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Stabilizing effect of polyols is sensitive to inherent stability of protein

Md. Zahid Kamal [1], Shoeb Ahmad [1], Nalam Madhusudhana Rao *

Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad-500007, India

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ABSTRACT

In studies on polyol-mediated protein stabilization, the polyols are the preferred variable and less importance is given to the intrinsic properties of the protein used. We investigated the stabilizing effects of glycerol on three *in vitro* evolved lipase mutants with varying stabilities and also in a broad pH range of 3.3–12.1. Significant linear negative correlation between increment in stability due to glycerol and prior stability suggests that stabilizing effects of glycerol depend on the prior stability of the protein. Polar/nonpolar surface area and charge do not have a bearing on the stabilizing effects of glycerol.

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1. Introduction

Increasing protein stability with the addition of polyols like sucrose, glycerol, trehalose and other organic osmolytes such as TMAO and amino acids is a widely used strategy [1]. Several models were proposed to understand the molecular basis of polyol-induced protein stabilization. Common amongst them are excluded volume effect [2–5], Wyman linkage function [6], preferential interaction and surface tension [7–9] and transfer free energy of protein's chemical groups [10]. All these models were very useful in explaining and predicting the outcome of many observations made on polyol-mediated protein stability [1,11,12]. Nevertheless, the models offer incomplete description on some data [1,13]. Interestingly, most of the experiments performed to measure the effect of polyols on protein stability restricted themselves with the variation in polyol concentration and its chemical nature. Protein–polyol interactions were seldom investigated keeping the properties of the protein in focus. Indeed many proteins including lysozyme, ribonuclease A, staphylococcal nuclease, α-chymotrypsinogen, trypsin inhibitor and cytochrome c etc. have been tested for stability in the presence of polyols [11,12,14]. These proteins differ widely in their sequence, structure, stability etc. to probe the role of intrinsic properties of a protein in its response to the presence of polyol. This information on the intrinsic properties of the protein on the phenomenon of protein–polyol interactions is sparse.

We attempted to explore polyol-mediated protein stabilization using three thermostable mutants of a lipase (Lip A from *Bacillus subtilis*) named TM, 2D9 and 4D3, which were generated using 1, 2 and 3 rounds directed evolution for higher thermostability. They differ from each other and the wild type lipase significantly in their stability properties. Each round of directed evolution added three new mutations to the previous one so that TM has three (L114P, A132D and N166Y), 2D9 has six (TM + F17S, N89Y and I157M), while 4D3 (2D9 + A15S, A20E and G111D) harbors a total of nine mutations. The crystal structures of all the four proteins were published and detailed information on the nature and location of additional interactions, stability properties and accessible surface area (ASA) parameters are available [15,16]. Although all the mutations are present on the protein surface, there is no significant variation in solvent-exposed surface area. Similarity goes to the extent of fractions of polar and nonpolar surface. In the native structures of both wild type and 4D3 (the most stable mutant), the polar and nonpolar exposed surface area, as calculated with Surface Racer [17], are ~45% and 55% respectively. In light of the above facts it is quite obvious to presume that the interaction of polyols with all these lipase mutants will be quantitatively similar to that of the wild type.

In addition to variations arising out of amino acid substitutions in these four proteins we have also employed pH change as another variable to change the stability properties of these proteins.

2. Materials and methods

Glycerol, sorbitol and polyethylene glycol were purchased from Merck (India) Ltd. Concentrations of polyol solutions were confirmed by refractive index measurements. All lipase variants were over-expressed and purified as described earlier [15,16]. Protein concentrations were estimated by the modified Lowry method [18]. Throughout the study, 50 mM buffer concentrations were used with the following buffers for the mentioned pH range: sodium acetate

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* Corresponding author. Tel.: +91 40 27192514; fax: +91 40 27160591.
E-mail addresses: zahid@ccmb.res.in (M.Z. Kamal), shoeb@ccmb.res.in (S. Ahmad), madhu@ccmb.res.in (N.M. Rao).

[1] Tel.: +91 40 27192552.

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buffer 3.3–5.8; sodium phosphate buffer 6.0–8.2 and 10.4–12.1 and sodium borate buffer 8.4–10.2. Charges on lipase at various pH values were calculated by H++ [19].

2.1. Far UV circular dichorism spectroscopy

Secondary structure of proteins were monitored by recording far UV CD spectra of 0.1 mg/ml protein in 50 mM buffer of appropriate pH, in a 0.1 cm path length cuvette using a JASCO J-815 spectropolarimeter between wavelengths from 250 nm to 200 nm. All the reported spectra were an average of four accumulations. Wavelength scans were carried out in the ellipticity mode at a scan speed of 50 nm/min, bandpass of 2 nm, at response time of 2 s and wavelength step of 0.5 nm. All spectra were corrected for buffer base line by subtracting the respective blank spectra recorded identically without the protein.

2.2. Thermal denaturation and stability measurement

Thermal denaturation experiments for all lipases at all pH values were performed on a CD spectropolarimeter equipped with Peltier-type temperature controller (PTC-348WI). Temperature scans were recorded at 0.05 mg/ml protein concentration in 0.5 cm path length cuvette at 215 nm wavelength. Data pitch and scan speed were 0.2 °C and 60 °C/h respectively. Melting temperature (T_m) for each thermal scan is estimated as the midpoint of thermal transition.

3. Results

3.1. Stabilization effect of polyols on the lipase mutants

We have carried out thermal denaturation experiments on the thermostable variants of lipase viz. TM, 2D9 and 4D3 along with the wild type protein at pH 7.2. Fig. 1 shows the thermal denaturation profile of various lipase variants at pH 7.2. Lipases show irreversible thermal denaturation behavior due to aggregation of the denatured protein. Thermal denaturation experiments were also performed on lipase variants at pH 7.2 in the presence of 40% (v/v) glycerol. For all the lipase variants, T_m in the absence and in the presence of glycerol was estimated. We have called T_m in the absence of polyol as "prior stability" to make a distinction in notation. As can be seen in Fig.2, wild type lipase having least prior stability (T_m) shows the largest increase in stability (ΔT_m) with the addition of glycerol, sequentially followed by TM, 2D9 and 4D3. Clearly, as the prior stability (T_m) of lipase variants increases, increment in stability decreases. Whenever tested 40% sorbitol, as a positive control, also gave similar change in T_m with the mutants and the wild type lipase. Because all the lipase variants are different from each other mainly in terms of their prior stability, we further explored the behavior of polyols-mediated stability increment as the function of prior stability. For this objective to be fulfilled other conditions were employed where the lipase structure is invariant but its stability has changed.

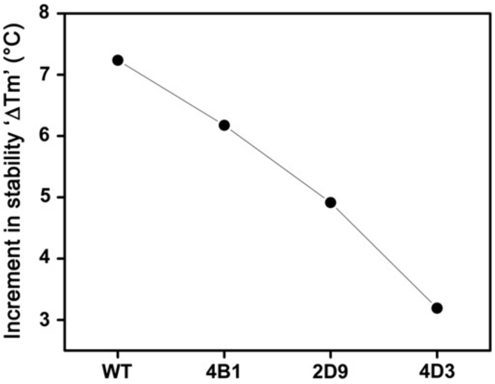

Fig. 2. Increment in stability (ΔT_m) of various lipases at pH 7.2, by addition of 40% (v/v) glycerol. 'ΔT_m' is the difference in 'T_m' estimated in the presence and absence of glycerol.

3.2. pH variations and stability in variants of lipase

Presence of ionizable amino acids renders the stability of proteins to be a dependent variable on the pH of the medium. Away from the pKi the protein accumulates charges, positive or negative, depending on the direction of pH change. Addition of charges destabilizes the protein due to electrostatic repulsion leading to loss in stability [20]. We have confirmed that the native secondary structure of lipase variants studied using far UV circular dichroism over the entire pH range was unaltered. Earlier in the lab, wild type lipase was crystallized between pH 4.0–5.0 and high resolution structural information was obtained [21,22]. The overall monomeric structure of the protein at low pH (pH 4.0) and high pH (pH 8.7) was identical with rms deviations in C_α position in the range of 0.314–0.509 Å. Fig.3 shows the far UV CD spectra of wild type lipase at extremes of pH, indicating the intact native structure of protein, while Fig. 4 shows the variation in charge and stability as a function of pH for the wild type protein. As evident, wild type lipase is having a zone of nearly equivalent stability between pH 5.5 and 9.5, beyond which stability decreases with increase or decrease in pH while the charge on it varies from >+10 to <−10 in the pH range studied (pH 3.3–12.1).

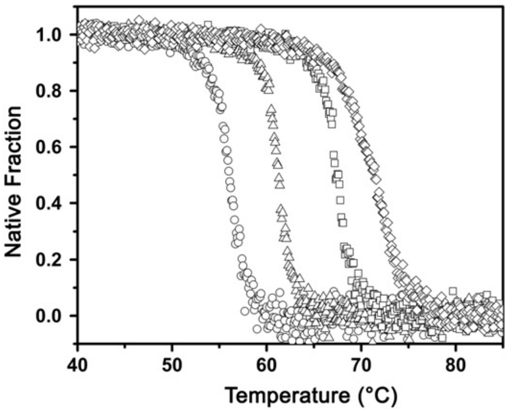

Fig. 1. Thermal denaturation profiles of lipase variants at pH 7.2. WT (o, 56.0 °C), TM (Δ, 61.2 °C), 2D9 (□, 67.4 °C) and 4D3 (◊, 71.2 °C). Melting temperatures of the lipase variants are given in the brackets.

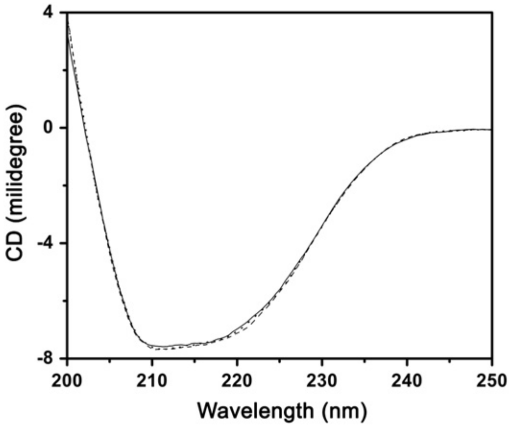

Fig. 3. Far UV CD spectra of wild type lipase at pH 3.6 (solid line), 7.8 (dot line) and 12.0 (dashed line). Spectra were recorded in 0.1 cm path length cuvette for 0.1 mg/ml protein in 50 mM buffer at 10 °C.

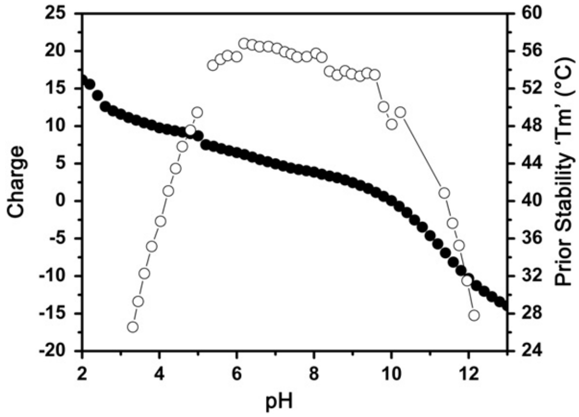

Fig. 4. Variation in charge (●) and prior stability 'T_m' (○) of wild type lipase as a function of pH. Charge was calculated using H++[19].

To see whether the extent of stabilization by polyols depends upon the prior stability of protein variants generated by pH, we selected three pH values viz. 3.6, 7.8 and 12.0. At pH 3.6 and 12.0, wild type lipase has very low and nearly equal stability (T_m~32.1 and 31.5 °C respectively) with charge ~+11 and ~−10.3 respectively, while at pH 7.8 it is in the high stability zone (~55.1 °C) with charge ~+4. We have used 60% (v/v) ethylene glycol, 40% (v/v) glycerol and 40% (w/v) sorbitol to increase protein stability. As can be seen in Fig. 5, these polyols increase the stability at both pH 3.6 and 12.0 to a larger extent compared to pH 7.8. Besides, increment in stability, in the presence of any of these polyols at pH 3.6 and pH 12.0, is comparable despite the fact that the difference in protein charge at these pH values is >21. These observations confirm the earlier made observation that stability increment by polyols depends upon prior stability. Surface charge does not appear to directly play any role in polyol-mediated protein stabilization.

To uncover the relationship between increment in stability brought by glycerol and prior stability, stability of all the lipase variants in the pH range of 3.3 to 12.1 was estimated both in the absence and presence of 40% (v/v) glycerol (Fig. 6a and b). In Fig.7 we have plotted T_m value of all lipase variants in this wide pH range against the increment in T_m value in the presence of 40% glycerol (i.e., ΔT_m). It is evident from the figure that the increment in stability (ΔT_m) by glycerol decreases linearly with an increase in prior stability (T_m). Such linear dependence of increment in stability by any polyol on prior stability has never been reported earlier. It will be apt to mention that our attempt to examine this relationship in terms of free energy stabilization (ΔG) could not succeed because of practical limitation of achieving complete denaturation by GdmCl and urea in the presence of glycerol.

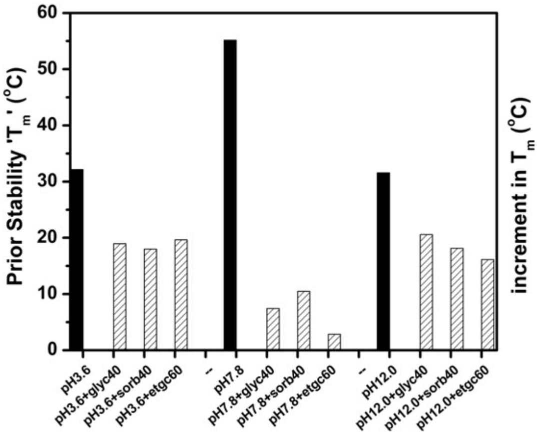

Fig. 5. Increment in stability of wild type lipase by polyols at three selected pH conditions. Solid bars represent prior stability (T_m) while mesh bar represents increment in stability (ΔT_m) with addition of polyols. glyc40 is 40% (v/v) glycerol, sorb40 is 40% (w/v) sorbitol and etgc60 is 60% (v/v) ethylene glycol.

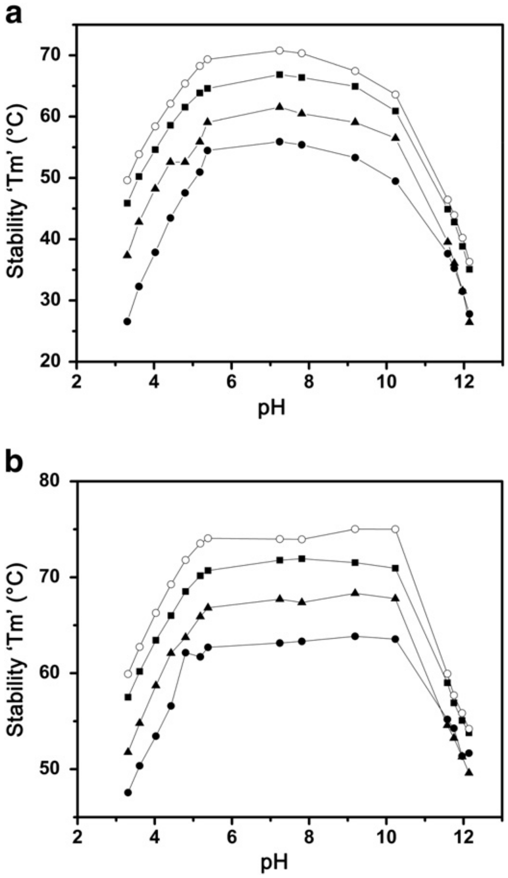

Fig. 6. Stability (T_m) of lipase variants at various pH in the (a) absence and (b) presence of 40% (v/v) glycerol. Wt (●), TM (▲), 2D9 (■) and 4D3 (○).

4. Discussion

As mentioned before mutants used in the present study show minimal variation in properties like volume, polar/nonpolar surface area etc. while variants created by pH although maintained their native structure, differ drastically in their surface properties like charge. However, in both cases, we noticed that irrespective of extent

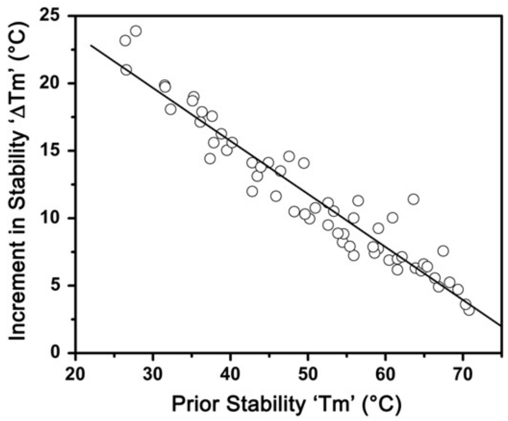

Fig. 7. Linear dependence of increment in stability (ΔT_m) of all lipase variants by 40% (v/v) glycerol as a function of prior stability (T_m). Straight line is the linear fit to the whole data points (correlation coefficient = ~0.93).

of dissimilarity in surface property (minimal amongst mutants while very large amongst variants created by pH changes), increment in protein stability is inversely dependent on prior stability only and not on surface property (Figs. 2, 5 and 7). Independence of increment in stability on protein surface properties also emphasizes the very fact that no specific interaction(s) between polyols and protein plays significant role in polyol mediated protein stabilization.

It is interesting for us to notice that similar kind of observations have been reported in scientific literature, although the role of prior stability has not been strongly emphasized. Kaushik and Bhat [23] noticed that trehalose brought about a larger increment in the melting temperature of RNaseA at pH 2.5 than at pH 7.0, although they attributed this behavior to increased hydrophobicity of the protein molecule at pH 2.5 due to protonation of acidic groups rather than high prior stability of protein at pH 7.0 than at pH 2.5. Similar behavior was noticed by Haque et al. [14,24] while working with lysozyme and RNase A at neutral and acidic pH. In contrast to previous studies which are limited to few pH values, our work on large number of lipase variants, created by directed evolution and wide pH range (3.3–12.1) helped us to establish an inverse linear relationship between increments in protein stability by polyol and prior stability of protein.

Why increment in stability by polyols depends upon prior stability? It is pertinent to mention that inverse linear relationship of increment in stability with prior stability does not directly infer that increment in stability depends upon prior stability, but points to the fact that both are related to some very basic protein behavior in an inverse way. Low stability implies less compact native structure. Cioni et al. [25] reported that sucrose brought higher degree of compactness to a less compact native structure and vice versa. Other studies on specific volume of proteins in the presence of polyols suggested that polyols induce a reduction in the size and number of voids in the protein [26,27]. Such change in native structure compactness is often missed by usual spectroscopic techniques and requires special attention. Our observation on the set of lipase variants can be described by the influence of polyols in increasing native structure compactness as this behavior does not depend upon protein's surface property but only on compactness of native protein structure which is very often correlated with protein stability. Investigation of the relationship between increment in compactness (or rigidity) of native protein structure by polyols with prior compactness will be worthy extension of present work.

5. Conclusion

Our study strongly suggests that prior stability of proteins plays a major role in polyol mediated stabilization while surface properties like polarity and charge do not directly contribute to it. This study is perhaps the first to show the inverse linear dependence of polyol/osmolyte mediated stability increase on prior stability of proteins. It appears that prior stability exerts its effect through native structure compactness, hence any factor like mutations and charge, which can increase/decrease native structure compactness (or stabilize/destabilize protein) can alter the polyol mediated stabilization behavior of proteins. We believe that these data may help refining the models that explain the polyol–protein interactions.

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