Engineering the loops in a lipase for stability in DMSO

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Received September 20, 2012; revised December 28, 2012; accepted January 2, 2013

Edited by Dr Stefano Gianni

Nearly 65% of the surface of a lipase, from Bacillus subtilis, is occupied by the loops. Since the loops are dynamic components of a protein, located on the surface and are tolerant to substitutions, we subjected all 91 amino acids of the loops to site saturation mutagenesis to identify mutations that improve the stability and activity of lipase in dimethyl sulfoxide (DMSO). Based on a novel screening system, we have identified six positions in the lipase, from a population of 18,000 transformants that contributed to higher activity in DMSO. We combined all the six mutations into one lipase gene (6SR), purified the protein to study its activity and structural properties. 6SR has shown eight times higher catalytic turnover in 60% DMSO and showed a marginal shift in DMSO tolerance. 6SR showed a similar secondary structure with little alteration in tertiary structure. The melting temperature of 6SR is lower than the wild type and binds the least to hydrophobic fluorescent probes, indicating that the surface has become more polar in nature. This study provides clues to the role of loop amino acids in modulating the activity in organic solvents.

Keywords: Bacillus subtilis lipase/dimethyl sulfoxide/loops/site saturation mutagenesis

Introduction

With exquisite substrate specificity and high reaction rates, enzymes offer tremendous advantages for chemical synthesis. In order to enable competitive manufacturing routes, most biocatalysts must be tailor-made for these processes (Wohlgemuth, 2010). Nonetheless, the practical applications of enzyme catalysis have been limited, owing to relatively poor stabilities and catalytic activities under the conditions that characterize industrial processes such as high temperatures, denaturing concentrations of non-aqueous solvents and extremes of pH (Klibanov, 1997). The behaviors of proteins in water-miscible and water-immiscible solvents are expected to be different (Timasheff, 1993). A large amount of phenomenological information was provided by the work of Klibanov on the behavior of proteins in water-immiscible solvents (Klibanov 1989, 1997, 2001). The denaturation by a water-miscible organic solvent is due to its ability to alter the delicate interactions between water–protein interactions by penetration of the organic solvent into the essential water layer or by pushing aside the essential water (Hutcheon et al., 2000). Consequently, chemical denaturants and non-aqueous solvents that change the properties of the hydration water can readily tip the balance in favor of the denatured state. Although many naturally occurring enzymes are used in organic solvents, their efficiency is severely reduced compared with its efficiency in water (Klibanov, 2001; Ogino and Ishikawa, 2001; Serdakowski and Dordick, 2007).

The effect of an organic solvent on structure and function had been studied in great detail by Klibanov and Dordick’s groups (Klibanov, 2001; Serdakowski and Dordick, 2007). It was observed that the loss in activity of an enzyme in organic solvents may not be due to loss of the structure of the protein in organic solvents. The presence of a solvent could bring about subtle changes in protein structure resulting in loss of activity. Unlike the knowledge base on the behavior of proteins in thermal stress and the role of amino acid substitutions that contribute to thermostability, similar information on the behavior of proteins in the presence of organic solvents is not available (Ahmad et al., 2008, 2012). An interesting question is: what kinds of amino acid substitutions are preferred for protein stability and activity in organic solvents? More success was reported in solvent engineering to improve the activity of enzymes in organic solvents rather than in catalyst engineering (Serdakowski and Dordick, 2007). Among catalyst engineering methods, lyopholization and crosslinking are considered beneficial in improving the value of enzymes in organic solvents (Haring and Schreier, 1999; Ru et al., 1999).

To design proteins to function in non-natural conditions, rational and directed evolution principles were used with considerable success (Arnold, 1990; Brustad and Arnold, 2011; Cobb et al., 2012). A ‘rational design’ approach involving site-directed mutagenesis requires the understanding of the structure of the protein and the structural basis for the property of interest. Alternatively, in the absence of structural information, directed evolution approaches, which rely on random mutagenesis followed by screening, have been extremely successful (Turner, 2009). Directed evolution has been used to enhance or alter various enzyme features, including thermal stability (Liao et al., 1986), stability in organic solvents (Chen and Arnold, 1991) and substrate specificity (Oliphant and Struhl, 1989). In this work on improving protein stability in organic solvents, the approach used is semi-rational, i.e. we focused on the loops of the protein and not the entire protein as in directed approaches, and performed site saturation mutagenesis, unlike site-specific mutagenesis as in rational approaches.

Non-regular secondary structural regions of the protein, the loops, are normally exposed to the solvent and are the most dynamic components of the protein. Loops have an important role in the stability of the protein and could also tolerate substitutions due to their unordered nature. We focused on loops with the intention to improve the stability of the protein in dimethyl sulfoxide (DMSO). In this study we chose Bacillus subtilis lipase A as our model protein. B. subtilis lipase (Lip A), a product of the lipA gene with a mass of 19.34 kDa, is a monomeric protein without any bound
ligand (Supplementary Fig. S1). Lip A preferentially hydrolyses C8 fatty acid esters and it is one of the three known lipases that do not possess interfacial activation (Van Pouteroyen et al., 2001; Kamal et al., 2012). Structurally, this lipase has a minimal α/β-fold and has a compact domain that consists of six β-strands in a parallel β-sheet, surrounded by five α-helices. Two high-resolution crystal structures of Lip A have recently been reported (Van Pouteroyen et al., 2001; Kawasaki et al., 2002). We have subjected all the 91 amino acids present in the loops to site saturation mutagenesis to create a mutant population. The stability properties of the mutants were screened for stability in the presence of DMSO. The screening consisted of three steps based on lipase activity on tributyrin or a chromogenic substrate, each eliminating the undesired clones from the mutant library. The properties of the recombined mutant lipase were compared with the wild-type lipase.

**Materials and methods**

Phenyl Sepharose 6 Fast Flow (High Sub) was from Amersham Biosciences Corp. (NJ, USA). DMSO, 4-nitro phenyl butyrate (pNPB) and hen’s egg white lysozyme were purchased from Sigma Chemical Co. (St Louis, MO, USA). Ethylene glycol and acetonitrile were from Merck & Co., Inc. (NJ, USA). Restriction enzymes NdeI and HindIII were purchased from New England Biolabs, Inc. (MA, USA). All other reagents used were of the highest grade available.

**Screening for lipase mutants with improved activity at high concentrations of DMSO**

The methods and primers used in the generation of the mutant library on loop amino acids were reported earlier (Ahmad et al., 2012). In brief, site saturation mutagenesis was performed using a pair of oligonucleotide primers at selected 90 positions of the loop regions in the wild-type lipase gene. The target amino acid position was coded by NNK (sense strand) and MNN (antisense strand), where N=A, G, C or T, K=G or T and M=A or C. The reaction was carried out by the overlap extension method using two vector-specific primers T7-promoter and T7-terminator, along with position-specific mutagenic primers, using pET22-WT as a template. The amplified products were digested with NcoI and HindIII followed by gel purification. The digested and purified products were ligated with similarly digested pET22b and used for the transformation of *Escherichia coli* BL21DE3, which directs the expression of protein in the periplasm upon induction with IPTG. Approximately 18 000 colonies, 200 transfomants for each position, were screened for mutants tolerant to large amounts of DMSO. The mutant library was cloned downstream of the *pelB* signal sequence in the pET22b expression vector to facilitate the periplasmic expression of lipase mutants in *E. coli* BL21 (DE3). Culture supernatants obtained from the mutant library were subjected to a three-step screening protocol based on lipase activity. In brief, glycerol stocks of a mutant library of ~18 000 clones were inoculated in 100 µl LB medium with 100 µg/ml ampicillin in 96-well microtitre plates (Tarsons) and grown for 6 h at 37°C, 180 rpm. Five microliters of each clone were inoculated into 200 µl LB medium with 100 µg/ml ampicillin and induced with 0.5 mM IPTG after the optical density of the culture reached 0.6 at 600 nm. The cultures were incubated at 37°C for 12 h post-induction and then spun at 4°C, 4000 rpm for 1 h to collect the culture supernatant.

In the first step, 5 µl of culture supernatants of the mutant library generated for each of the 91 amino acid positions were spotted on a LB agar petri dish containing 100 µg/ml ampicillin and were incubated overnight at 37°C for the colonies to grow. Each petridish contained 90 spots of mutant clones (the result of site saturation mutagenesis at one codon) and six spots of the wild type as the control. 0.2% (weight/volume) agarose was dissolved in phosphate buffer pH 7.2, and after cooling, 75 ml was immediately mixed with 0.8% (weight/volume) tributyrin emulsified in gum Arabic and 80% DMSO along with 1 mM IPTG as the inducer. This mixture was uniformly overlaid and was allowed to solidify on the petridish. An identical plate without DMSO in agar was used as a control plate to assess activity. The petridishes were incubated for an additional 12 h. Lipase hydrolytic activity was observed as the formation of a halo or zone of clearance around the colonies secreting the lipase that hydrolyze tributyrin in the surrounding medium. Since colonies of different sizes were formed in each plate, the difference of the radius of the halo from the center of the colony and the radius of the colony was measured from recorded photographs prepared using Adobe Photoshop CS3 image analysis software and the mutant clones that showed a larger difference in the above parameter than wild type, i.e. clones that formed larger halos than wild-type colony, were selected for the next step of the screen.

After the initial screening of 18 000 clones on petridishes, a stringent liquid phase assay was performed with the selected clones. The mutant clones selected in the first step were inoculated from the stored library of transformants in individual wells of 96-well microtitre plates containing 200 µl of LB medium with 100 µg/ml ampicillin. Plates were incubated at 37°C with shaking at 200 rpm for 6 h. These primary cultures were used to inoculate fresh medium in identical plates and were incubated at 37°C with shaking at 200 rpm. After 6 h, cultures were induced by adding IPTG in each well to a final concentration of 1 mM and incubated under the same conditions further for 12 h. Cells were harvested by spinning the plates at 4000 rpm for 30 min at 4°C in a 5810R Eppendorf centrifuge. From each plate, the supernatant was carefully separated without disturbing with pellet and transferred into two plates. One set was mixed with 100 mM Tris.Cl pH 7.2 buffer (the control plate) and another was mixed with a buffer containing 60% DMSO. A substrate stock solution of 2 mM pNPB in acetonitrile was prepared and added to a final concentration of 0.2 mM pNPB in the reaction mixture. A kinetic assay was performed after the addition of pNPB by following changes in absorbance at 410 nm in an ELISA spectromax 190 (Molecular Devices, USA) spectrophotometer plate reader. Clones hydrolyzing pNPB faster than wild type were selected from this step by comparison of the initial rates of hydrolysis between the control plates and the DMSO plate and by analysis using Softmax Pro software (Molecular Devices, USA).

The selected clones were reconfirmed by performing the pNPB hydrolysis assay using tube cultures. The clones were grown in 5 ml of LB medium containing 100 µg/ml ampicillin and induced with 1 mM IPTG after the mid-log phase was reached. After 12 h, the culture supernatant was
separated and the pNPB hydrolysis assay as mentioned in the above paragraph was performed at 25°C in 1 ml reaction volume and monitored at 410 nm in a UV-Vis Perkin Elmer spectrophotometer attached with a Peltier thermostat.

Identification of lipase mutants by DNA sequencing
Plasmid DNA from *E. coli* DH5α cells was isolated using a Qiaprep® spin Miniprep kit according to the manufacturer’s instructions. Sequencing reactions consisted of 50 ng of template DNA, 2.5 pmol of each of the following primers: T7 promoter primer or T7 terminator primer (5′-GGC CGT TAT TGC TCA GCG G-3'), sequencing buffer and BigDye™ reagent (Applied Biosystems). Reactions were carried out for 30 cycles of 94°C for 10 s, 50°C for 5 s followed by 60°C for 4 min in a polymerase chain reaction (PCR) thermal cycler. Sequencing was performed with an ABI 3730 sequencer analyzer (Applied Biosystems).

Recombination of single mutations in lipase
Recombination of the six single mutants was performed using overlap extension PCR. Fragments of the lipase gene containing the mutations were amplified using specific primers. The amplified fragments containing the mutations were overlapped and extended to form the full-length recombinant gene in a PCR using primers-1F (5′-GGC CGT TAT TGC TCA GCG G-3') and T7 terminator. The amplified products were gel eluted using a QIAGEN gel extraction kit. Restriction digestion of amplified full-length products were gel eluted using a QIAGEN gel extraction kit. For protein over-expression and purification, structural genes of wild-type and single mutants, cloned in pET22b vector, were introduced into E. coli expression in BL21(DE)3 cells and plated on LB agar containing ampicillin 100 μg/ml. Plasmids were isolated from the resultant colonies, and the DNA sequence of the recombinant was confirmed by sequencing.

Purification of lipase
For protein over-expression and purification, structural genes of wild-type and single mutants, cloned in pET22b vector, were PCR amplified using primers 1F and T7 terminator. The amplified products were digested with *NdeI* and *HindIII* and ligated with similarly digested pET21b. Wild-type and mutant proteins, cloned in pET21b, were purified upon expression in BL21(DE)3 *E. coli* cells and plated on LB agar containing ampicillin (100 μg/ml). Plasmids were isolated from the resultant colonies, and the DNA sequence of the recombinant was confirmed by sequencing. The amplified products were digested with *NdeI* and *HindIII* and ligated with similarly digested pET21b. Wild-type and mutant proteins, cloned in pET21b, were purified upon expression in BL21(DE)3 *E. coli* cells as described earlier (Acharya et al., 2004). All the mutants bind to a Phenyl Sepharose matrix and get eluted in the conditions similar to that of wild-type protein.

In brief, BL21(DE)3 *E. coli* culture pellet was resuspended in TE buffer (10 mM Tris-1 mM EDTA) pH 8 and lysed with lysozyme (10 mg for 1-l culture pellet). The cell lysate was sonicated in an ultrasound sonicator (Bruker Laboratories) and centrifuged at 15,000 rpm, 4°C and 30 min in a Beckmann centrifuge. The supernatant was loaded on a Phenyl Sepharose high-performance matrix pre-equilibrated with 100 mM potassium phosphate buffer pH 8. After washes with 10 mM phosphate buffer and 30% ethylene glycol, the lipase was eluted in fractions with 80% ethylene glycol and the absorbance was monitored at 280 nm. As it was observed that most of the mutants have a tendency to aggregate at high concentrations, like the wild type, the lipase-containing fractions (that were confirmed by activity assay for each fraction) were diluted with phosphate buffer, pooled and dialyzed extensively against 2 mM glycine buffer pH 10 for wild type and pH 8.5 for mutants. Mutants were observed to aggregate during dialysis at pH 10; thereby the pH was adjusted to 8.5 where aggregation was observed to be relatively low. To get >95% purity, the lipase solution was taken in binding buffer (50 mM bicine—NaOH buffer pH 7.7) and loaded on a cation exchange matrix-Mono-S (Amersham Biosciences) pre-equilibrated with the same binding buffer. Elution of the bound protein was performed by using a linear gradient of NaCl from 0 to 1 M in the corresponding binding buffer. Active fractions were pooled, extensively dialyzed overnight against 2 mM glycine-NaOH pH 10 for wild-type and pH 8.5 for mutants at 4°C and stored at −20°C. Purity was checked by running the sample proteins on a 15% urea-sodium dodecyl sulfate polyacrylamide gel electrophoresis and visualized by Coomassie brilliant blue staining. Protein quantitation was done by the modified Lowry method (Markwell et al., 1978).

Circular dichroism of lipase
Circular dichroism spectral measurements were performed using a JASCO J-815 spectropolarimeter equipped with a Jasco Peltier-type temperature controller (CDF-426S/15). Far-UV spectrum measurements were performed in the range of 200–250 nm using 0.2 mg/ml protein concentration in a 0.2 cm path length cuvette. Near-UV circular dichroism (CD) spectra were recorded in the range of 250–310 nm using a 1 mg/ml protein solution in a 1 cm path length cuvette. 50 mM sodium phosphate pH 7.2 was used as the buffer in the above spectral recordings. All spectra reported are the averages of three accumulations. Spectra were recorded in ellipticity mode at a scan speed of 50 nm/min, a response time of 2 s, a bandwidth of 2 nm and a data pitch of 0.2 nm. All spectra were corrected for buffer baseline by subtracting the respective blank spectra recorded identically without protein. The mean residue ellipticity was calculated using the equation

$$[\Theta]_{MRW} = \frac{(M_r \times \Theta)}{(10 \times l \times c)}$$

where $\Theta$ is the ellipticity in degrees, $l$ is the path length in centimeters and $c$ is the concentration in grams/milliliter. The mean residue weight ($M_r$) of 115 was used (Kelly and Price, 1997).

Thermal unfolding of lipase
Thermal unfolding of lipase mutants was monitored by circular dichroism in a JASCO J-815 spectropolarimeter fitted with a Jasco Peltier-type temperature controller (CDF-426S/15). The protein concentration used was 0.05 mg/ml, in 50 mM sodium phosphate buffer pH 7.2 with a path length of 0.2 cm. Temperature-dependent unfolding profiles were obtained by heating protein at a constant rate of 1°C per minute from 25 to 75°C and measuring the change in ellipticity at 222 nm. Fraction of unfolded protein was calculated by analysis of spectra with non-linear curve fitting using a two-state transition model ($y = A_2 + (A_1 - A_2)/(1 + \exp(x - x_0)/dx)$, where $A_1$, the initial value; $A_2$, the final value; $x_0$, the center of the transition; and $dx$, time constant) in Origin 7.0 software (Origin Lab). Initially, we have performed optimization runs

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to assess the dependence of $T_m$ on the protein concentration and temperature ramp.

Activity measurements of lipase

All enzyme kinetic parameters were determined at 25°C by measurements performed on a spectrophotometer attached with a Peltier temperature controller (Lamda-35 attached with a PTP-1 Peltier temperature programmer, Perkin Elmer) using pNPB as the substrate. A concentrated substrate stock of pNPB was made in acetonitrile. Different volumes of the substrate were added from stock to 100 mM Tris.Cl buffer pH 7.2 to get varying concentrations in the range of 0.05–2 mM, at which the initial rate of substrate hydrolysis was monitored by monitoring the increase in absorbance at 405 nm. The values for $K_m$ and $k_{cat}$ were derived from the corresponding Lineweaver–Burk plots.

Specific activities of lipase mutants at room temperature (25°C) were determined using 0.2 mM pNPB as the substrate. pNPB is a preferred substrate due to its high solubility in water as compared with other long chain substrates (p-nitrophenyl esters) and also due to very less spontaneous hydrolysis as compared with other substrates under identical conditions. In brief, 970 µl of 100 mM Tris.Cl buffer pH 7.2 was incubated at the desired concentration of DMSO for 5 min for equilibration in a 1-cm cuvette within the cuvette holder to which 1 µg of enzyme was added and incubated further for 1 min. Reaction was started by adding 10 µl of PNPB from 100× stock (2 mM PNPB dissolved in acetonitrile) and the rate of increase in absorbance at 410 nm was recorded. At each concentration of DMSO in the reaction mixture, spontaneous hydrolysis of pNPB was also monitored and was used to subtract from the absorbance change in the presence of enzyme.

Binding of 4,4′-bis(1-anilinonaphthalene 8-sulfonate) to lipase

4,4′-Bis(1-anilinonaphthalene 8-sulfonate) (bis-ANS) binding was monitored by adding bis-ANS to a final concentration of 10 µM to 0.05 mg/ml protein in 50 mM sodium phosphate buffer pH 7.2. A stock solution of bis-ANS was prepared in methanol and the concentration was determined by absorbance at 385 nm using the extinction coefficient, $\varepsilon_{385} = 16,790$ M$^{-1}$ cm$^{-1}$. Samples were excited at 390 nm, while emission was recorded in the range of 400–600 nm. Excitation and emission band passes were set at 5 nm, respectively, while the scan speed was 50 nm/min.

Results

Generation of DMSO tolerant lipase mutants

A three-step protocol was followed for screening lipase mutants, generated on 91 amino acid positions of the loops, in DMSO as explained in experimental procedures. The solid phase assay was standardized for the percentage of agarose for overlay and the amount of tributyrin substrate to obtain reproducible halos with the wild-type colonies (Fig. 1). In addition, the overlay mixture was optimized for the presence of different percentages of DMSO. To have a better discrimination between halo size between wild type and mutants, different concentrations of DMSO (0–80%) in overlay mixtures were tested. Although the wild-type lipase activity was lost more than half at 60% DMSO, as measured by the spectrophotometric assay, the halos formed around wild-type colonies at this DMSO concentration do not correspond to 60% reduction in size of halo compared with halo formed by wild type in the absence of DMSO. The formation of halos is based on longer times of incubation of lipase with the substrate, compared with short-time spectrophotometric assays; hence a correlation between halo size and solution activity was established. To be able to discriminate the mutant population from wild type, the plates were overlaid with 80% DMSO. As controls for every petridish, wild-type clones were spotted in six replicates so as to account for differences in the colony formed after spotting. Photographs of the plates at a certain time point, usually after overnight incubation, were taken and the radius was calculated from the images using standard imaging software. Based on the difference in halo size, 720 clones were identified to be positive from the petridish assay and these were subjected to a liquid phase assay in microtitre plates. Many of the clones identified were expected to be duplicates due to degeneracy in the codon. Fifty-nine mutants were identified as positive by using a criterion of 2–3-fold increase in residual activity compared with the wild type in the presence of 60% DMSO.

Fig. 1. Screening and specific activities of wild-type lipase and mutants in the presence of 60% DMSO. Top panel: A representative image of halos observed in a petridish screen. The halos formed at the lower left corner are due to wild-type lipase replicates. Bottom panel: Specific activities of the purified single mutants identified from the site-saturation approach.
These clones were further reconfirmed in a tube assay by pNPB hydrolysis in the presence of 60% DMSO.

Out of 91 positions, activity was severely compromised upon mutagenesis at 12 locations. These are positions 10, 11, 14, and 19 in the loop connecting β3 with αA; position 30 on the loop connecting αA with β4; 78 on the loop connecting β5 with αC; 92 on the loop connecting αC with β6; 104 and 106 on the loop connecting β6 and β7; 136 on the loop connecting β7 with αE and 141 and 145 on the loop connecting αE with β8. Out of these 12 positions, 11, 14, 30, 92, 104 and 145 were occupied by glycine in wild-type protein. Substitution of glycine by any other residue might have compromised the required conformational freedom, either affecting the folding of the protein to its native state or perturbing the local dynamics of the protein, thus severely affecting the activity of the mutant enzyme. This indicated the importance of glycine at these locations in maintaining the native tertiary structure of the protein. Out of 59 clones, six amino acid substitutions were obtained, namely I12L, W42L, A68S, P119S, Y139K and L140F/V, which resulted in a 2- to 3-fold increase in activity at 60% DMSO in the screen.

**Enhanced activity of lipase mutants in 60% DMSO**

Six of the single mutants of lipase, showing highest activity in the tube assay, were purified. The wild-type lipase undergoes a drastic reduction in activity by 50% when placed in 60% DMSO. The purified single mutants all showed a ~2–3-fold enhancement in catalysis in 60% DMSO as seen in Fig. 1. Interestingly, these mutants showed higher activity even in the absence of the organic solvent. The best mutant among the selected clones was P119S that showed more than 3-fold enhancement in activity at 60% DMSO than wild type lipase. We have obtained two positive substitutions at position 140. L140F has shown better residual activity in 60% DMSO compared with L140V; hence in further studies only L140F is taken.

**Recombination of six beneficial mutations in lipase**

All the six mutations selected, i.e. I12L, W42L, A68S, P119S, L140F and Y139K, were recombined, as described in the methods, into one gene, named 6SR. While the best single mutant (P119S) showed ~3.5-fold increase in activity in 60% DMSO, 6SR showed ~8-fold increase in activity under similar conditions. 6SR exhibited higher activity than wild-type lipase in the entire range of DMSO concentrations tested and specifically shows enhancement also at lower concentrations of DMSO (Fig. 2). The catalytic parameters, obtained with pNPB, of the recombinant 6SR compared with all single mutants and wild type 60% DMSO are given in Table I. The catalytic potential (k_{cat}/K_{m}) of 6SR was found to be eight times higher than wild type in 60% DMSO.

6SR retains its higher residual activity till 60% DMSO but its stability is not shifted to higher DMSO concentrations. Recombinant 6SR was tested for activity in other organic solvents as well and it was observed that it is active over the wild type in all the concentrations of the solvents tested—methanol, acetonitrile, isopropanol and dimethyl formamide (DMF) (Supplementary Fig. S2). However, in these solvents it does not show any enhancement in activity over control activity, i.e. in the absence of the solvent.

**Secondary and tertiary structure of the mutants**

Circular dichroism is a very useful technique for following the status of the secondary and tertiary structure of proteins on denaturation. Circular dichroism of proteins recorded in the far-UV region (200–250 nm) is a direct measurement of the percent of secondary structure in proteins (Kelly and Price, 1997). The single mutants and 6SR had a similar secondary structure profile as seen by far-UV CD as seen from Fig. 3. Since DMSO absorbs in the 200–250 nm region, we have taken the tertiary spectra of the wild type and 6SR in 60% DMSO in the region of 300–250 nm (Fig. 3). The data show that there is a decrease in ellipticity content in the 6SR but it retained the details of the tertiary CD profile of the wild type.

**Thermostability of the mutants**

Thermostability of the variants was measured by monitoring the temperature-induced unfolding by circular dichroism. The apparent melting temperature, T_{m}^{app}, was used as the parameter to compare as the wild type and the mutants undergo irreversible thermal denaturation. As seen from Fig. 4, the T_{m}^{app} of the single mutants was lower than that of wild-type lipase. A68S, P119S had a melting temperature 0.5°C lower than wild type in all the concentrations of the solvents tested—DMF (Supplementary Fig. S2). However, in these solvents it does not show any enhancement in activity over control activity.
than wild type, whereas I12L, Y139K, L140V, L140F had 1–2 °C lower $T_m^{\text{app}}$. The mutant W42L had a similar $T_m^{\text{app}}$ as wild-type lipase. The combination of these single amino acid changes in the recombinant 6SR led to a decrease in its $T_m^{\text{app}}$ to 50 °C, a decrease of 5 °C compared with the wild-type lipase. With all the lipases, including these mutants and mutants reported earlier, we found that the unfolding is not reversible. The temperature-mediated unfolding leads to aggregation and loss of protein from the sample. Thermal unfolding data show that the mutants evolved from the wild-type lipase for enhanced activity in DMSO are less thermally stable. Thermal stability in a protein is strongly linked to the dynamics of a protein. Several protein engineering studies reported an increase in thermal stability in a protein by reducing the $B$-factor in a protein (Reetz et al., 2006). While such a simple relation may not always be applicable, it is believed that thermal stability may emerge from multiple mechanisms. MD simulation studies in several solvents with a lipase suggested that the overall dynamics of a protein are reduced in organic solvents compared with water (Trodler and Pleiss, 2008). The decrease in thermal stability of 6SR suggests that the mutations may not be contributing to any additional structural stability and were conducive to function in DMSO.

**Binding of the hydrophobic dye bis-ANS to lipase**

Tertiary structure of the lipase mutants was probed by measuring their affinity to the hydrophobic dye bis-ANS (Smoot et al., 2001). bis-ANS is a valuable probe to investigate 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 the proteins (Sharma et al., 1998). As seen in Fig. 4, the wild-type lipase binds bis-ANS strongly with an emission maximum at ~500 nm whereas the single variants I12L, P119S, Y139K, L140F except W42L bind more strongly to bis-ANS dye with a minor blue shift in the emission maximum. Most importantly, 6SR binds very weakly to the hydrophobic bis-ANS to an extent even lesser than W42L with a blue shifted emission maximum. One consequence of mutations in 6SR is altered affinity to bis-ANS,
Discussion

Several studies on the application of directed evolution methods to improve the organic solvent stability of proteins include the entire gene for mutational purposes (Song and Rhee, 2001; Wong et al., 2004; Reetz et al., 2009). In this study, we have restricted the segments of the gene that code for loops of a lipase and subjected these positions to site saturation mutagenesis. A three-tier screening protocol for screening a population of 18,000 clones, derived from site saturation mutagenesis of 91 amino acid positions, resulted in six mutations that are better in their activity than the wild type in 60% DMSO. The combined mutant, 6SR, from six positive mutations, was found to have eight times higher catalytic potential in 60% DMSO compared with the wild type. Catalytic turnover of wild-type lipase has decreased by 3-fold in 60% DMSO compared with 0% DMSO, whereas with 6SR this decrease is marginal. These mutations have contributed to higher activity and also to structural integrity to the protein to retain activity in 60% DMSO. To understand the basis of this enhanced activity of 6SR, we have measured the distances of each of the substitutions to the three catalytic amino acids, i.e. S77, D133 and H156 (Supplementary Table S1). Since none of these mutations are at a distance to directly influence the activity, we could not implicate them in enhancement of catalytic activity. In our earlier study we have investigated another mutant of lipase, with 12 mutations and no overlap with the mutations reported in this study, and found that pre-organization of the active site in this mutant compared with the wild type was responsible for higher activity in the mutant (Ahmad et al., 2012). It is commonly reported in protein engineering studies that substitutions at positions far from the active site have influenced the activity of enzymes (Farinas et al., 2001). The basis of such enhancements was speculated but not supported. 6SR binds less to bis-ANS, indicating a more polar surface and has less thermal stability compared with the wild type. Three substitutions, A68S, P119S and Y139K, increase the surface polarity of 6SR. In an earlier report on lipase mutant with different sets of mutations we have shown that the mutations have caused discontinuities in surface hydrophobic patches, while not altering the total accessible surface hydrophobicity (Kamal et al., 2012). The decrease in binding of bis-ANS to 6SR compared with wild type could be due to the dominant effect of P119S on bis-ANS binding and enhanced polarity of the lipase surface and may also be due to subtle long distance effects of these mutations as seen in the case of activity enhancement. The secondary structures of the individual mutants and 6SR were identical to the wild type as assessed by CD. Moreover, the mutations, when mapped on the crystal structure of wild-type lipase, are located on the entire protein (Fig. 5). The location of the mutations indicates that for the stability of a protein in DMSO, the entire protein may be involved. The effects of each mutation were found to be marginal but cumulative in 6SR. A similar systematic evaluation of catalytic activity by each mutation was reported earlier (Chen and Arnold, 1991). This demonstrates that minimal changes in the structure are sufficient for enhancing catalytic activity in the organic solvent. Amino acid substitutions are known to be tolerated more if they occur in the loops than in the core, where any substitution may interfere with the packing (Wintrode and Arnold, 2001).

The mutations P119S, Y139K, A68S support the notion that replacement of solvent-exposed non-polar side chains (Y139 and A68) with highly polar charged residues on the surface seems to be favorable in increasing the activity in organic solvents (Zumárraga et al., 2007). P119 is a proline residue in the longest loop of the protein that may reduce the flexibility of the loop. Mutating this residue to a polar and small amino acid serine resulted in a 3-fold increase in activity in DMSO. It would be tempting to speculate here that the release of constraints on a loop, which is functionally and structurally important for catalysis, would lead to activation in the presence of organic solvents. The enhanced dynamics could contribute to the loss of thermal stability as revealed in melting curves. Although the catalytic parameters $k_{cat}$ and $K_m$ show subtle but significant alterations upon the introduction of single mutations, the overall specific activity showed an improvement of 1.5–2-fold as compared with wild-type enzyme in aqueous medium. Compared with the wild type, the mutants reported here resulted in an improvement of activity but a loss in thermal stability. The influences of these mutations resulted in a loss of rigidity required for thermostability. The effective screening and combination of mutations has resulted in a lipase mutant with both enhanced catalytic activity at high concentrations of DMSO and other solvents and also high specific activity in aqueous medium. The contributions of the mutations isolated in our study have primarily contributed to the activity but not significantly to DMSO stability. These mutations are dissimilar to other reported mutations that improve the activity in terms of their contributions to thermostability (Wintrode and Arnold, 2001).

Fig. 5. Map of mutated positions on the crystal structure of wild-type lipase. The mutated amino acid residues are colored in brown and the active site residues are colored in red and blue. PDB accession code 1I6W was used in generating the lipase model using PYMOL software.
The improvement in activity of the recombinant 6SR has also rendered the protein less thermostable, suggesting that the structural basis for these properties could be different.

The first report of the successful application of directed evolution for activity in non-aqueous media (Chen and Arnold, 1991) identified 10 amino acid substitutions. These substitutions are on the active site face of subtilisin and among these three were replacements with glycines. Subsequent reports of the application of directed evolution on subtilisin E and p-nitrobenzyl esterase resulted in mutants with substantial improvement in activity in DMSO and DMF (Chen and Arnold, 1993). These reports were on applying four cycles of mutations/screening cycles whereas in the present study only one cycle was attempted. Improved half life in DMSO but not higher activity was reported in directed evolution approaches on phospholipase A1 (Song and Rhee, 2001). More recently, an organic solvent tolerant lipase was evolved from Pseudomonas aeruginosa LST-01 by directed evolution, for DMSO (Kawata and Ogino, 2009). The lipase was subjected to random mutagenesis followed by screening in DMSO and a better variant having higher activities was obtained. The mutations were located mostly on the surface of the lipase. Directed evolution of cytochrome P-450 monooxygenase for activity in DMSO resulted in a 10-fold increase in activity (Roccatano et al., 2006). Fungal laccases, with applications in bioremediation and organic synthesis, were redesigned by directed evolution to give six mutations spread on the surface and gave tremendous increment in its activity in ethanol and acetonitrile (Zumárraga et al., 2007). Results of directed evolution for organic solvent resistance on mammalian cytochrome P-450 2B1 demonstrate that DMSO tolerance could be further improved on variants that have higher specific activity (Kumar et al., 2006). More recently, an organic solvent tolerant lipase was evolved from Pseudomonas aeruginosa LST-01 by directed evolution, for DMSO (Ogino et al., 2009). Compared with these studies, the activity enhancements reported here even up to 60% DMSO in a single round of mutational study is significant.

A detailed look at the nature of substitutions and their location that imparted stability in DMSO and DMF suggests that most of the mutations are surface located. The reported mutations in this study on lipases show a similar trend. However, regarding the nature of the substitution reported in these studies, the substitutions are largely polar than the amino acid they replaced. In one study a substitution with increasing hydrophobic amino acids at D248 seems to have stabilized a protein in a graded fashion. Our study corroborates that to improve the stability of a protein in a water-miscible organic solvent the substitution of more polar surface amino acids may help.

In conclusion, a focus on loop amino acids to improve the stability of protein in water-miscible organic solvents has given 8-fold improvements in activity of lipase in DMSO after one round of selection. Largely, more polar nature of the substitutions suggests that such mutations are preferred for stability in DMSO. That amino acid positions far from the active site have contributed to the stability suggests that stability in organic solvents involves the entire protein.

Acknowledgments

The authors acknowledge Dr Shoeb Ahmad for the generation and screening of the mutant library and Dr Zahid Kamal for his advice during the preparation of the manuscript. The work was supported by a CSIR Network Grant (NWP0044).

Supplementary data

Supplementary data are available at PEDS online.

References