Stability studies on a lipase from *Bacillus subtilis* in guanidinium chloride
2.0 Introduction:

Lipases are hydrolytic enzymes involved in the metabolism of glycerides and often also exhibit phospholipase, cutinase and amidase activities (Petersen and DrablØs, 1994). The abundance of lipases, their unique properties and wide substrate specificities have led to their widespread application in modification of fats and as additives in detergent formulations. Owing to their high regio- and enantioselectivity, lipases are used as biocatalysts in organic chemistry (Jaeger et al., 1999). Lipases preferentially work on substrate aggregates than monomers and thereby, exhibit 'interfacial activation' (Verger, 1997). Interfacial activation, which allows enhancement of activity in the presence of substrate interfaces, is a unique property of lipases and phospholipases, and distinguishes them from esterases. The unusual kinetics of these enzymes owing to the aggregated state of the substrates they hydrolyze, have intrigued enzymologists for a long time. During the last few years, many labs have actively pursued engineering of lipases for improved functions. More than 12 lipases from various sources have been crystallised and extensive information on lipase engineering has been documented (www.led.uni-stuttgart.de).

Applications based on lipases critically depend on their stabilities in solution. Structural alterations in proteins due to their interaction with chaotropes such as GdmCl and urea or on exposure to high temperatures have immensely helped our understanding of protein stability and folding (Privalov, 1992). There have been a few studies on the stability of lipases as a function of temperature, detergents, organic solvents and chaotropes (Jutila et al., 2000; Melo et al., 2000; Tombs and Blake, 1982; Zhu et al., 2000; Zhu et al. 2001). However, despite widespread interest in lipases and the availability of crystal structure information of lipases from many sources (Jaeger et al., 1999), the number of reports on biochemical and structural studies of lipases in solution are relatively few.
Among bacterial lipases, lipase from *Pseudomonas aeruginosa* has been extensively studied. Recently, the crystal structure of another bacterial lipase, the lipase LipA from *Bacillus subtilis*, has been reported at 1.4 Å resolution (Pouderoyen *et al.*, 2001). This protein belongs to the homology family 1.4 of bacterial lipases. The crystal structure of the *B. subtilis* lipase revealed a compact minimal α/β hydrolase fold without a lid and has corroborated several biochemical observations on this protein. The *B. subtilis* lipase has interesting and unique properties (Lesuisse *et al.*, 1993) that set it apart from other lipases. With a reported MW of 19,348, it is one of the smallest lipases known. Loss of activity on incubation with the serine reagent, PMSF, and the recently available crystal structure showed that the "classical" lid covering the active site, found in most lipases, is absent in this enzyme. Due to the absence of the 'lid' this lipase does not show 'interfacial activation' which essentially results from the rearrangement of the helices constituting the 'lid' on interaction with an interface, consequently exposing the active site. In this respect, the *B. subtilis* lipase is similar to the *Fusarium solani* cutinase (Martinez *et al.*, 1992), the acetylxylan esterase from *Penicillium purpurogenum* (Ghosh *et al.*, 1999) and the *Pseudomonas aeruginosa* lipase (Misset *et al.*, 1994). It has been classified as a lipase because it shows maximum activity with medium chain esters (C8) and unlike esterases, can hydrolyse long-chain esters. The *B. subtilis* lipase with pi of 9.9 is a basic protein showing optimum activity at pH 10 and is remarkably stable in alkaline conditions (Dartois *et al.*, 1992). In addition, in the *B. subtilis* lipase, the conserved lipase active site motif G-X1-S-X2-G is replaced by A-X1-S-X2-G [17]. The *B. subtilis* lipase hydrolyses p-nitrophenyl esters and triglycerides at sn-1 and sn-3 position with a preference for C8 fatty acids (Lesuisse *et al.*, 1993).

The lipase from *B. subtilis* has a strong tendency to aggregate, which considerably affects the protein yields during purification, specific activities of the pure protein from one batch to another and the biochemical properties of the protein (Lesuisse *et al.*, 1993). Strong binding to hydrophobic columns and its tendency to aggregate
in solution suggest that the protein has considerable surface hydrophobicity. The crystal structure too shows a solvent-exposed, hydrophobic active site cleft. The enzyme has considerable stability in organic solvents and shows full retention of activity after 24 hr incubation at 20°C in up to 30% dimethylformamide or methanol or up to 60% in dimethylsulfoxide (Lesuisse et al., 1993). Presence of dimethylsulfoxide (<30%) has been shown to have a stimulating effect on the enzyme activity. These properties of the enzyme along with the wide range of substrates it can hydrolyze make it an attractive candidate for use in organic synthesis. Stability of the enzyme is of crucial importance in such applications. A convenient way to investigate protein stability is to study its behaviour in denaturants such as urea or GdmCl. In this work, we have studied the aggregation behaviour of the B. subtilis lipase in solution, the interaction of the protein with the hydrophobic dye, bis-ANS, and the changes in the conformation and the surface hydrophobicity of the protein in GdmCl.

2.1 Materials and methods:

2.1.1 Materials

Bis-ANS, ANS, and fluorescein diacetate were purchased from Sigma Chemical Co., USA. GdmCl and acrylamide were purchased from Serva, Gmbh and Co., Heidelberg. All other reagents were of analytical grade.

2.1.2 Preparation of the lipase

The purification of the protein from the overproducing strain B. subtilis BCL1051 was carried out essentially as described earlier (Lessuisse et al, 1993) with minor modifications. B. subtilis BCL1051 was grown aerobically for 16-18 hrs at 37 °C in 2l Erlenmeyer flasks, each containing 500 ml of medium of the following composition: 2.4 % yeast extract, 1.2 % tryptone, 0.4 % gum Arabic, 0.4 %
glycerol, 0.017 M KH$_2$PO$_4$, 0.072 M K$_2$HPO$_4$, 50 mg/ml kanamycin sulfate. The culture medium was inoculated at 1% from 10 ml precultures. After harvesting the cells by centrifugation at 6000 rpm for 30 min, the culture supernatant was pumped at a flow rate of 30 ml/hr onto a Phenyl Sepharose Fast Flow High sub column (Pharmacia) (20 ml column volume per 1l culture) equilibrated with 100 mM potassium phosphate, pH 8.0. The column was washed at a flow rate of 50 ml/hr first with 10 mM potassium phosphate, pH 8.0 and then with 30% ethylene glycol in 10 mM potassium phosphate, pH 8.0. Elution was performed at a flow rate of 50 ml/hr with 80% ethylene glycol in 10 mM potassium phosphate, pH 8.0. 2 ml fractions were collected and the fractions containing protein (detected by absorbance at 280 nm) were checked for enzyme activity. The active fractions were pooled and dialyzed against 2 mM glycine-NaOH, pH 10.0. The dialyzed protein was diluted 1:1 with 50 mM Bicine-NaOH, pH 8.5 (buffer A) and loaded onto a MonoS HR5/5 (Pharmacia) column, pre-equilibrated with buffer A, using a Superloop (Pharmacia) on a FPLC (Pharmacia) system. The protein-bound-column was washed thoroughly with the buffer A to remove unbound proteins. The protein was eluted using a linear gradient with buffer A to buffer B (50 mM Bicine-NaOH, pH 8.5, 1 M NaCl). The enzyme eluted around 300 mM NaCl as a single peak. The active fractions eluted from the MonoS column were dialyzed overnight against 2 mM glycine, pH 10.0 and concentrated using an Amicon concentrator fitted with a YM10 membrane (10 kD cutoff). Purity of the protein was checked on a 12% SDS-PAGE gel containing 5 M urea (Lessuisse et al, 1993). The protein was > 95% pure on a Coomassie stained gel.

2.1.3 Unfolding studies:

The lipase (at the concentrations indicated in the figure legends) was incubated for 12 hours at room temperature (25°C) in 50 mM glycine buffer, pH 10 (except for activity measurements), in different concentrations of GdmCl before making the various spectroscopic and activity measurements.
2.1.4 Static light scattering

Static light scattering was monitored in a Hitachi F4010 fluorescence spectrometer with the excitation and emission monochromators both set at 600 nm. The excitation and emission band passes were 5 nm. 53 µM (1 mg/ml) protein solution was used for this study. Bis-ANS was added at a concentration of 10 µM.

2.1.5 Dynamic light scattering

All dynamic light scattering measurements were carried out at 25°C using 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 5.25.44 software package was used for data analysis. The translational diffusion coefficient \(D_t\) of the sample particles was determined by measuring the fluctuations in the intensity of scattered light with an autocorrelation function. The hydrodynamic radius \(R_h\) of the particles was calculated using the Stokes-Einstein equation \(D_t = \frac{k_b T}{6\pi \eta R_h}\), where \(k_b\) is the Boltzman constant, \(T\) is the absolute temperature and \(\eta\) the solvent viscosity. The viscosity for different GdmCl solutions were determined (Fasman, 1976) and used for the analysis. Protein concentrations of 0.5 mg/ml were used for the measurements. All solutions were passed through 0.02 µm filters (Anodisc 13, Whatman) before taking measurements.

2.1.6 Bis-ANS binding studies

Bis-ANS is a valuable probe to investigate the surface hydrophobicity of proteins. Upon binding to hydrophobic pockets, the fluorescence of bis-ANS increases significantly, which can be used to titrate the surface hydrophobicity of proteins (Krishna Sharma et al., 1998; Smoot et al. 2001; Shi et al., 1994 and Rosen and Weber, 1969). Samples containing bis-ANS were excited at 390 nm and emission
was monitored from 400-600 nm. Both excitation and emission bandpasses were 5 nm and scan speed was 120 nm.min$^{-1}$. A stock solution of bis-ANS was prepared in methanol and the concentration was determined by absorbance at 385 nm using an extinction coefficient, $\varepsilon_{385} = 16,790 \text{ cm}^{-1} \text{ M}^{-1}$ (Krishna Sharma et al., 1998).

Bis-ANS titrations were done as described in ref 21. All the fluorescence intensities were corrected for inner filter effect using the following equation (Lakowicz, 1999):

$$F_{\text{corr}} = F_{\text{obs}} \times 10^{\left(\frac{A_{\text{ex}} + A_{\text{em}}}{2}\right)}$$

Where $A_{\text{ex}}$ and $A_{\text{em}}$ are the measured absorbance at the excitation and emission wavelengths, respectively. Samples were prepared in 50 mM glycine buffer pH 10, containing varying amounts of bis-ANS. Each tube contained 10.3 $\mu$M protein. Bis-ANS spectra was taken 5 min after incubation with the protein.

For determining the binding of bis-ANS to the protein at various GdmCl concentrations, bis-ANS was added at a final concentration of 10 $\mu$M to protein samples that had been preincubated in the appropriate concentrations of GdmCl for 12 hours and the emission spectra were recorded after 5 min.

2.1.7 Assay for lipase activity

Enzyme assays were done at room temperature in Tris.HCl buffer, pH 10, using fluorescein diacetate as substrate by essentially following the method described earlier (Thomas et al., 1979). Tris.HCl buffer was used for activity measurements due to the very high rates of spontaneous hydrolysis of the substrate in glycine buffer, pH 10. In the conditions used the rates of spontaneous hydrolysis were not more than 1% of the enzymatic hydrolysis. A freshly made stock solution (20 mM)
of the substrate in acetone was added to aqueous buffer to give a 10X substrate solution. The final concentration of the substrate in the reaction mix was 20 μM. Fluorescein diacetate (FDA) is hydrolyzed by the lipase to generate a highly fluorescent product. The excitation monochromator was set at 490 nm and product formation was monitored by following the change in the fluorescence emission with time at 514 nm. The excitation and emission band passes were set at 5 nm. Protein concentration of 0.5 μM was used for the activity assays. 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 fluorescein diacetate (FDA), i.e., in the absence of enzymes, were measured and used for correcting the hydrolysis rates obtained with enzymes. p-Nitrophenyl acetate (PNPA) was also tested but found to have unacceptably high rates of spontaneous hydrolysis at pH 10, even in Tris.HCl buffer. Triton X-100-solubilized p-nitrophenyl oleate was also not preferred as substrate for activity measurements, since simultaneous changes occurring in the micellar organization in the presence of GdmCl are known to confound activity measurements (Acharya and Rao 2002a).

2.1.8 Intrinsic tryptophan fluorescence

Fluorescence spectra were recorded using a Hitachi F4010 fluorescence spectrophotometer. Intrinsic tryptophan fluorescence spectra were recorded between 310-400 nm by exciting the sample at 295 nm. The excitation and emission bandpasses were set at 5 nm. All spectra were recorded in the corrected spectrum mode. All spectra were corrected for buffer base line by subtracting the respective blank spectra, without the protein, recorded in identical conditions.

Fluorescence polarization measurements were performed using a Hitachi polarization accessory. The excitation wavelength was 295 nm and emission was
recorded at the $\lambda_{\text{max}}$. Excitation and emission bandpasses of 10 nm were used. Polarization values were calculated using the equation

$$P = \frac{(I_{\text{lv}}-G_{\text{lv}})}{(I_{\text{lv}}+G_{\text{lv}})}$$

Where $I_{\text{lv}}$ and $I_{\text{vh}}$ are the measured fluorescence intensities with the excitation polarizer oriented vertically and the emission polarizer oriented vertically and horizontally respectively. $G$ is the grating correction factor and is equal to $I_{\text{hv}}/I_{\text{hh}}$ where $I_{\text{hv}}$ and $I_{\text{hh}}$ are the measured fluorescence intensities with excitation polarizer oriented horizontally and the emission polarizer oriented vertically and horizontally respectively.

Quenching of tryptophan fluorescence by the polar quencher, acrylamide, was studied. Excitation and emission slit widths were set at 5 nm. The excitation wavelength was 295 nm and the emission was monitored at the $\lambda_{\text{max}}$. Stocks of acrylamide were prepared in buffer containing required concentration of GdmCl. Small aliquots of this stock were added to the protein sample up to a final concentration of 0.5 M. Fluorescence spectra were recorded after each addition. The emission intensities were corrected for the increase in volume resulting due to addition of quencher. The fluorescence intensities were also corrected for the inner filter effect according to published methods (Eftink and Ghiron, 1981) using the equation

$$F_{\text{corr}} = F_{\text{meas}} \times 10^{0.23[\text{Acrylamide}]/2}$$

where $F_{\text{corr}}$ and $F_{\text{meas}}$ are the corrected and measured fluorescence intensities, respectively. [Acrylamide] is molar concentration of acrylamide.

$$I_0/I = 1 + K_SV[Q]$$
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.

2.1.9 Circular dichroism studies

CD spectra were recorded using a JASCO J-715 spectropolarimeter. All spectra recorded are the average of 5 accumulations. For far- and near-UV CD, 0.65 and 1 mg/ml solutions of protein were used, respectively. For far-UV CD a 0.01-cm pathlength sandwich-type cuvette was used whereas near-UV CD was recorded using a 1-cm pathlength cuvette. The CD spectra were recorded in the ellipticity mode at a scan speed of 50 nm/min and a bandpass of 2 nm. The wavelength step was 0.5 nm with a response time of 2 s. All spectra were corrected for buffer base line by subtracting the respective blank spectra recorded identically without the protein.

Mean residue ellipticity was calculated using the equation

$$[	heta]_{MRW} = \frac{M_r \times \theta}{10 \times l \times c}$$

where $\theta$ is the ellipticity in degrees, $l$ is the pathlength (in cm) and $c$ is the concentration (in g/ml). For calculation of the mean residue weight ellipticity, a mean residue weight ($M_r$) of 115 was used (Kelly and Price, 1997).
2.2 Results

2.2.1 Light Scatter

Hydrodynamic radius of a protein is a sensitive indicator of changes in the size of a protein. The size of a protein is most frequently monitored by size exclusion chromatography (SEC). SEC is difficult to perform on the *Bacillus subtilis* lipase since the retention times do not reflect its size accurately, possibly due to affinity to the matrix and also strong tendency of the protein to aggregate (Lesuisse *et al.* 1993 and Pouderoyen *et al.*, 2001). Hence, we studied the size of the native proteins by light scatter techniques. Light scatter provides size information on molecules in true solution conditions and is considered to be the method of choice especially when interactions between protein and a separation matrix, as in size-exclusion chromatography, is suspected. A protein undergoing denaturation in the presence of GdmCl would demonstrate profound changes in the size or radius of the protein. Dynamic light scatter (DLS) would provide information on the polydispersity (heterogeneity) and percent mass in addition to the intensity of the scattering particles.

The lipase was incubated overnight at room temperature at 0.5 mg/ml concentration in varying amounts of GdmCl before the measurements were made. The *B. subtilis* lipase, in the absence of GdmCl, exists as heterogenous aggregates with an average radius of nearly 6 nm (Fig. 2.1A). The lipase spontaneously aggregates at room temperature. In buffer the aggregation of lipase was slow and continues till turbidity is observed due to precipitation of protein (~2-3 days). Typical DLS data obtained with the native protein and protein incubated in GdmCl are presented in Fig. 2.1A. The hydrodynamic radius corresponding to maximum intensity in fig 2.1A has been plotted as a function of GdmCl concentration (Fig. 2.1B). The native protein appears to be very polydisperse; with
**Fig 2.1  Dynamic Light Scatter of the *B. subtilis* lipase.**

(A) Dynamic light scatter profiles of the native protein and in various concentrations of GdmCl. The change in the hydrodynamic radius (B) and the % polydispersity (C) of the protein as a function of GdmCl concentration. Protein concentration used was 0.5 mg/ml. Percent polydispersity is calculated as (Standard deviation/ Mean) \cdot 100.
the hydrodynamic radius varying from 1.7nm to 15nm. This indicates that aggregates of up to 100 monomers could be forming after overnight incubation. In low concentrations of GdmCl (0.5M), relatively homogeneous monomers of 2.16 nm radius (Fig 2.1 A and B) are obtained. With subsequent increase in GdmCl the protein again increases in size and reaches a maximum radius of 4 nm by 2 M GdmCl that remains unchanged till 6 M GdmCl. Polydispersity decreases with increase in GdmCl (Fig. 2.1A and C) suggesting increasing uniformity in size of the protein as it denatures. However, there is a small but unusual increase in polydispersity around 1.5-1.75 M GdmCl (Fig 2.1C).

2.2.2 Interaction of bis-ANS with the lipase

Bis-ANS is a polarity-sensitive molecule that fluoresces intensely in hydrophobic environment and is practically non-fluorescent in water (Rosen and Weber, 1969). Based on their environment responsive properties, probes such as 8-anilino-1-sulfonic acid (ANS) and bis-ANS have been extensively used to characterize the exposed hydrophobic pockets in proteins and to identify molten-globule intermediates in folding and unfolding pathways of proteins. Fig 2.2A shows that on binding to lipase, the fluorescence of bis-ANS increases significantly, more than 5-fold, and is also blue shifted to 510 nm. ANS, a mono-anionic fluorescent probe, shows marginal increase, approximately 10%, in its fluorescence on binding to lipase (data not shown). Titration of the lipase with bis-ANS demonstrated cooperative (sigmoid) binding of bis-ANS to the protein (Fig 2.2B). The cooperative behavior suggests that binding of the first bis-ANS molecule to lipase, which may have lower affinity, enhances the binding of subsequent ligand molecules.

Figure 2.2C shows the data on extent of binding of bis-ANS after 5 min to lipase incubated overnight in various concentrations GdmCl. Bis-ANS binding decreases on increasing GdmCl concentration from 0 M to 0.5 M GdmCl. On increasing
**Fig 2.2**  **Bis-ANS binding to *B. subtilis* lipase.**

(A) Fluorescence of 10 μM bis-ANS alone (dot-dashed line) in buffer; bound to native protein (solid line), to protein in presence of 0.5M (dotted line) and 1.75 M (dashed line) GdmCl. (B) Titration of 10.34 μM of native protein with increasing amounts of bis-ANS (1.25-30 μM). (C) Relative fluorescence intensity at 500nm of bis-ANS bound to the lipase at different GdmCl concentrations. Scans were started 5min after adding bis-ANS to the protein sample. Protein concentration used was 0.1mg/ml).
GdmCl concentration further, bis-ANS fluorescence increases again and is highest at 1.5-1.75 M GdmCl. Since bis-ANS is a hydrophobic ligand, binding of bis-ANS to the native lipase was expected, based on the tendency of lipase to aggregate and more importantly, the crystal structure of the lipase, which shows a large solvent accessible hydrophobic patch on the surface molecule.

Since both aggregation and binding of bis-ANS could involve hydrophobic surfaces on the lipase, we tested whether bis-ANS alters the aggregation of lipase. The aggregation of lipase in buffer can be monitored by changes in light scatter. On incubating at room temperature, lipase shows strong tendency to aggregate. However, in the presence of 10 μM of bis-ANS, the tendency to aggregate was significantly reduced (Fig. 2.3). Even in the samples incubated overnight, binding of bis-ANS to lipase would reduce further aggregation and within three hours would reach a constant fluorescence value.

2.2.3 Activity and intrinsic fluorescence of lipase in GdmCl

To characterize the lipase during unfolding we have estimated the activity of lipase incubated overnight in various concentrations of GdmCl, using a soluble fluorescent substrate, fluorescein diacetate (FDA). The activity steadily decreased till the enzyme was completely inactivated by 3 M GdmCl (Fig. 2.4).

The intrinsic fluorescence of a protein is contributed primarily by the tryptophan and tyrosine residues. Intrinsic fluorescence is a sensitive indicator of the conformational changes experienced by a protein. The *B. subtilis* lipase has two tryptophan residues (at positions 31 and 42) and 9 tyrosine residues. One can selectively excite the tryptophans at 295 nm. The emission spectrum would represent the averaged emission properties of the two tryptophan residues. The native-enzyme has an emission peak at 337 nm when excited at 295 nm (Fig. 2.5 A and C). Upon denaturation in 6 M GdmCl the emission peak was red shifted to 350 nm and the relative fluorescence intensity of lipase was doubled (Fig. 2.5A).
Aggregation kinetics of *B. subtilis* lipase and prevention of aggregation on binding to bis-ANS.

Time dependent change in the scatter of the protein (1 mg/ml; 53 μM) in absence (-■-) and presence (-●-) of 10 μM bis-ANS.
Fig 2.4 Activity of *B. subtilis* lipase in different concentrations of GdmCl.
The enzyme activity is expressed as rate of change of fluorescence per minute.
The error bars represent the standard deviation for data obtained from three independent experiments. The protein concentration used was 6 μg/ml.
Fig 2.5 Denaturation of *B. subtilis* lipase by GdmCl monitored by intrinsic tryptophan fluorescence.

(A) Intrinsic tryptophan fluorescence of the protein (0.1 mg/ml) in 0 M (solid line), 0.5 M (dotted line), 1.75 M (dashed line) and 6 M (dot-dashed line) GdmCl. (B) Ratio of relative fluorescence intensities at 350 nm and 337 nm (RFI350/RFI337) as a function of GdmCl concentration. (C) Wavelength of maximum emission (excitation at 295 nm) as a function of GdmCl concentration. The error bars represent the standard deviation obtained from three independent experiments.
The considerable increase in fluorescence intensity of the fully unfolded protein over the native one suggests that the fluorescence of the tryptophans in the native proteins is strongly quenched, and unfolding leads to dequenching. The emission maximum sharply increases at 1.5 M GdmCl and the profile of $\lambda_{\text{max}}$ vs. GdmCl was suggestive of a two state transition. Ratio of relative fluorescence at 337 nm and 350 nm ($F_{350} / F_{337}$) vs. GdmCl also has a similar 2-state profile (Fig. 2.5B).

Polarization/anisotropy measurements of tryptophan fluorescence reveal the average angular displacement of tryptophans that occurs between absorption and subsequent emission of the photon. On unfolding, a protein is expected to lose many of the interactions responsible for maintenance of the native structure. Unfolding can, therefore, be monitored by the decrease in rigidity of the side-chains. The tryptophans, on progressive unfolding, are expected to experience more rotational diffusion, which would reflect in their polarization values. We have monitored the tryptophan polarization as a function of GdmCl concentration (Fig. 2.6A). Sharp decrease in polarization values between 1 M and 2 M GdmCl suggests that simultaneous to exposure to polar surroundings, tryptophans experience considerable motional freedom.

We have also investigated the changes in conformation by assessing the accessibility of tryptophans to the polar quencher, acrylamide (Fig. 2.6B). Accessibility to polar quenchers is a sensitive probe to assess the relative solvent accessibility of fluorophores in a protein. The quenching was plotted as Stern-Volmer plots and the coefficient of quenching ($K_{SV}$) was estimated. The quenching of tryptophan fluorescence increases sharply between 1 M and 2 M GdmCl, in agreement with the shift in $\lambda_{\text{max}}$ values of tryptophan fluorescence.
Denaturation of *B. subtilis* lipase by GdmCl monitored by tryptophan fluorescence polarization and quenching.

Polarization of tryptophan fluorescence emission as a function of GdmCl concentration. Protein concentration used was 1mg/ml. (B) Acrylamide quenching of tryptophan fluorescence. Slopes of the Stern-Volmer plots ($K_{SV}$) plotted as a function of GdmCl concentration. Protein concentration used was 0.1mg/ml.
2.2.4 Circular dichroism of lipase

Circular dichroism is a very useful technique to follow the status of secondary and tertiary structure of proteins on denaturation. Circular dichroism of proteins recorded in the far UV region (190-240 nm) is a direct and fairly dependable measurement of the amount of secondary structure in proteins (Kelly and Price, 1997). Fig. 2.7 A and B show the CD spectra of lipase in various concentrations of GdmCl between 205 and 320 nm. There are perturbations in the far-UV CD spectrum in as low as 0.5 M GdmCl. There is a sharp decrease in the secondary structure content of the protein on going from 1 M to 2 M GdmCl (Fig. 2.7A inset). The intermediate at 1.75 M GdmCl retains considerable amount of the native secondary structure (43%). By 2M GdmCl the far-UV CD spectrum resembles that of a random coil structure.

Near-UV CD of proteins (250-320 nm) arises due to the contribution from tryptophanyl, tyrosyl, phenylalanyl, cystinyl and certain prosthetic groups. Near-UV CD spectra in proteins predominantly arise due to the interactions of the aromatic amino acid side chains with each other in space when they are less than 10Å apart (Kelly and Price, 1997) and, therefore, can be observed only in well-structured proteins. It is a sensitive indicator of perturbations in three-dimensional conformation of proteins. The near-UV CD spectrum of the native protein is shown in Fig. 2.7B. At 0.5 M GdmCl, there is a small decrease in the ellipticity at all wavelengths in the near UV CD spectrum but hardly any loss in the fine structure. The 1.75 M GdmCl intermediate however, shows complete loss of ellipticity indicating complete loss of tertiary structure. All the above measurements performed on lipase, strongly suggest that between 1 to 2 M GdmCl, lipase undergoes cooperative changes in its structure affecting its aggregation, activity, CD, bis-ANS binding and intrinsic fluorescence.
Fig 2.7  Denaturation of *B. subtilis* lipase by GdmCl monitored by circular dichroism.

Far UV (A) and near UV (B) CD spectra of the protein in 0 M (——), 0.5 M (· · · · ·), 1.5 M (—), 1.75 M (— · —), 2 M (— · — ·) and 4.75 M (— · — · ·) GdmCl. The inset in (B) is the expanded view of the 260-300nm region.
2.3 Discussion

In this report we have investigated the stability of lipase from *B. subtilis* in the protein denaturant, GdmCl, by various spectroscopic techniques. Activity of lipase and tertiary structure of lipase, as expected, were most susceptible to denaturation. Aggregation affects the activity of the lipase marginally, since on going from 0 to 0.5 M GdmCl, though there was drastic reduction in aggregation, activity was slightly affected. The quantum yield of two tryptophans increases on denaturation, indicating that tryptophans in native protein are quenched, and also red-shifted indicating exposure to solvent. The \( \lambda_{max} \) of native tryptophan fluorescence at 337 nm suggests the tryptophans may not be entirely buried in the protein. Polarization changes with GdmCl suggest that the lipase loses rigidity in GdmCl with a sharp transition between 1-2 M GdmCl. Similar studies on tryptophan fluorescence on lipase from *Humicola lanuginosa* also showed that in the presence of GdmCl steady state anisotropies decreased revealing that the protein had become less compact (Zhu et al., 2000). At 1 M GdmCl, the radius of lipase increases sharply, reaching a value twice that of the native protein by 2 M GdmCl. Circular dichroism values and the environment of two tryptophans demonstrate global changes in the protein between 1 M and 2 M GdmCl. By 2 M GdmCl, the protein is completely denaturated, as indicated by all spectroscopic values reaching values observed with protein present in 6 M GdmCl. It was found that the lipase could be refolded from 6M GdmCl on 100-fold dilution, with complete recovery of activity (data not shown).

The lipase apparently unfolds by passing through an intermediate that has remarkable affinity to bis-ANS. This intermediate, which appears around 1.5-1.75 M GdmCl, apparently exposes extensive hydrophobic pockets on its surface. It is characterized by bis-ANS binding and also shows small but distinct increase in the polydispersity of size, suggesting that it may have tendency to form aggregates:
Bis-ANS is a polarity sensitive probe and has been extensively used to study surface hydrophobicity, molten globules, subunit-subunit interfaces etc. Bis-ANS binds to the native enzyme in a cooperative manner suggesting that bis-ANS induces structural changes in lipase upon binding, which may expose more hydrophobic pockets on the protein surface. The active site of lipases, in general, is predominantly hydrophobic and in this lipase it is solvent-exposed (Pouderoyen et al. 2001 and Lesuisse et al. 1993). Surface hydrophobicity in lipase, which is implicated in bis-ANS binding, is also involved in aggregation of lipase. Bis-ANS, as demonstrated in this study, could be a very useful probe to investigate the structural properties of aggregation-prone proteins, especially lipases.

Lipase from B. subtilis is one of the smallest lipases and does not have the catalytic lid that is essential for interfacial activation in the presence of oil-water interfaces. Lipases with 'lid' are relatively hydrophilic, because the hydrophobic substrate-binding site is covered by the helices forming the structural 'lid'. The helices move only on interaction with substrate aggregates. This movement has been shown to be accompanied by exposure of a large number of hydrophobic amino acids and burial of some hydrophilic ones. In Rhizomucor meihei lipase the movement of a 15 amino acid 'lid' has been shown to expose 800Å² of hydrophobic surface, which is 8% of the total molecule surface. Similarly, in the Candida rugosa lipase 1000 Å² of hydrophobic surface gets exposed on lid movement (Cygler and Schrag, 1997). The conformation of the 'lidless' B. subtilis lipase is similar to the open conformation of the lipases with 'lids' with hydrophobic grooves near the active site exposed to solvent. The considerably high hydrophobicity of the lipase compared to other water-soluble proteins, strongly determines its solution properties. Light scatter experiments showed that lipase at room temperature aggregates spontaneously till it reaches sufficiently large aggregates to precipitate out of solution (after 3 days). Presence of small amounts of bis-ANS slows down the aggregation of lipase, suggesting that hydrophobic pockets on the surface are involved in both bis-ANS binding and aggregation.
Similar behaviour of the hydrophobic probe ANS has been reported (Kundu and Guptasarma, 1999) where ANS has been shown to prevent thermal aggregation of carbonic anhydrase. It has been speculated that hydrophobic molecules like ANS could mimic molecular chaperones like α-crystallin in their ability to bind to hydrophobic surfaces and thereby prevent intermolecular association (Kundu and Guptasarma, 1999).

In characterization of lipases from bacteria the emphasis has been largely on crystal structure determination, expression and export of lipases. Very few attempts to study the solution structures and stabilities of bacterial lipases were made. Given the increasingly widespread use of lipases as biocatalysts in a variety of industrial and synthetic procedures, the need to understand the structure and stability of lipases in non-physiological conditions has been recognized. A series of papers describing the stability of an industrially important lipase Humicola lanuginosa (Lipolase from Novo Nordisk) and its three tryptophan variants in the presence of detergents, GdmCl, iso-propanol and high temperatures, have recently appeared (Jutila et al. 2000; Zhu et al. 2000 and Zhu et al. 2001). The B. subtilis lipase with its unique properties of stability at alkaline pH and in considerably high concentrations of organic solvents (Lesuisse et al. 1993), together with the facility of overproduction and easy purification make it an attractive enzyme for industrial optimization. Surface hydrophobicity as demonstrated in this report plays a significant role in determining the behaviour of the protein in both its native state and during its denaturation. We have investigated the aggregation behaviour of the lipase and some of the factors that affect it. Understanding the aggregation behaviour of an industrial enzyme is of importance in order to control and minimize the losses that occur due to protein aggregation and precipitation. Further efforts in this direction would be to study the stability of this lipase in other relevant non-physiological conditions like high temperature, organic solvents, detergents etc.