FEBS Letters 476 (2000) 296–300 FEBS 23848

Protein stabilization through phage display

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Received 29 March 2000; received in revised form 23 May 2000

Edited by Thomas L. James

Abstract RNase S consists of two proteolytic fragments of RNase A, residues 1–20 (S20) and residues 21–124 (S pro). A 15-mer peptide (S15p) with high affinity for S pro was selected from a phage display library. Peptide residues that are buried in the structure of the wild type complex are conserved in S15p though there are several changes at other positions. Isothermal titration calorimetry studies show that the affinity of S15p is comparable to that of the wild type peptide at 25°C. However, the magnitudes of $\Delta H°$ and ΔC_p are lower for S15p, suggesting that the thermal stability of the complex is enhanced. In agreement with this prediction, at pH 6, the T_m of the S15p complex was found to be 10°C higher than that of the wild type complex. This suggests that for proteins where fragment complementation systems exist, phage display can be used to find mutations that increase protein thermal stability.

Key words: Phage display; Fragment complementation; Thermal stability; Epitope mapping

1. Introduction

Although the various interactions that stabilize a folded protein with respect to the unfolded state are qualitatively well understood, increasing protein stability through rational design remains a challenging task [1]. Random mutagenesis and combinatorial methods such as DNA shuffling and phage display have been used with great success in engineering new activities or enhanced binding affinities [2,3]. The lack of an appropriate, general in vivo screen has made it difficult to use such methodologies to increase protein thermal stability, especially for monomeric proteins. However, in cases where the protein forms a complex with other proteins or ligands, phage display in combination with DNA shuffling has been used to select for mutants with enhanced binding affinity and in some cases enhanced stability [4–6]. Another study [7] has used resistance to proteolysis as the basis for selecting stably folded proteins via phage display though this methodology has not been used to generate mutants structurally more stable than the wild type protein. In the present work, we show that, for a monomeric protein where a fragment complementation system exists, phage display can be used to select for mutations that lead to enhanced thermal stability. The experimental system consists of the protein:peptide complex ribonuclease S (RNase S). This is obtained by cleavage of ribonuclease A (RNase A) by subtilisin to give two fragments, S peptide (residues 1–20) and S protein (residues 21–124). The fragments can be reconstituted to give the complex RNase S which has an identical structure and reactivity to RNase A. We have used a synthetic phage display library to screen for peptides that bind with high affinity to S protein. A complex of the optimal binder with S protein was found to have a T_m 10°C higher than that of the wild type complex.

2. Materials and methods

2.1. Materials

S protein and biotinylated S protein employed in the phage display and phage enzyme-linked immunosorbent assay (ELISA) experiments were obtained from Novagen, Bad Soden, Germany. Synthetic S15 phage peptide (S15p) was produced in the peptide synthesis service facility of the Center for Molecular Biology, Heidelberg, Germany, purified by high performance liquid chromatography and analyzed by mass spectroscopy for purity. The concentration of S15p was determined using an ε_{280} of 11.1 mM^{-1} cm^{-1} [8]. Preparation and quantitation of RNase S and S protein were carried out as described previously [9,10]. RNase A (type XII A), Subtlisin Carlsburg, cCMP and streptavidin were obtained from Sigma.

2.2. Phage display, S15p peptide and S protein purification

The 15-mer phage display library was an amplification of a library initially created by Nishi et al. [11] using the phage display vector fUSE5 [12]. The library was presented as fusion to filamentous phage XIII protein. The selection followed the basic procedures as given by Smith and Scott [13], differences are indicated in the following: all pannings were done in 96 well Maxisorp Immunoplates (Nunc, Wiesbaden, Germany), blocking was done with blocking buffer (10% (v/v) fetal calf serum, 0.1% (w/v) milk powder, 1% (w/v) bovine serum albumin, 0.05% (v/v) Nonidet NP40, 0.02% (w/v) NaN_3 in phosphate-buffered saline (PBS)). Washing was done eight times with PBS containing 0.5% (v/v) Nonidet NP40. Acid elution was done as described. For the first panning, 1 μg of S protein per ELISA well was coated overnight at 4°C in 0.1 M $NaHCO_3$. The phage library was applied 1:1 diluted in blocking buffer overnight at 4°C before acid

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Abbreviations: RNase A, bovine pancreatic ribonuclease; S20, residues 1–20 of RNase A; S15, residues 1–15 of S20; S protein, residues 21–124 of RNase A; RNase S, complex of S protein+S20 peptide; S15p, phage peptide selected from the phage display library; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; cCMP, cyclic cytidine monophosphate; CD, circular dichroism; ITC, isothermal titration calorimetry; $\Delta H°$, change in molar enthalpy due to complex formation; $\Delta G°$, change in molar free energy due to complex formation; ΔC_p, change in molar heat capacity; T_m, the temperature at which fraction of the peptide:protein complex is half of the total protein concentration

0014-5793/00/$20.00
PII: S0014-5793(00)01725-7

elution, neutralization and reinfection was done. For panning cycles 2–4, an indirect approach was employed: phages were incubated overnight at 4°C with 1 μg biotinylated S protein in 200 μl 1:1 blocking buffer. Phages binding the S protein were isolated by panning on microtiter wells coated with 1 μg/well streptavidin (Sigma, Munich, Germany) for 30 min at room temperature before acid elution was done as described. After the fourth panning, clones were randomly selected, picked and grown in microtiter plates. Culture supernatants were subjected to phage ELISA as described [14] using 1 μg/well S protein as antigen. Sequencing of fUSE5 clones derived from the library was done using the sequencing oligonucleotide primer 5′-CCCTCATAGTTAGCGTAACG with the T7 sequencing system (Pharmacia, Freiburg, Germany). Reaction products were separated on buffer gradient polyacrylamide gels.

2.3. Sequence alignment

A BLAST [15] search for RNase A homologues in the Swiss-Prot [16] database was carried out. One hundred sequences with an *E* score less than 10^{-5} were chosen out of ~85 000 sequences in the Swiss-Prot database. A BLAST search with S15p as the query sequence against this data set was carried out to pick the sequences of nearest neighbors of S15p in the RNase family. The sequence of S15p was aligned with its nearest neighbors from the RNase family using CLUSTAL W [17].

2.4. RNase activity and binding affinity measurement

The RNase activity was assayed by examining the hydrolysis of cytidine 2′-3′-phosphate (cCMP) to cytidine 3′-phosphate as described [18]. The assay was carried out at a fixed S protein concentration (15 μg/ml or 1.3 μM) and peptide concentrations ranging from 1.3 to 13 μM. Binding thermodynamics of S15p to S protein were determined by isothermal titration calorimetry (ITC) in the temperature range of 5–35°C [10,19].

2.5. Thermal denaturation studies

The concentration dependence of the thermal denaturation temperature was examined by circular dichroism (CD) spectroscopy. Thermal denaturation was monitored by measuring mean residue ellipticity at 222 nm using a Jasco J715 spectrophotometer interfaced with a Jasco PTC-348WI peltier device, in a 1 mm path length cell. The heating rate was 1 K/min. The S protein concentration was varied from 10 to 50 μM. In all cases, the peptide concentration was 10% higher than that of S protein. The experiments were performed in 5 mM acetate/10 mM NaCl, pH 6 (ITC conditions) and PBS: 1 mM KH_2PO_4, 8 mM K_2HPO_4, 3 mM KCl (panning conditions). The fraction of unfolded protein (fu) was calculated from the mean residue ellipticity by the standard relationship $fu = (\theta - \theta_N)/(\theta_U - \theta_N)$ where θ is the value of the mean residue ellipticity at any temperature, θ_N and θ_U represent the mean residue ellipticity of values at temperatures where the fully folded and fully unfolded states exit. In our case, fully folded state corresponds to the S protein:peptide complex and the unfolded state corresponds to complete dissociation of the complex to unstructured S protein and peptide.

3. Results and discussion

3.1. Random peptide phage display

Four rounds of panning on S protein were done with a random 15-mer library of peptides presented as an amino-terminal fusion to filamentous phage pVIII [11]. After the fourth round, 72 clones were randomly picked and subjected to phage ELISA on S protein. Of the 72 clones, 10 showed binding signals more than 10 times stronger than background. These 10 clones were sequenced. Four clones contained identical sequences, six clones contained multiple phage. The latter clones were retransfected for subcloning. In subclones of all those six clones, the same DNA sequence was identified again. The translated peptide sequence of the 10 inserts was NRAWSEFLWQHLAPV. The binding of phages presenting this sequence to S protein in ELISA could be competed by synthetic peptides representing the amino-terminal 15 amino acids of human or bovine RNase A (data not shown). We conclude that the screening of the peptide library resulted in the isolation of a highly specific binder for S protein.

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 1       ---QDR-MYQRFLRQHVD-PDETGGN---
 2       ---QDR-MYQRFLRQHVD-PDATGGNDA-
 3       ---QDG-MYQRFLRQHVH-PEETGGSDR-
 4       ---QDNSRYTHFLTQHYD-AKPQGRDDR-
 5       ---KDEDRYTHFLTQHYD-AKPKGRDGR-
 6       ---QDNYRYIKFLTQHYD-AKPTGRDYR-
 7       -----ETRYEKFLRQHVDYPKSSAPDSR-
 8       -----ETRYEKFLRQHVDHPRTLGLMGH-
 9       ----KESAAAKFERQHMDPSMSSASSSN-
10       ----KESAAAKFERQHMDPSPSSASSSN-
11       ----KESAAAKFERQHMDPSASSISSSN-
12       ----KESAAAKFERQHMDPSTSSASSSD-
13       ----KESAAAKFRRQHMDSGSSSSGNPN-
14       ----KETAAAKFERQHMDPAPAAAXXQN-
15       ------QNWAKFQEKHIPNTSNINCNTI-
16       ------QNWAKFQEKHIPNTSNINCNTI-
17       ------QNWAKFKEKHIRSTSSIDCNTI-
18       ------QDWSSFQNKHIDYPETSASNPN-
S15p     -----NRAWSEFLWQHLAPV---------
19       ----GVPTYQDFLYKHMDFPKTSFPSNAA
                   *  :*
S15      ----KETAAAKFERQHMDS-
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Fig. 1. Multiple alignment of S15p with N-terminal sequences of 19 closest homologues in the RNase A family. The sequences are (1) bovine BL4 RNase, (2) pig RNase 4, (3) human RNase 4, (4) human angiogenin, (5) bovine angiogenin, (6) mouse EF-5, (7) snapping turtle RNase, (8) chicken angiogenin, (9) fallow deer RNase, (10) rain deer RNase, (11) European elk RNase, (12) axis porcinus RNase, (13) roe deer RNase, (14) Virginia white tailed deer RNase, (15) Japanese sialic acid binding protein, (16) sialic acid binding protein, (17) bullfrog liver RNase, (18) common Iguana pancreatic RNase, (19) chicken RNase ('*' and ':' indicate fully and partially conserved residues, respectively). The N-terminal sequence of RNase A (S15) is also shown.

Most interestingly, Schultz et al. [20] obtained an identical S protein binding peptide from phage display. This probably occurred because the phage libraries employed in both studies were different amplifications from the same stock library with an initial complexity of about 2×10^8 created by Nishi and coworkers [11]. This indicates that the bottleneck in obtaining optimal binding peptides is not the screening procedure but the initial complexity of the original library. For peptides longer than 6–7 amino acid residues, this limitation results in a dramatic decrease of the fraction of all possible sequences which are physically represented in the real library. With a calculated complexity of 3×10^{19}, the probability for the occurrence of one particular 15-mer peptide sequence in the phage suspension used for panning is one to a trillion. Hence, the probabilities of finding the most optimal binder out of all possible sequences is extremely low in any given phage display library. What is selected is probably the most optimal binder of the sequences present in the primary *Escherichia coli* transformants after ligation of the random oligonucleotides into the fUSE vector. In the light of these observations, the conclusion of Schultz et al. [20] that phage display allows identification of the exact amino acids that tether specific protein subdomains together, and that this can be achieved for non-contiguous binding motifs using a 15-mer random peptide library, has to be taken with caution. Further, random peptide libraries offer new interaction motifs, so the result does not necessarily allow the identification of essential residues in the

original partner. For example, the X-ray crystal structures of an anti-p24 (HIV-1) monoclonal antibody Fab fragment in complexes with three different unrelated peptides demonstrated that although all peptides bind to the same site, the non-homologous peptides adopt different binding conformations and also form their critical contacts with different antibody residues [21]. There are two possible ways to technically overcome this restriction in the future. First, the library size could be increased. So far, the only possibility to achieve this is to avoid the step of bacterial transformations, which limits the complexity to about 10^{10}. For scFv antibody libraries, this has recently been demonstrated using ribosomal display, allowing the synthesis as well as the selection to be performed entirely by in vitro reactions [22,23]. A second possibility, allowing the use of established phage display protocols, might soon arise from the opportunity to generate surface expression libraries of helical peptides on the major coat protein of filamentous phage. Here, the theoretical complexity can be adjusted not to exceed the calculated complexity by keeping some amino acid positions constant, in particular at the face of the helix which does not contact the binding partner [24]. In this approach, however, it is a necessary to carefully evaluate how the structural and sterical restrictions imposed on the peptide affect the binding. In any case, keeping the physical library size as close as possible to the calculated complexity is required to obtain an optimal binding motif.

3.2. Sequence alignment and activity assay

The alignment of S15p with N-terminal residues of the closest homologues in the RNase A family of proteins is shown in Fig. 1. Residues F8, Q11 and H12 of RNase A are conserved in 99% of the members of the family including S15p (data not shown). It is important to note that Q11 and H12 are the catalytic residues [25,26], which are selected even though the peptide screening was performed based on binding affinity and not on activity. Earlier studies have shown that F8 and M13, two bulky hydrophobic residues, in RNase S contribute significantly to the stability of RNase S [27,28]. S15p has leucine in place of M13. Although Leu is not found at this position in RNase A homologues, earlier calorimetric studies [10] show that M13L mutation does not affect stability or activity. K7 and R10 of RNase A are proposed to be involved in binding of the RNA back bone during catalysis [29].

To analyze the interaction of the phage selected protein motif with S protein, a synthetic peptide (hereafter referred to as S15p) with the above sequence was employed for further studies. The activity of its complex with S protein was assayed using fixed concentrations of S protein and increasing concentration of peptides (Fig. 2A). For the wild type peptide S20, the rate of hydrolysis of cCMP by the complex increases steadily in a hyperbolic fashion indicating reconstitution of the wild type protein. The S15p:S protein does not show any activity even at a 10:1 molar ratio. The lack of activity may be due to replacement of Lys (K7 of RNase A) by Glu in the peptide (Fig. 1). Although K7 of RNase A is not part of the active site, it has previously been shown to be critical in binding negatively charged RNA molecules [29] and is con-

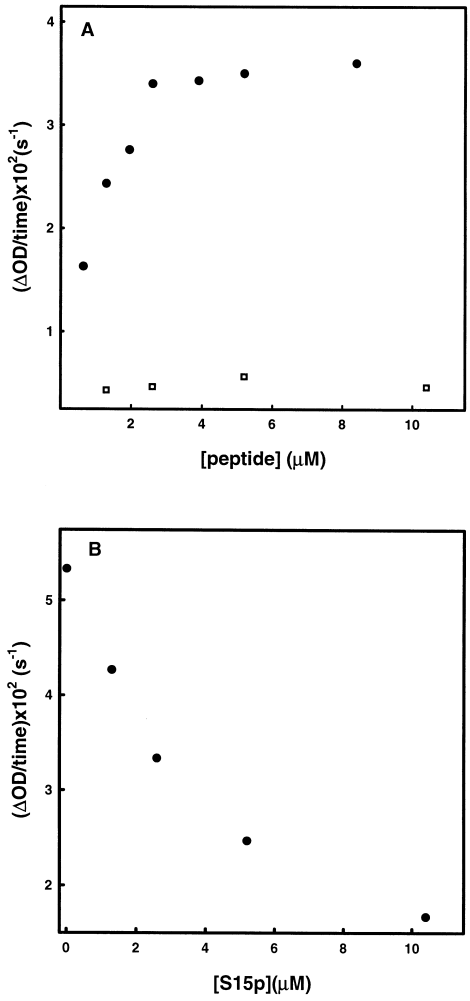

Fig. 2. Activity assay of the complex. A: Activity assay. The rate of change of OD_{284} due to the hydrolysis of cCMP (0.1 mg/ml of cCMP in 0.1 M Tris, 0.1 M NaCl, pH 7.1) as a function of time is shown for a fixed concentration of S protein (1.3 μM) and increasing concentrations of S15p and S20. Closed circles and open squares represent the data for S20 and S15p, respectively. B: Inhibition assay. The inhibition of RNase activity by the addition of S15p. The reaction was performed with 1.3 μM of S protein and 2.6 μM of S20 and increasing concentrations of S15p.

Table 1
Thermodynamics of S15p binding to S protein by ITC

Temperature (°C)	[S15p] (μM)	[S protein] (μM)	$K\times10^{-7}$	$\Delta G°$ (kcal/mol)	$\Delta H°$ (kcal/mol)
5.7	52	320	–	–	−3.10 ± 0.10
10.0	40	300	–	–	−4.13 ± 0.10
15.4	36	320	1.60 ± 0.70	−9.60 ± 0.25	−6.10 ± 0.10
20.0	50	500	1.30 ± 0.10	−9.60 ± 0.06	−11.51 ± 0.10
25.0	55	500	0.95 ± 0.13	−9.60 ± 0.07	−12.53 ± 0.10
30.0	49	460	0.66 ± 0.08	−9.50 ± 0.07	−22.05 ± 0.20
35.0	49	460	0.03 ± 0.01	−9.20 ± 0.08	−29.85 ± 0.30

served in 54% of the RNase A homologues. Only one member of the set of 100 aligned sequences (chicken angiogenin) has a negatively charge Asp at this position and is active [30]. Other notable differences between the S15p and typical RNase A sequences are two Trp residues of S15p. Trp4 of S15p is seen in 4% of the aligned sequences, all of which show catalytic activity [31]. Unlike S15p, none of the existing homologues contains a second Trp corresponding to position 9 of S15p.

An inhibition assay was performed to see if S15p binds to S protein at the same site, as does the wild type peptide. Constant amounts of S protein and S20 were equilibrated with increasing amounts of S15p before adding cCMP (Fig. 2B). Fig. 2B shows the drop in the rate of hydrolysis of cCMP with increasing concentration of S15p. S15p competes with S20 for the binding site on the S protein. This is consistent with the observation that most residues involved in contacting the protein remain unchanged. Enzymatic activity performed with RNA substrates also confirmed that S protein:S15p complex possesses negligibly weak activity compared to RNase S under similar conditions (data not shown). The S peptide is widely used as an affinity tag for purification of recombinant proteins by affinity chromatography against immobilized S protein [32]. One drawback is that it cannot be used if the protein of interest is a riboprotein or RNA binding protein. In contrast, S15p could be used in such cases as well.

3.3. Binding affinity measurement by ITC and thermal denaturation of the complex

The binding of S protein to S15p is an exothermic process (Table 1 and Fig. 3). Each molecule of S protein binds a single molecule of S15p. Since S15p binds tightly to S protein, estimates of $\Delta G°$ are unreliable at low temperatures. The temperature dependence of $\Delta H°$ in the range of 5–25°C was fitted to yield the heat capacity change on binding of peptide to S protein using the following equation as previously described [28].

$$\Delta H(T) = \Delta H^{\circ}(T_O) + \Delta C_p(T - T_O) \quad (1)$$

The ΔC_p and $\Delta H°$ are calculated to be -0.58 ± 0.08 kcal/mol/K and -12.80 ± 1.00 kcal/mol, respectively, for $T_O = 298$ K. The magnitudes of these parameters are significantly lower than corresponding values for the wild type peptide [28]. In an earlier analysis [20], a similar value of $\Delta H°$ of -10.4 kcal/mol at 293 K was reported, though no determination of ΔC_p or of thermal stability of the complex was carried out. A plot of the free energy of folding/binding as a function of temperature is known as the stability curve [33]. The curvature of the stability curve is expected to decrease and consequently the thermal denaturation temperature (T_m) is expected to increase with a decrease in the magnitudes of $\Delta H°(T_O)$ and ΔC_p. Experimental measurements confirmed that the T_m of the S protein:S15p complex is substantially higher than that of wild type RNase S (Table 2 and Fig. 4). Wild type RNase S contains the S20 peptide. The binding affinity as well as the T_m with this peptide are very similar to that of the S15 peptide whose binding has been characterized in more detail [10]. The thermal unfolding studies were carried out at pH 6 (under conditions identical to those used for titration calorimetry) and at pH 7 (under conditions similar to those used in the phage display selections). Since the binding reaction is a bimolecular process, T_m is concentration dependent and T_m increases as the concentration of the complex increases. However, the difference in T_m between S20 and S15p is relatively independent of concentration and varies from about 11 K at pH 6 to 7 K at pH 7. Since the structures of the RNase S complex and RNase A are identical, any mutation that increases the thermal stability of the complex should result in an increase in stability of RNase A. Attempts are currently underway to prepare an

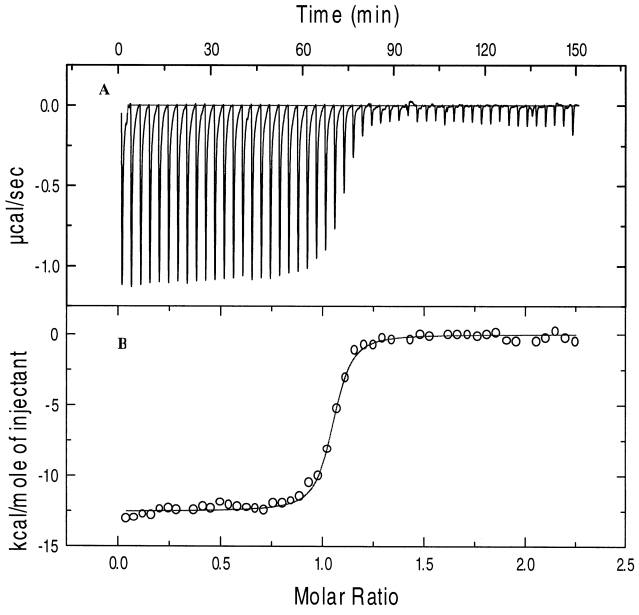

Fig. 3. Exchange of heat on binding of S15p to S protein. A: A representative isothermal calorimetric titration of 500 μM of S protein into 55 μM of S15p at 25°C 50 mM acetate/100 mM NaCl buffer, pH 6. B: Exothermic heats exchanged per mol of injectant as a function of mol ratio of S protein to S15p. The data were fitted to a single-site binding model to obtain $\Delta H°$ and K of -12.5 kcal/mol and 9.5×10^6, respectively.

Table 2
Temperature of thermal denaturation monitored by CD

Protein concentration (μM)	Wild type RNase S T_m (K)	S protein:S15p complex T_m (K)
Acetate buffer pH 6		
10	312	322
25	313	324
50	315	326
Phosphate buffer pH 7		
10	317	321
25	318	324
50	319	327

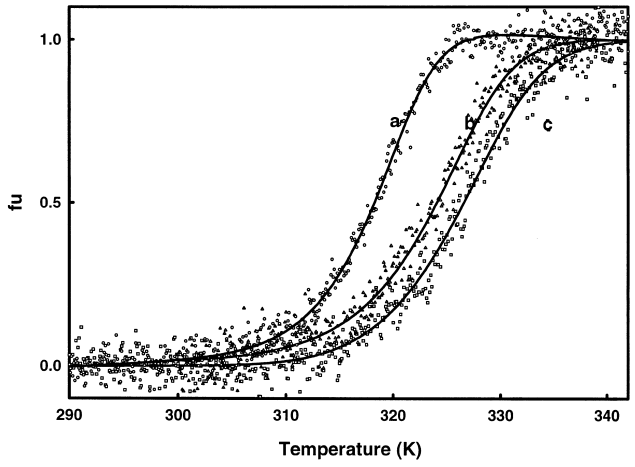

Fig. 4. Thermal melts of protein:peptide complexes monitored by CD at pH 7. The fraction of complex present is plotted as function of temperature. Curve a presents the 25 μM RNase S (empty circle). Curves b and c represent 25 μM (empty triangle) and 50 μM (empty square) of S protein:S15p complex, respectively. Data were fit as described [35].

RNase A derivative that contains the mutations present in the S15p peptide and to structurally characterize the mutant complex.

The present work shows that, despite the restrictions imposed by the library complexity as discussed above, phage display can be used to engineer substantial changes in the thermal stability of the complex where an efficient complementation system exists and where one of the partners is relatively small. In this situation, it can even be an advantage to employ completely random libraries instead of DNA shuffling and random mutagenesis since it is possible to obtain completely new stabilizing motifs containing a large number of sequence changes. In an elegant and detailed study, phage display was used previously to increase the thermal stability of a single chain antibody [6]. The approach involved DNA shuffling and exposure of phage to elevated temperatures followed by panning against the antibody ligand. Of several mutants obtained, one was shown to have an increase in T_m of 4 K. However, the methodology was quite involved, moreover that the protein of interest has a known ligand. In the present work, without employing a ligand, we have been able to achieve an increase in T_m of as much as 11 K by using conventional phage display combined with fragment complementation. It is now relatively straightforward to design complementation systems by engineering single Met residues into a protein of interest followed by cleavage with cyanogen bromide [34]. Hence the methodology used in this paper should be generally applicable to the stabilization of many monomeric proteins.

Acknowledgements: This work was supported by grants from DST and DBT (Government of India). We are very grateful to Dr. Ying Zhang for his kind gift of an aliquot of the 15-mer random peptide phage library and to Jutta Bergmann for her initial studies on phage ELISA. We are very grateful to E.K.F. Bautz for his continuous support and encouragement. We thank the Bioinformatics Centre and the SERC at the Indian Institute of Science for access to database and alignments.

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