td>
</tr>
<tr>
<td>CTAB</td>
<td>0.87</td>
<td>28.1</td>
<td>2.97</td>
<td>2.10</td>
</tr>
</tbody>
</table>

Table 1—Effect of chain length of ethanolamine surfactants on $\alpha$-CT catalyzed reaction of PNPA

Fig. 4—Effect of ethanolamine surfactant concentration on the catalytic rate constant [(▲) $C_{16}$DMEAB, (■) $C_{12}$DMEAB, (●) $C_{10}$DMEAB]
histidine, which, in turn, is activated by the aspirate. The subsequent cleavage of the ester bond (deacylation step) is realized by reacting with a water nucleophile activated by a general base, namely, the imidazole group of the His residue. The presence of surfactant can modify each one of the above states either by competing with the active site or by changing the conformation of the enzyme. However, further studies needed to gain deeper understanding of micellar enzymology.

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References
12 Abuin E, Lissi E & Duarte R (2005) J Colloid Interface Sci 283, 539-543