Structural changes in Triton X-100 micelles in guanidinium chloride: Implications in lipase assays
5.0 Introduction

Micelles are thermodynamically stable aggregates formed when amphiphilic molecules are dispersed in water above a well-defined concentration known as the critical micelle concentration (CMC) (Tanford, 1980). Micelles are often considered to be a separate but uniformly distributed pseudophase (Romsted, 1979). Industrial applications of detergent micelles arise from their ability to solubilise molecules that are sparingly soluble in water (Falbe, 1987; Christian and Scamehorn, 1995). The solubilization is a consequence of the presence of a hydrophobic domain in the surfactant aggregates, which acts as compatible microenvironment for the location of hydrophobic solubilizates. The solubility of hydrophobic solutes has been found to be orders of magnitude higher than their solubility in water in the absence of the surfactant. The extent of solubilization depends on the chemical characteristics of surfactant and solubilizates as well as on the composition of the solution and temperature (Mackay, 1987). In studying enzymes with non-polar or amphiphilic substrates, detergents are often used to solubilise the substrates. Non-ionic detergents, like Triton X-100, are used as substrate diluents for enzymes such as phospholipases, lipases, etc. that act upon substrate aggregates (Dennis, 1974; Buxeda et al., 1991; Carman and Dowhan, 1979; Redondo et al., 1995; Simons et al., 1997; Wilcox et al., 1991; Burdette and Quinn, 1986). In non-ionic micelles, the Stern layer present in ionic micelles with charged head groups is replaced by a diffuse palisade layer. The palisade layer is made up of a large number of ethylene oxide units and can be large relative to the apolar core that is made up of octylphenyl moieties in case of Triton X-100 (Robson and Dennis, 1977).

Ionic surfactant micelles in aqueous media are known to catalyse organic reactions through a combination of one or more of the following: a) favorable medium effects owing to the lower polarity of the micelle interior compared to water; b) stabilization of transition state by electrostatic interaction with the
surfactant headgroup; c) lowering of pKₐ of ionic reagents, again by electrostatic effects and d) partitioning of substrates such that they are present at much higher concentration in the micelle than in bulk solution, thereby leading to an increase in the rate of bimolecular reactions (Brown et al., 1980). According to the empirical rules formulated by Hartley, anionic micelles increase and cationic micelles decrease protonation of non-ionic bases and non-ionic micelles have little effect (Hartley, 1948). Rate enhancements of bimolecular reactions between organic and hydrophilic reactants by ionic micelles in water are primarily ascribed to equilibrium incorporation of both reactants into the micelles, which are treated as a pseudophase, distinct from the bulk solvent. The pseudophase ion exchange model provides, within limits, a unified quantitative interpretation for micellar effects of ionic micelles over a wide range of experimental conditions (Romsted, 1979). The hydrolysis of p-nitrophenyl esters has been extensively used to study the effect of micelles on reaction rates. There have been fewer studies on catalysis by non-ionic micelles because of the lack of any dramatic effects on reaction rates. Catalysis by non-ionic reversed micelles has been studied (Kon-no et al., 1987 and references therein) and is primarily due to the effect of solubilization in the micellar core on the properties of the reactants and in some cases, stabilization of transition states due to interaction with the ether oxygen atoms and/or to the OH group of the surfactant. Micellar catalysis would have a confounding influence on catalysis rate measurements on enzymes, whose substrates are solubilised by micelles.

The stability and structure of micelles is known to depend critically upon the solvent properties, temperature and the presence of additives (Meguro et al, 1987 and references therein). The thermodynamics of formation and structures of Triton X-100 micelles is well-studied using light scatter (Kushner and Hubbard, 1954; Brown et al, 1989), small angle X-ray (Paradies, 1980), NMR(Brown et al, 1989; Ribeiro and Dennis, 1976), viscosity (Kushner and Hubbard, 1954) and fluorescence methods (Brown et al., 1989). Several insights into the structure of
the micelles were obtained by solvent perturbation studies (Meguro et al., 1987). The effect of urea, a well-known protein denaturant, on micellar structure has been extensively studied (Schick, 1964; Emerson and Holtzer, 1967; Briganti et al., 1991; Costantino et al., 2000; Ruiz and Sanchez, 1994; Benjamin, 1966; Balasubramanian and Mitra, 1979). Urea was shown to increase the critical micellar concentration and decrease the mean micellar hydrodynamic radius of non-ionic micelles. However, very few reports (Benjamin, 1966; Balasubramanian and Mitra, 1979) discuss the effects of guanidinium chloride (GdmCl), another popular protein denaturant, on micelle structure. Solvent additives such as urea and GdmCl, can affect macromolecular structure by direct interaction with the macromolecule or by indirect action through effects on the structure and properties of the solvent or by a combination of both these mechanisms (Makhatadze and Privalov, 1992). To the best of our knowledge there are no reports that address the issue of the effect of protein denaturants on the enzymatic and non-enzymatic hydrolysis of substrates solubilized in non-ionic detergents. Such studies are important since micelle-solubilized substrates are extensively used in studies with enzymes like lipases and phospholipases that preferentially act upon substrate aggregates (Dennis, 1974; Buxeda et al., 1991; Carman and Dowhan, 1979; Redondo et al., 1995; Simons et al., 1997; Wilcox et al., 1991).

Lipases (triacylglycerol hydrolases EC 3.1.1.3) catalyze the hydrolysis and synthesis of glycerol esters. Apart from their natural substrates, they also catalyze the hydrolysis, synthesis and transesterification of other esters of varying chain lengths and containing different functional groups (Woolley and Petersen, 1994). Activities of lipases are affected greatly by the nature and the composition of interfaces, hence “interfacial quality” is an often used term in lipase studies (Verger, 1997). In studies involving perturbations of lipase structure such as with salts, chaotropes, temperature etc., it is expected that the substrate aggregates would also be influenced by these perturbations. A careful evaluation of the consequences of the perturbations on micelle structure and their contributions to
activity measurements should be made. In our investigations on lipase substrates, we tested a water-insoluble and a water-soluble p-nitrophenyl ester as substrates of lipase. To solubilise the insoluble ester we have used the non-ionic surfactant, Triton X-100. We observed anomalous enhancements in the rate of hydrolysis of the micelle-solubilized ester, in the presence of GdmCl, but not with the water-soluble ester. Chaotropes such as GdmCl and urea are known to affect structures whose formation is driven by hydrophobic interactions (Makhatadze and Privalov, 1992). To understand and account for any contribution of GdmCl-induced alteration in the micelle structure towards the rate enhancements observed with lipases, we probed the changes in the Triton X-100 micelle in GdmCl by studying the non-enzymatic hydrolysis of PNPO and by fluorescence and dynamic light scattering (DLS) techniques.

5.1 Materials and Methods

5.1.1 Materials

*Candida rugosa* lipase, potassium chloride, potassium iodide, sodium thiosulfate and Triton X-100 were purchased from Sigma Chemical Co., USA. Lipase from *Bacillus subtilis* was purified from the overexpressing strain BCL1051 (Pouderoyen *et al.*, 2001). GdmCl was obtained from Serva, Germany. PNPA was purchased from Sisco Research Laboratories, India. DMA was purchased from E.Merck, India. PNPO was synthesized by conventional means from an equimolar solution of oleic acid and p-nitrophenol in dichloromethane using an equivalent amount of *N,N*-dimethylcyclohexylcarbodiimide (DCC) and a catalytic amount of 4-*N,N*-dimethylaminopyridine (DMAP). All the reagents used for synthesis were from Spectrochem, India and were of the highest analytical grade. Diphenylhexatriene (DPH), 2-anthroyloxy stearic acid (2-AS), 9-anthroyloxy stearic acid (9-AS) and 12-anthroyloxy stearic acid (12-AS) were from Molecular Probes, USA.
5.1.2 Enzyme assays

All enzyme assays were carried out at room temperature (~25°C) in 0.05 M Tris.Cl buffer, pH 7.2. Appropriate amounts of the PNPO ester and Triton X-100 were weighed out in a glass vial and mixed with a magnetic stirrer till the ester was completely dissolved in Triton X-100. Magnetic stirring was maintained and buffer was added slowly while stirring to prepare a 10X stock solution containing 2 mM p-nitrophenyl oleate and 200 mM Triton X-100. Substrate solutions prepared in this way were optically clear. Where PNPA was used as the substrate, a concentrated stock solution, prepared in acetone, was added to aqueous buffer to get a 2mM aqueous stock solution (10X). The concentration of acetone in the stock solution was 1%. Assays were done with freshly prepared substrate solutions.

1 unit is defined as the amount of enzyme that hydrolyses 1μmol of substrate in 1min. With PNPA as substrate, the specific activities of the *B.subtilis* lipase and *C.rugosa* lipase were 16U/mg and 5U/mg protein respectively.

The enzyme was incubated for 6 hours in appropriate GdmCl concentrations in a volume of 900 μl. 100 μl of the substrate solution was added to initiate the reaction. Reaction rates were determined by following the increase in absorbance at 405 nm due to the formation of p-nitrophenolate as one of the products.

Lipase activity was measured as initial reaction rates (<5% of total substrate hydrolysis) in order to avoid the possible inhibition that might take place due to appearance of the reaction products. Background hydrolyses of the p-nitrophenylesters, i.e., in the absence of enzymes, were measured and used for correcting the hydrolysis rates obtained with enzymes.
5.1.3 CMC determination

CMC values of the micellar solutions in the presence of different concentrations of GdmCl were determined using the fluorescent probe, DPH, as described earlier (Chattopadhyay and London, 1984). The fluorescence of DPH, a hydrophobic probe, is enhanced several fold when it partitions into the hydrophobic core of micelles. Fluorescence of DPH increases abruptly at the CMC. The break in the fluorescence vs. surfactant concentration curve indicates formation of micelles, and the corresponding concentration of Triton X-100 was assigned as the CMC. Briefly, 1μl of 10 mM DPH dissolved in tetrahydrofuran was added to various amounts of detergent dissolved in a total volume of 2 ml of aqueous solution (0.05 M Tris.Cl, pH 7.2) containing appropriate amounts of GdmCl. Tubes were incubated for 30min (unless mentioned otherwise) in the dark at room temperature before measuring fluorescence. Tris.Cl at 0.05 M or tetrahydrofuran do not have any effect on the CMC values. The fluorescence of the mixed micelle samples was again recorded after 1hr incubation to determine if hydrolysis of PNPO has any effect on the CMC values.

5.1.4 Dynamic light scattering

All dynamic light scattering measurements were carried out at 25°C in a DynaPro-MS/X instrument from Protein Solutions Inc., U.S.A. The measurements were made at a fixed angle of 90° using an incident laser beam of 830 nm. The DYNAMICS graphical size analysis software provided with the instrument was used for data analysis. The viscosity and refractive index values for different GdmCl solutions were determined (Fasman, 1976) and used for the analysis. All the solutions contained 20 mM Triton X-100 in 0.05 M Tris.Cl, pH 7.2, and appropriate concentrations of GdmCl. The mixed micellar solutions were prepared as described above.
5.1.5 Fluorescence studies

All fluorescence measurements were carried out at room temperature with a Hitachi spectrofluorimeter (F-4010). Samples were prepared in 0.05M Tris.Cl pH7.2. Stock solutions (2 mM) of the anthroyloxy stearic acid probes were prepared in methanol. Appropriate volumes of each probe were taken in a glass vial and dried under a slow, constant stream of nitrogen. The samples were then kept in vacuum for at least three hours. Triton X-100 was weighed out and stirred for 30' with a magnetic stirrer to allow mixing with the probe. A 10X aqueous stock was made by slowly adding buffer to make up the volume to yield a final concentration of the probe and Triton X-100 of 0.05mM and 20 mM respectively. The solutions were vortexed and allowed to stand for at least 30'. Working stocks were prepared in appropriate concentrations of GdmCl.

Wavelength scans were done with the excitation and emission slits set at a bandpass of 3 nm each. Excitation wavelength used was 365 nm. Emission was recorded from 400-600 nm. All the spectra were recorded in the 'correct spectrum' mode.

Fluorescence polarization measurements were performed using a Hitachi polarization accessory. Excitation and emission band passes of 5 nm were used. The excitation wavelength was 365 nm and the emission was monitored at the $\lambda_{max}$. Polarization values were calculated using the equation

$$P = \frac{(I_{vv}-Gl_{vh})}{(I_{vv}+Gl_{vh})}$$

Where $I_{vv}$ and $I_{vh}$ are the measured fluorescence intensities with the excitation polarizer oriented vertically and the emission polarization oriented vertically and horizontally respectively. $G$ is the grating correction factor and is equal to $l_{vh}/l_{hh}$ where $l_{hv}$ and $l_{hh}$ are the measured fluorescence intensities with excitation
polarizer oriented horizontally and the emission polarizer oriented vertically and horizontally respectively. All fluorescence intensities are corrected for background scatter with samples without the fluorophore.

Fluorescence quenching studies were carried out with the non-polar quencher, DMA, and the negatively charged, polar quencher, iodide. Excitation and emission band passes of 5 nm were used. The excitation wavelength was 365 nm and the emission was monitored at the $\lambda_{max}$. Concentrated solutions of the quenchers were added to the samples and fluorescence was measured after 5 min. No change in fluorescence was observed on longer incubations. All samples were continuously stirred in the cuvette. For quenching with potassium iodide (KI), isoionic strength between samples was maintained by adding KCl. Sodium thiosulfate was added to the iodide stock solutions. The quenching data were analyzed according to the conventional Stern-Volmer equation

$$\frac{I_0}{I} = 1 + K_{sv}[Q]$$

Where $I_0$ and $I$ are the fluorescence intensities in the absence and presence of the quencher, $K_{sv}$ is the Stern-Volmer constant and $[Q]$ is the total quencher concentration.

5.2 Results and Discussion

5.2.1 Enzyme catalyzed hydrolysis of p-nitrophenyl esters in guanidinium chloride

Lipases are hydrolytic enzymes that are distinguished from esterases by their ability to act on aggregated substrates. The stability of the aggregate and the quality of the interface are expected to have an influence on the activity of these enzymes. The physiological substrates of lipases are triacylglycerols. Long chain
p-nitrophenyl esters (PNPe) have been extensively used to monitor lipase activity since the product, p-nitrophenolate possesses high extinction coefficient ($\varepsilon_{405} = 18.3 \text{ cm}^{-1}\text{mM}^{-1}$). These long chain esters are solubilized in Triton X-100 for the enzyme assays. The PNPe- Triton X-100 mixed micellar system has become very popular because it allows continuous spectrophotometric monitoring of the hydrolysis reaction with high sensitivity and provides a good physicochemical system for interpreting lipolytic activity because the surface of micelles is reproducible and easy to handle.

Activity assays of lipase from *B. subtilis* were carried out with PNPA and PNPO as substrates. With PNPA, a water-soluble ester, the activity of the enzymes decreased with increasing GdmCl concentration. By 2 M GdmCl more than 80% of the activity of lipase was lost. PNPO is a long chain (C$_{18}$), water insoluble ester, unlike PNPA. It was solubilized in Triton X-100 (see Experimental) and the mixed micelles were used for the enzyme reactions. In the presence of GdmCl, with PNPO, the activity of the *B. subtilis* enzyme increases to a maximum at 0.75 M GdmCl and then falls on further increase in GdmCl concentration (Figure 5.1A). A similar observation, though to a lesser extent, has been made with the *C. rugosa* enzyme, where the activity peaks at 0.1 M GdmCl (Figure 5.1B). Dual behavior of the *B. subtilis* lipase towards soluble and insoluble substrates was interesting because on the one hand assays with the soluble substrate indicates denaturation of the enzyme on addition of GdmCl whereas, on the other hand, assays with a micellized substrate points to activation of the enzyme at intermediate concentrations of the denaturant followed by complete denaturation at higher concentrations. Beyond 0.75 M GdmCl very little active lipase was remaining. Lipase from *Humicola lanuginosa* also demonstrates enhanced activity in the presence of GdmCl up to 1.5 M GdmCl and decreases on increase of GdmCl. In that report Carboxy-DFDCA, a soluble substrate, was used in lipase assays and the “loosening” of the tertiary structure was suggested to result in increased activity of the enzyme in the presence of GdmCl (Zhu et al., 2001). Decrease in
Fig 5.1  (A) *Bacillus subtilis* lipase catalyzed and (B) *Candida rugosa* lipase catalyzed hydrolysis of PNPO (■) and PNPA (●).

Concentration of enzyme used was 0.7 μg/ml and 0.5 μg/ml. The rates of hydrolysis of both esters at 0 M GdmCl have been assigned as 1 and rates of hydrolysis of the esters in GdmCl are plotted relative to their respective rates at 0 M GdmCl. The reactions are carried out at 25°C in 0.05M potassium phosphate buffer, pH7.2. PNPO is present as a mixed micelle with Triton X-100, whereas PNPA is dissolved in the aqueous buffer. The background hydrolysis in absence of enzyme has been subtracted.
activity of lipase, or any enzyme, with increasing GdmCl is expected since the structure of the protein is progressively lost in GdmCl. To evaluate the observed enhancements of lipase activity in low GdmCl concentrations, direct effects of GdmCl on the micelle structure also should be evaluated. The driving force for micellization is hydrophobic interactions and compounds of the urea or GdmCl class are known to affect hydrophobic forces, hence, it is expected that the structure of the PNPO-Triton X-100 mixed micelles will be affected by GdmCl (discussed below).

5.2.2 Non-enzymatic hydrolysis of the p-nitrophenyl esters in guanidinium chloride

We studied the rates of non-enzymatic hydrolysis in different concentrations of GdmCl in an effort to determine if there were any changes in the inherent susceptibility of the esters to hydrolysis, which may occur due to any change in the orientation, conformation, and/or hydration state of the substrate in the presence of GdmCl that facilitates enzymatic breakdown. Unlike the enhancing effects observed with cationic micelles (Romsted, 1979), e.g., cetyltrimethylammonium bromide (CTAB), wherein the cationic head group of the surfactant increases the hydroxide ion concentration in the micelle interfacial region, non-ionic micelles are incapable of enhancing the hydrolysis of PNPO by similar mechanisms.

The spontaneous hydrolysis experiments were performed at pH 8 since the non-catalytic hydrolysis rates of PNPO in the conditions used for the enzyme reactions (pH 7.2) are too low to be measured. The rates of hydrolysis of both PNPO and PNPA were enhanced on increasing the pH, which is consistent with a base-catalyzed hydrolysis mechanism. The rates of hydrolysis of $1 \times 10^{-5}$ and $4 \times 10^{-4}$ μmoles per min reflect 40-times more hydrolysis of PNPA compared to PNPO, in the absence of GdmCl. This is expected because PNPA is soluble and, therefore, the ester bond is accessible to the solvent. On the other hand, PNPO was
entrapped in Triton X-100 micelles and the ester bond is relatively inaccessible to water. Since water is a reactant in the hydrolysis reaction, accessibility of the ester bond to water is expected to play a determining role in the rate of hydrolysis.

The hydrolysis behavior of the two esters on addition of GdmCl was also very different. The rate of hydrolysis of PNPO increased with increasing concentration of GdmCl (Figure 5.2). The increase from 0 M to 6 M GdmCl was nearly 74-fold. On the contrary, there was very little increase (<2-fold) in the rate of hydrolysis of soluble PNPA on increasing the GdmCl concentration from 0M to 6M. That the rate of hydrolysis of PNPA was essentially unchanged by GdmCl indicates that GdmCl does not play a direct role in the hydrolysis of the ester. Observed difference in the behavior of a soluble and an insoluble ester points to a possible perturbatory role of GdmCl on the structure of mixed micelles. Accessibility of PNPO, present in a micelle, to water is very limited, which is reflected in the low rates of hydrolysis in the absence of GdmCl. On addition of GdmCl, the structure of the micelle may be changing in a way to make the ester more prone to hydrolysis. Since water is one of the reactants, the enhancement could be resulting from a greater accessibility of the ester moiety to the aqueous solvent. If this were indeed true, it could be a major, if not the only, contributing factor to the differences in hydrolysis rates of lipases observed towards the soluble and insoluble substrates in GdmCl (Fig.5.1). The altered accessibility of PNPO in GdmCl also may be responsible for the enhanced activities seen with lipase up to 0.75 M GdmCl. Although approximately 50% of the lipase activity was lost in 0.75M GdmCl, enhanced accessibility of the ester bond of PNPO in micelle for lipase may be responsible for the observed increase of activity. Unlike lipase from H. lanuginosa, lipases from B. subtilis and C. rugosa do not show any stimulation of activity with soluble substrates in GdmCl. Since lipases from C. rugosa and B. subtilis are enzymes with and without a lid on the active site respectively (Pouderoyen et al., 2001; Grochulski et al., 1993) the observed stimulation in
Fig 5.2 Non-enzymatic hydrolysis of PNPO (■) and PNPA (●) carried out at 25°C in 0.05M potassium phosphate buffer, pH 8.0. PNPO is present as a mixed micelle with Triton X-100 whereas PNPA is dissolved in the aqueous buffer. At 0 M GdmCl, the rate of release of p-nitrophenol from PNPO and PNPA is $1.10^{-5}$ and $4.10^{-4}$ μmoles.min$^{-1}$, respectively. The rates of hydrolysis of both esters at 0 M GdmCl have been assigned as 1 and rates of hydrolysis of the esters in GdmCl are plotted relative to their respective rates at 0 M GdmCl.
activity in GdmCl is not dependent on the ability of enzyme to demonstrate interfacial activation (Verger, 1997).

Chaotropes, being water-structure breakers, profoundly affect micelle formation. Both urea and guanidinium salts have been shown to affect the thermodynamics of micellization (Benjamin, 1966). Urea has been shown to (a) increase the critical micellar concentration of non-ionic and ionic surfactants; (b) decrease the mean micellar hydrodynamic radius of ionic surfactants and (c) raise the cloud point temperatures of aqueous solutions of non-ionic surfactants (Schick, 1964; Emerson and Holtzer, 1967; Briganti et al., 1991; Costantino et al., 2000; Ruiz and Sanchez, 1994; Benjamin, 1966; Balasubramanian and Mitra, 1979). The effect of GdmCl on micelles has been much less studied (Benjamin, 1966; Balasubramanian and Mitra, 1979). We decided to investigate the structural changes that the micelle would undergo in GdmCl. We examined the effect of GdmCl on the size and CMC of Triton X-100 and its mixed micelle with PNPO. Also, we investigated the extent of water penetration into the micelle by using different fluorescent probes. The spectral behavior of these probes as well as their accessibility to various classes, ionic and non-polar, of quenchers was examined at different GdmCl concentrations.

5.2.3 Critical micelle concentration

In order to determine the effect of GdmCl on CMC of Triton X-100 (and its mixed micelle with PNPO), the fluorescence of DPH as a function of surfactant concentration at different GdmCl concentrations was monitored. CMC of pure or mixed micelles, at various concentrations of GdmCl, was identical within experimental error, suggesting that presence of PNPO molecules, at the concentration used for the present study, does not alter the aggregation behavior of Triton X-100. This is in agreement with earlier reports (Burdette and Quinn, 1986) where, using a different method for CMC determination, it was shown that
esters at similar ratios does not alter CMC. The CMC values for the mixed micelles obtained on recording fluorescence after 10 min incubation and that after 1 hour incubation did not show any change indicating that any spontaneous hydrolysis of PNPO under these experimental conditions does not affect the CMC determination.

The CMC of Triton X-100 in absence of GdmCl was found to be 0.3 mM, which is in good agreement with values reported in literature (Chattopadhyay and London, 1984). The CMC increases with increasing concentration of GdmCl (Figure 5.3) to a value of ~7.34 mM in 6 M GdmCl. Increase in CMC of Triton X-100 has also been observed in urea (Ruiz and Sanchez, 1994). However, GdmCl seems to have a stronger effect than urea on CMC values. At the same molar concentrations, shifts in CMC value were greater for GdmCl than for urea. For instance, the CMC value at 3 M urea is reported as ~0.7 mM whereas, at 3 M GdmCl the CMC value is ~1.75 mM. Similar observations have been made in studies with proteins (Makhatadze and Privalov, 1992) where GdmCl is considered to be a stronger perturbant of protein structure than urea. GdmCl, like urea, probably acts by solvating the monomer in water thus reducing the contribution of hydrophobic interactions in micellization. The CMC values of non-ionic surfactants depend on the balance of forces between the van der Waals interactions in the hydrophobic groups and the opposing hydration of the ethylene oxide chains. Enhanced solvation of octylphenyl chain and/or increased solubility of the non-polar tail, in the presence of GdmCl solutions, may be responsible for the higher CMCs.

5.2.4 Size

The size of Triton X-100 micelles (and its mixed micelle with PNPO) was determined in various concentrations of GdmCl by dynamic light scattering measurements. The hydrodynamic radius for the Triton X-100 micelle in absence
Fig 5.3 Critical micelle concentration (CMC) of Triton X-100 as a function of GdmCl concentration (n=3).
of GdmCl was found to be 4.27 nm, which is in good agreement with values reported in literature (Charlton and Doherty, 2000). The hydrodynamic radius steadily decreased (Figure 5.4) with increasing GdmCl concentrations dropping to 1.36 nm at 6 M GdmCl. With increasing GdmCl the scattering intensity decreased to half and the percent polydispersity increased. Increase in polydispersity shows that as the concentration of GdmCl increases the micellar solution becomes more heterogeneous and micelles of different sizes are expected. Decrease in the size of non-ionic micelles was also observed in urea but of a much lesser magnitude than in GdmCl. The PNPO -Triton X-100 mixed micelles were 14% larger in size than the pure Triton X-100 micelles and decrease to 2.47 nm in 6 M GdmCl (data not shown).

One important difference between GdmCl and urea is that GdmCl is ionic, whereas urea is not. It is important to differentiate the ionic effect of GdmCl from its chaotropic behavior. Sodium chloride and potassium chloride were shown to increase the size of Triton X-100 micelles predominantly by increasing the hydration of the micelle and to a lesser extent by increasing the aggregation number (Charlton and Doherty, 2000; Phillies and Yambert, 1996). Decrease in size in GdmCl was in contrast to effects of sodium or potassium chloride and qualitatively similar to the effect of urea.

Micelle formation is driven by two opposite effects a) hydrophobic interaction of the non-polar tails, which puts a lower limit on the size of the micelle b) steric repulsions of the head group, which limits the size of the micelle (Tanford, 1980). Urea has been shown to decrease the size of a non-ionic micelle by increasing the hydration of the ethylene oxide chain, thereby increasing the steric repulsions (Briganti et al., 1991). The effects of urea were opposite to those of temperature and salts like NaCl and KCl on Triton X-100 micelles. Presence of salts and/or increased temperature is known to cause dehydration of polyoxyethylene (POE) chains leading to decreased steric repulsion between head groups and thus, aids
Fig 5.4 Dynamic light scattering study of Triton X-100 micelles in GdmCl. Variation of hydrodynamic radius (▲), % polydispersity (■) and % total scatter (●) as a function of GdmCl concentration. The error associated with these measurements was smaller than the size of the symbol.
micelle growth. The effects of GdmCl on size of Triton X-100 micelles were similar to that of urea. Asymmetric light scatter measurements would provide more information on transitions in shape of micelles, e.g., ellipsoid to oblate etc. However in the present investigations contributions of shape changes to the hydrodynamic radius in the presence of GdmCl are not ruled out.

5.2.5 Fluorescence Studies

Use of fluorescence probes has provided important insights into the process of micelle formation and the structure of micelles. At very low, non-perturbant mole fractions fluorescent probes can report their microenvironment precisely. We have used a set of anthroyloxy fatty acids where an anthroyloxy group is located at various positions along a stearic acid chain (Thulborn et al., 1979). Since quenching depends upon the molecular proximity of the fluorophore and the quencher, measurement of quenching of fluorophores of known location by small molecule quenchers determines the location of the fluorophores. The position of the anthroyloxy moiety with respect to carboxyl group in a fatty acid, which could be varied, would act as a "dipstick" to probe the biophysical properties of fluorophore environment. Fluorescence properties of anthroyloxy probes are sensitive to the polarity of their environment (Blatt et al., 1981). In our study, the ester moiety being hydrolyzed would be located in the hydrophobic core very close to the hydrophobic core-palisade layer interface. The anthroyloxy group in 2-AS is also expected to be located close to the hydrophobic core-palisade layer interface and would, therefore, report the polarity changes in this region. The anthroyloxy stearic acid molecules are expected to be similar to PNPO with respect to their packing in the micelle as both the molecules are C18 chains with the difference being the unsaturation at position 9 of the oleic acid moiety in PNPO. Such a probe is, therefore, more suited than a free fluorophore to study changes in the conformation/environment of the substrate molecule in the micelle. Positions of the emission maxima have been used to study the changes in the polarity experienced.
by the probe. Fluorescence quenching has been done to study the accessibility of the fluorophores to non-polar and polar quenchers. Polarization of the fluorescence of the anthroyloxy moiety would report the restrictions to motion experienced by the probe and since, in this case the probe is linked to a stearic acid chain, it would also report the restrictions in rotational motion of the stearic acid chain.

2-AS, 9-AS and 12-AS were incorporated into the Triton X-100 micelles at a low ratio of 1:4000 to minimize perturbation of the micelle structure. At these ratios no more than one probe is present per micelle, thereby avoiding any artifacts due to dimerization of the probe molecules. Table 5.1 shows the emission maximum of the three probes in various GdmCl solutions when excited at 365 nm. The fluorescence parameters of the anthroyloxy probes have been shown to be independent of viscosity (Thulborn et al., 1979) of the surrounding medium. $\lambda_{\text{max}}$ values of these anthroyloxy probes have been shown to report the polarity of the surrounding medium (Thulborn et al., 1979; Blatt et al., 1981). In the absence of GdmCl the $\lambda_{\text{max}}$ shows a red shift from 12-AS to 2-AS. This agrees with an earlier report (Blatt et al., 1985) where similar shifts were observed and were attributed to the polarity gradient being experienced by the probes in the micelle. Similar studies with SDS (Blatt et al., 1981) show that polarity differences experienced by the anthroyloxy moieties result in a blue shift of 11 nm from 2-AS to 12-AS. However, in Triton X-100 the corresponding blue shift is only 4 nm. The probes are thus experiencing a much steeper gradient in SDS micelles than in Triton X-100. In the presence of GdmCl there is a considerable red shift in the $\lambda_{\text{max}}$ of 2-AS probe and not in the $\lambda_{\text{max}}$ of 9-AS and 12-AS. Since the anthroyloxy in 2-AS would be located near the interface of the hydrophobic core and the palisade layer, the red shift suggests increased polarity due to greater water penetration into this region. Since, GdmCl disturbs the hydrophilic/lipophilic balance, as is evident from the considerably increased CMC values, deeper penetration of the POE chains into the micelle can also be envisioned. The polarity changes occurring at the
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<th>[GdmCl] (M)</th>
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Table 5.1
micellar interface in the presence of GdmCI does not extend into the micelle interior since the \( \lambda_{\text{max}} \) of 9-AS and 12-AS remain unchanged. In as high as 6 M GdmCl the \( \lambda_{\text{max}} \) values of 9-AS and 12-AS are unchanged indicating the integrity of the nonpolar core even at such high concentrations of the chaotrope. As mentioned earlier, 2-AS would most closely report the changes in the vicinity of the ester moiety in PNPO. The increased polarity in the vicinity of the anthroyloxy moiety in 2-AS could mean greater water penetration in this region. Since, water is one of the reactants in a hydrolysis reaction, greater water penetration would mean increased accessibility of one of the reactants at the site of the reaction. This would result in an increase in the rates of hydrolysis. The increased rate of non-enzymatic hydrolysis in the presence of GdmCl may be due to increased polarity at the micellar interface and greater water penetration near the reaction center.

Another spectral property that reports changes in microenvironment is fluorescence polarization. The polarization values for the probes in Triton X-100 in the absence of GdmCl are in the order of 2AS>9AS>12AS. This trend agrees with earlier reports (Blatt et al., 1985) and shows that proximity of the probe to the interface restricts its motion. Changes in polarization of the three-anthroyloxy probes in Triton X-100 micelles in various GdmCl concentrations are presented in fig. 5.5. The polarization values of all three probes increase with increase in GdmCl concentration. However, the changes in polarization are more prominent in 2-AS, which is near the interface, than in 9- and 12-AS, which are located deeper into the hydrophobic core. Increase in fluorescence polarization indicates restricted rotation of the fluorophore. Since the fluorophore, in this case, is covalently linked to the stearyl chain, it could also reflect the restriction in motion of the C18 chain that traverses the length of the hydrophobic core. The increase in polarization of 12-AS is in contrast to the unchanged polarization seen with 2-methylanthracene (2-MA) in Triton X-100 in urea. However, the anthroyloxy moiety is attached to a stearic acid chain that is anchored at the interface and probably
Fig 5.5 Polarization of 2-AS (■), 9-AS (●), 12-AS (▲) incorporated in Triton X-100 micelles as a function of GdmCl concentration.
samples more volume of the micelle than does 2-MA. Any change in the rotational motion of the stearic acid chain would result in a polarization difference and might not necessarily be due to increased microviscosity in the vicinity of the fluorophore. The polarization changes reported by the anthroyloxy stearic acid probes would, therefore, be more global. Considering that the size of the micelle reduces dramatically in GdmCI, it may be quite possible, as speculated for the lattice organization of the hydrophobic chains in a micelle, that anthroyloxy fatty acids may have several reversals in direction in their packing within the core, which might have a role in restricting their mobility.

We have also investigated the accessibility of the anthroyloxy probe, 2-AS, in the presence of GdmCI, using polar and non-polar quenchers. Quenching by dimethylaniline (DMA), a non-polar quencher, is known to be diffusion controlled in low viscosity solvents (Blatt et al., 1981). In absence of GdmCI, the extent of quenching by DMA follows the order 12AS> 9AS> 2AS which is expected since DMA, being non-polar, preferentially partitions into the more non-polar regions of the micelle. Fig 5.6A shows the Stern-Volmer plots of DMA quenching of 2-AS in various GdmCI concentrations. Small positive deviations observed in Stern-Volmer plots may arise from reduced partitioning of quencher at higher quencher concentrations and also due to proximity of the quencher to the fluorophore at the time of excitation, often termed as “sphere of action”. The decreasing slopes of the plots with increasing GdmCI may be either due to localization of the quencher deeper into the micelle, away from the region sampled by the fluorophore in 2-AS, or due to efflux of the quencher out of the micelle. To distinguish between the two possibilities, similar quenching studies were done with 9- and 12-AS. Fig. 5.6B shows the variation of the Stern-Volmer quenching constants for 2-, 9-, and 12-AS as a function of GdmCI. The decreased quenching, in GdmCI, of all the three fluorophores, located at various depths in the micelle, is best explained by increased solubility of the quencher in the bulk solvent. This is consistent with the ability of GdmCI to solubilize nonpolar molecules and is further supported by the
Fig 5.6 (A) Stern-Volmer plots for the quenching of 2-AS in Triton X-100 by DMA in 0 M (■), 1 M (●), 2 M (▲), 4 M (▼) and 6 M (▲) GdmCl.

Fig 6 (B) Stern-Volmer quenching constants (K_{SV}) for the quenching of 2-AS (▲), 9-AS (■), 12-AS (●) incorporated in Triton X-100, by DMA, plotted as a function of GdmCl concentration. The errors associated with determination of K_{SV} are plotted.
observations in red shifts of the fluorescence of the anthroyloxy moieties in 2-AS and increase in CMC values in the presence of GdmCl. Urea decreases the quenching of 2-methylanthracene in Triton X-100 micelles by DMA. Decreased partitioning of DMA into Triton X-100 micelles in the presence of urea was suggested to be reason for the observed decrease in quenching by DMA in urea.

We have performed quenching experiments using the polar quencher, iodide. With increase in GdmCl concentration we observed increased quenching of the fluorescence of the anthroyloxy moiety by iodide. Till 1M GdmCl, the Stern-Volmer quenching constant ($K_{SV}$) values increased and beyond 1M GdmCl a decrease was observed (Figure 5.7). Increase in the $K_{SV}$ values indicate greater accessibility of the polar quencher to the anthroyloxy group in 2-AS. This corroborates the data on red shifts of the $\lambda_{max}$ values and the enhanced rates of non-enzymatic hydrolysis presented earlier that show greater water penetration in the micelle in the presence of GdmCl. Similar quenching patterns were also reported with 9-methylanthracene as the fluorophore and iodide as the quencher in Triton X-100 in the presence of urea (Ruiz and Sanchez, 1994). At higher concentration of GdmCl, iodide quenching decreases. A similar observation with urea was interpreted as lesser accessibility between quencher and fluorophore due to the presence of a thicker solvation shell in presence of high concentrations of the chaotrope.

In the presence of additives such as GdmCl and urea the micellar structure is profoundly affected. Increase in CMC and corresponding decreases in size of the micelle in GdmCl solutions indicate that hydrophobic/hydrophilic balance of micelle is altered. Rate enhancements of alkaline hydrolysis in cationic micelles provided an excellent system to model micellar catalysis. Primarily the rate enhancements were due to the concentration of the reagents to the micellar pseudophase and possibly a specific catalytic effect. Non-ionic micelles have little effect on the rate constants of reactions of a variety of polar molecules as predicted. The present
Fig 5.7 Stern-Volmer quenching constants ($K_{SV}$) for the quenching of 2-AS incorporated in Triton X-100, by iodide plotted, as a function of GdmCl concentration.
report demonstrates a dramatic increase in the alkaline hydrolysis of PNPO in Triton X-100 in the presence of the additive, GdmCl. GdmCl per se does not hydrolyse p-nitrophenyl esters as demonstrated by unaltered hydrolysis rates of PNPA in GdmCl. The seventy fold increase in GdmCl is attributed to the enhanced penetrance of water into the interior of the micelles, which otherwise has restricted accessibility to water. The enhanced accessibility of PNPO in a micelle also reflected in the observed 2.5 fold increase in the activity of lipase till 0.75 M GdmCl. Enhanced penetrance of water was corroborated by red shift in 2-AS fluorescence, enhanced quenching by iodide and increased CMC in GdmCl. Given the structures one would expect both ester bond of PNPO and anthroyloxy of 2-AS experience similar environment.

Fluorescence quenching and PNPO hydrolysis measurements of Triton X-100 micelles in GdmCl describe a micelle wherein polarity extends farther into the hydrophobic core than in an 'unperturbed' micelle. The shape of Triton X-100 micelles is still unresolved. Robson and Dennis have discussed a spherical, non-classical micelle model (Robson and Dennis, 1977) where a sharp boundary does not exist between the hydrophobic and hydrophilic regions. They have also argued that if a classical micelle model were considered, an oblate rather than a prolate micelle would be more consistent with experimental observations. Though our observations do not imply any particular shape of a micelle; it is possible that the packing of POE chains in the micelle core may be more favorable in GdmCl. Thus POE chains may extend less into the aqueous phase. Since the hydrodynamic radius of the micelle is largely due to the hydration of POE chains in Triton X-100, any decrease in the exposed POE chains would decrease the radius of the micelle. However, it is also possible that decrease in the size of the micelle could also be due to reduced aggregation number or attendant shape changes in the micelle in the presence of GdmCl. Quenching studies indicate decreased solubility of a non-polar quencher in the interior of the micelle and increased quenching by a polar quencher in GdmCl. In the presence of GdmCl micelle of Triton X-100 has
reduced hydrodynamic radius and enhanced accessibility of micelle interior to water and other polar molecules such as iodide ion. Scheme 5.1 illustrates the expected structural changes in the micelle in GdmCl indicating smaller micelle with penetrance of water deeper into the micelle.

There is a general agreement that micellar catalysis, especially by ionic micelles, is explained by pseudophase ion exchange model, wherein the catalysis is mediated by local concentration effect and the medium effect. Local concentration effect, the concentration of the both reactants in the micellar pseudophase will be higher from those of the bulk phase, can be quantified by a partitioning coefficient term. In the present report the primary reason for enhanced hydrolysis of PNPO in Triton X-100 micelles in the presence of GdmCl is due to increased availability of water, one of the reactants, at the reaction site. The changes in the structure of the micelles, as demonstrated by various techniques, in the presence of GdmCl, provides a structural basis for the altered partitioning of water into the micellar interior.

It is interesting to note that the anthroyloxy probe, 2-AS, and PNPO, probably experience similar environment in a micelle, respond to the altered water penetrance by red shift in fluorescence emission and enhanced hydrolysis, respectively. By placing the hydrolysable ester group at various distances from a hydrophilic head group, similar to anthroyloxy fatty acids, the polarity of their environment could be quantified by the extent of hydrolysis. In an unperturbed micelle the deeper hydrolysable bond would be less hydrolyzed compared to the one located more towards the interface.
Scheme 5.1
5.3 Conclusions

Dramatic enhancements in the lipase-catalyzed and non-enzymatic hydrolysis of PNPO, solubilized in Triton X-100, in the presence of additive GdmCl prompted us to investigate the structure of Triton X-100 micelle in GdmCl. Non-enzymatic hydrolysis of the soluble ester, PNPA, was practically independent of GdmCl concentration. GdmCl decreases the micelle size and restricts the mobility of the anthroyloxy stearic acid molecules. Twenty-five fold increase in CMC suggests that GdmCl increases the solubility of the surfactant monomers. Red shift of the emission maxima of the anthroyloxy group located nearer to the interface indicates enhanced hydrolysis may be due to increased water penetration into the micelle. Iodide quenching of fluorescence also confirms this observation. The effects of GdmCl on micellar structure were similar to the effects of urea. This report demonstrates that perturbation of micelle structure in GdmCl leads to increased availability of water at the site of hydrolysis, thereby increasing the rates of both non-enzymatic and enzymatic hydrolysis. These studies bring to the fore the importance of micellar “catalysis” even in non-ionic micelles in the presence of additives such as GdmCl.