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Misfolding-assisted Selection of Stable Protein Variants Using Phage Displays

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We describe a phage display strategy, based on the differential resistance of proteins to denaturant-induced unfolding, that can be used to select protein variants with improved conformational stability. To test the efficiency of this strategy, wild-type and two stable variants of α_1-antitrypsin (α_1AT) were fused to the gene III protein of M13 phage. These phages were incubated in unfolding solution containing denaturant (urea or guanidinium chloride), and then subjected to an unfavorable refolding procedure (dialysis at 37°C). Once the α_1AT moiety of the fusion protein had unfolded in the unfolding solution, in which the denaturant concentration was higher than the unfolding transition midpoint (C_m) of the α_1AT variant, around 20% of the phage retained binding affinity to anti-α_1AT antibody due to a low refolding efficiency. Moreover, this affinity reduced to less than 5% when 10 mg/mL skimmed milk (a misfolding-promoting additive) was included during the unfolding/refolding procedure. In contrast, most binding affinity (>95%) remained if the α_1AT variant was stable enough to resist unfolding. Because this selection procedure does not affect the infectivity of M13, the method is expected to be generally applicable to the high-throughput screening of stable protein variants, when activity-based screening is not possible.

Keywords: α_1-Antitrypsin, Phage display, Protein misfolding

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Introduction

The biomolecular engineering of proteins has accelerated at an unprecedented rate since the introduction of the directed evolution technique (Arnold, 2001; Kolkman and Stemmer, 2001). The most notable advantage of this random mutagenesis technique is that it does not require detailed structural information of a given biomolecule, which is essential for site-directed mutagenesis and computational design. Random mutagenesis, when combined with an appropriate screening system, often produces unique novel valuable mutations of interest. There are a number of successful examples of the identification of variants with improved functions, *i.e.*, higher thermostability (Giver *et al.*, 1998), higher tolerance to organic solvent (Chen and Arnold, 1993), altered substrate specificity (Oue *et al.*, 1999; Buchholz and Stewart, 2001), or inverted enantioselectivity (May *et al.*, 2000). However, screening methods based on the functional activities of individual clones are generally slow and laborious. Our interest was in the development of a high-throughput screening method that allows the rapid and laborsaving selection of stable protein variants from randomly mutated libraries.

The phage display technique is one of the most powerful approaches known for selecting and engineering peptides and proteins (Rodi and Makowski, 1999; Li, 2000). Jung *et al.* (1999) demonstrated that *Escherichia coli*-specific bacteriophage M13 shows remarkable stability against high temperature or protein denaturants like guanidinium chloride (GdmCl), which allows screening experiments by phage display to be performed under a wide range of chemical conditions. We investigated unfolding/refolding conditions capable of discriminating stability differences between fusion proteins without affecting the infectivity of M13 phage, and devised a more elaborate screening method based on the misfolding-assisted selection of stable protein variants in which stability is tunable (Fig. 1).

To determine whether the devised method is capable of discriminating between the different conformational stabilities

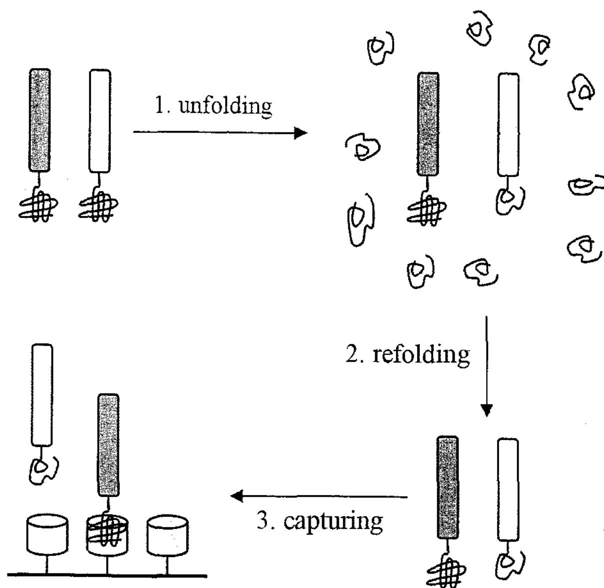

Fig. 1. Schematic diagram illustrating the misfolding-assisted protein selection process. Grey phage carries more stable protein variant than white phages. 1) During the unfolding process, phages are incubated with denaturing agent and misfolding-promoting additive (skimmed milk), which results in the unfolding of unstable fusion protein and additive. 2) During the refolding process, denaturing agent is slowly removed by dialysis at high temperature (37°C). 3) During the capturing process, only intact stable variants are recognized by binding protein.

of fusion protein variants, wild-type and two thermostable variants of α_1-antitrypsin (α_1AT), a prototype serine protease inhibitor (Whisstock *et al.*, 2000; Silverman *et al.*, 2001; Shin and Yu, 2002; Kang *et al.*, 2004), were displayed on M13 and their refolding efficiencies under the unfavorable refolding conditions were compared. We also investigated whether the devised system is capable of differentiating a highly stable conformation of α_1AT, *i.e.* a 'latent' form without biological activity (Erickson *et al.*, 1985; Hekman and Loskutoff, 1985; Mottonen *et al.*, 1992; Lawrence *et al.*, 1994; Im *et al.*, 2002). Our results show that the devised screening method is useful for selecting proteins with improved conformational stability in cases where activity-based screening is impractical because the target protein is not directly assayable, or when a ligand or an antibody that can discriminate variants is unavailable.

Materials and Methods

Plasmid construction and phage preparation. α_1AT gene in pFEAT30 vector (Kwon *et al.*, 1994) was subcloned into the SpeI/XhoI sites of pComb3H (Stoop *et al.*, 2001), which was then used to transform *E. coli* XL1-Blue cells. Phage production was carried out as described elsewhere with slight modification (Barbas *et al.*, 1991; Su *et al.*, 2003). Briefly, transformed cells were grown at 37°C in 50 mL of super broth medium (30 g/L tryptone, 20 g/L yeast extract, 10 g/L MOPS, pH 7.0) supplemented with 10 mg/mL tetracycline and 50 mg/mL ampicillin. When the cell OD_{600} reached about 0.4, 2×10^{11} pfu of M13K07 helper phage was added to the culture broth. After incubation for an hour, kanamycin (70 mg/mL) was added and the culture was incubated overnight. After centrifugation of the culture broth, phage particles in supernatant were precipitated with 0.25 volumes of polyethylene glycol (PEG) solution (20% PEG 8000, 2.5 M NaCl) and the phage pellets obtained were resuspended in 10 mL of phosphate-buffered saline (PBS; 50 mM potassium phosphate, 150 mM NaCl, pH 7.2). The resulting phage solution was stored at 4°C for further experiments.

ELISA. MaxiSorp ELISA plates (Nalge Nunc Int., Rochester, USA) were coated with 20 mL of 1 mg/mL anti-α_1AT antibody (Sigma Chemical Co., St. Louis) in PBS for 2 h at room temperature and blocked with 5% skimmed milk in PBS for an hour (Cho *et al.*, 2003). Phage solution in PBS (100 mL, 2.5×10^{10} cfu/mL) was preincubated with the same volume of 5% skimmed milk solution for 30 min and then added to the precoated ELISA plates. After incubation for an hour, plates were washed ten times with PBS containing 0.05% Tween 20 and five times with PBS. For detection, a commercial recombinant phage detection module (Amersham Biosciences, Uppsala, Sweden) was used.

Infection titer. *E. coli* XL1-Blue cells were cultivated in 10 mL 2xYT (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, pH 7.0) supplemented with tetracycline (10 mg/mL). Serial dilutions (10 mL) of phage solution in LB medium were added to 90 mL of *E. coli* XL1-Blue culture broth (OD_{600}~0.8) and incubated at 37°C for 20 min. Numbers of infected bacteria were measured by plating the solution on LB agar containing 50 mg/mL tetracycline and 50 mg/mL ampicillin.

Unfolding/refolding of α_1AT displayed on M13. For unfolding, phage solution (100 mL, 2.5×10^{10} cfu/mL) was incubated in skimmed milk (final concentration = 10 mg/mL) and denatured at 37°C for 3h. The solution was dialyzed overnight against 4 L of PBS containing 0.05% NaN_3 at 37°C. The dialysate was PEG-precipitated and the phage pellet obtained was resuspended in 100 mL PBS for ELISA. To convert α_1AT to its latent form, phage solution containing 0.1% NaN_3 was preincubated at 37°C for a week before the unfolding and refolding procedures (Lomas *et al.*, 1995).

Results

α_1AT can be functionally displayed on the surface of M13 phage. α_1AT was chosen as a fusion protein because owing mutational studies have well characterized its conformational stability (Lee *et al.*, 1996; Im *et al.*, 1999; Lee *et al.*, 2000). In addition to wild-type α_1AT, two similarly stable variants (M7 carrying seven hydrophobic core mutations and 366A/M7 carrying a F366A point mutation in a M7 background) were displayed on M13 phage. Both M7 and 366A/M7 mutations have been reported to increase stability versus the native conformation by 8 kcal/mol (Lee *et al.*, 1998; Im *et al.*, 2002). The 366A mutation, has also been found to facilitate

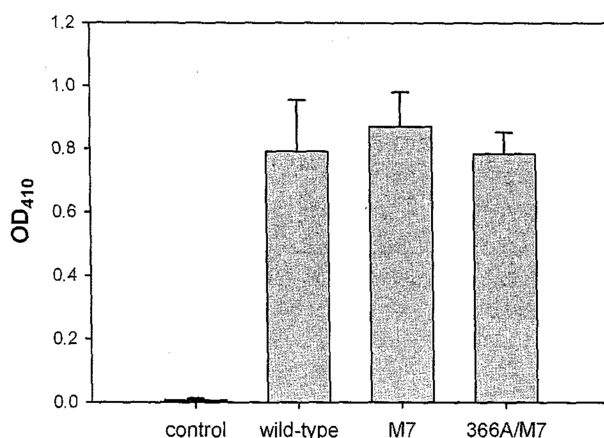

Fig. 2. ELISA signals for M13 phages displaying α_1AT variants. Control assays were carried out with M13 but without fusion protein. Phages were loaded at 2.5×10^9 cfu per well. Error bars represent the standard deviations of four separate phage preparations.

conversion to the hyperstable latent form (stability increase of 20 kcal/mol) upon prolonged incubation at physiological temperature (Im *et al.*, 2002). Anti-α_1AT antibody was used to capture phages displaying functional α_1AT and ELISA was performed using anti-M13 antibody conjugated with horseradish peroxidase. Much higher signals were detected from three M13 phages displaying α_1AT than from the control M13 without fusion protein (~0.8 AU at 410 nm compared to 0.01 AU; Fig. 2); the signal from control M13 was indistinguishable from the background signal. These findings demonstrate that all α_1AT variants were correctly displayed on M13 phage.

α_1AT-displaying phages are efficiently removed by unfolding/refolding treatment. All three α_1AT variants are completely unfolded in 6 M urea solution (Lee *et al.*, 1998; Im *et al.*, 2002), and consistent with this previous finding, we found that the ELISA signals of all three proteins greatly decreased when phages were treated with 6 M urea prior to refolding (Table 1). The ELISA signal reduction is attributed to the inefficient refolding of α_1AT to native form at the refolding conditions adopted. Compared to an efficient refolding procedure (*i.e.* fast dilution of unfolded protein solution in cold denaturant-free buffer), dialysis at 37°C induced more misfolding, presumably due to build-up of folding intermediates (data not shown). Table 1 also shows that the addition of skimmed milk to the unfolding solution was highly effective at inducing misfolding during the refolding procedure. When the phage was incubated in the unfolding solution without skimmed milk, around 20% retained binding affinity to anti-α_1AT antibody. In contrast, refolding efficiency reduced markedly to less than 5% when 10 mg/ml skimmed milk was included as a misfolding-promoting additive. Concomitant misfolding of the protein additive probably aggravates the misfolding of the unfolded fusion protein by forming heterogeneous aggregates (white protein aggregates were visible after the refolding process). These results demonstrate that harsh refolding conditions minimize the likelihood of the correct refolding of unfolded fusion protein.

Selection pressure has little effect on the infectious activity of phages. In the phage display system, information on target identification is retrieved from the phagemid encoding the target. Therefore, reinfection titer should be insensitive to selection pressure. It has been reported that the reinfection titer of M13 remains unaffected by overnight incubation even in 6 M GdmCl (Jung *et al.*, 1999), as was the case for our system. Moreover, dialysis at high temperature and high protein concentration (10 mg/ml) also had little effect on reinfection activity. Table 1 shows that reinfection titer was not significantly affected by unfolding/refolding procedures, whereas ELISA signals were greatly reduced after unfolding/refolding treatments. These results suggest that selection pressure operates almost exclusively on foreign fused protein, and not on viral capsid protein.

The screening system discriminates between the stabilities of α_1AT variants. We investigated whether the developed method is capable of discriminating stability differences between fusion protein variants (Table 2). Because the C_m values of the wild-type and M7 α_1AT are 0.7 and 2.0 M GdmCl (Seo *et al.*, 2002), respectively, and the C_m value of

Table 1. Reinfectivity and intact α_1AT presentation after unfolding/refolding treatments[a]

	Reinfection efficiency (%)		ELISA signal (%)[c]	
	- Skimmed milk[d]	+ Skimmed milk[e]	- Skimmed milk	+ Skimmed milk
Control[b]	97	98	-	-
Wild-type	90	93	17	3
M7	92	95	26	4
366A/M7	93	96	21	4

[a]The unfolding step was carried out in 6 M urea. Refolding was done by dialyzing the unfolding solution at 37°C.
[b]M13 phage without fusion protein.
[c]This represents the relative value of the signal taken with M13 phage before unfolding/refolding treatment.
[d]Unfolding/refolding was carried out without skimmed milk.
[e]Unfolding/refolding was carried out with skimmed milk (final concentration = 10 mg/mL).

Table 2. Stability of the α_1AT variants

Incubation[a]	Reinfection titer (%)	ELISA signal[b] (%)		
	Control[c]	Wild-type	M7	366A/M7
1.0 M GdmCl	98	7 ± 4	92 ± 7	90 ± 5
2.5 M GdmCl	95	4 ± 2	5 ± 4	7 ± 5
Latency induction and then 2.5 M GdmCl[d]	96	4 ± 2	7 ± 4	29 ± 3

[a]The unfolding step was carried out by incubating phage solution in 1 or 2.5 M GdmCl containing 10 mg/ml skimmed milk.
[b]Values are (means ± standard deviation) obtained from two or three separate experiments.
[c]M13 phage without fusion protein.
[d]Prior to unfolding, phage solution was preincubated at 37°C for a week.

366A/M7 is expected to be relatively similar to that of M7, only the wild-type is unfolded in 1.0 M GdmCl and all three variants are unfolded at 2.5 M. Consistent with these C_m values, only the wild-type showed a marked ELISA signal reduction (3% of the control) after unfolding treatment in 1.0 M GdmCl, whereas all three showed similar signal reductions (2~5%) when the concentration of GdmCl was increased to 2.5 M (Table 2). Reinfection titer was also found to be insensitive to unfolding/refolding procedures in the control experiment.

The 366A/M7 variant is expected to slowly convert into a latent form, which is highly stable to denaturing agent (C_m value of 3.4 M GdmCl) (Im *et al.*, 2002). Therefore, 31% of the ELISA signal of the 366A/M7 variant after a week of preincubation under latency inducing conditions is attributable to the accumulation of a hyperstable form resistant to the unfolding/refolding procedure (Table 2). The wild-type and M7 variant α_1AT presumably remained as intact native forms during this preincubation, and were later misfolded (3~4% ELISA signal) during the unfolding/refolding procedure (2.5 M GdmCl). Some portion of wild-type α_1AT may have converted into aggregate during the preincubation, however, this aggregate is indistinguishable from misfolded form that may have accumulated during the unfolding/refolding treatment and does not contribute to the ELISA signal.

Discussion

Protein refolding *in vitro* is usually prone to misfolding and aggregation, which reduces target protein recovery yields from inclusion bodies (Clark, 2001). Instead of adopting normal procedures optimized for high refolding yield, our selection strategy relies on the use of poor refolding conditions to promote the maximal misfolding of unwanted unstable proteins (Fig. 1). Protein variants vulnerable to a given unfolding pressure have a low probability of being correctly refolded to the native structure under unfavorable refolding conditions, e.g., dialysis in the presence of misfolding-promoting protein additive at high temperature. In contrast, a stable protein variant with a conformational stability that is high enough to survive an unfolding treatment, is not affected by unfolding/refolding procedures, and thus can subsequently be removed by utilizing a functional interaction between it and a binding protein. However, a robust protein display system that can tolerate harsh treatment is an essential prerequisite of the success of this strategy. Phage displays are ideal for this purpose owing to their higher stability to denaturing agents than cell-based display methods (Wittrup, 2001).

We describe a misfolding-assisted protein selection strategy based on a phage display approach. Jung *et al.* (1999) pioneered the application of denaturant-induced unfolding to the phage-display method for the selection of protein variants with improved stability. They treated a phage library with GdmCl and then diluted the treated sample before the next round of biopanning. In such a situation, selectivity largely depends on the irreversibility of target protein folding. This method could not be used for the selection of α_1AT variants because the rapid dilution of unfolded α_1AT in denaturant-free buffer usually causes efficient refolding (Kwon *et al.*, 1995; Lee *et al.*, 1996). However, dialysis at 37°C resulted in only about 20% refolding, and in the presence of 10 mg/ml of skimmed milk yieleded ,5% refolding (Table 1). By adopting harsh refolding conditions, we were able to increase selectivity by >20-fold (Considering <5% refolding recovery of the unfolded α_1AT variants *versus* the >95% ELISA signal from α_1AT variants that remain intact throughout unfolding/refolding treatment). Another advantage of our method is that it provides a convenient way of selecting variants with a stability greater than a certain criterion, because the selection pressure is easily adjusted by changing the denaturant concentration in the unfolding solution. For example, 1 M GdmCl can be chosen to screen variants that are more stable than wild-type α_1AT, and 2.5 M GdmCl is suitable for screening those more stable than M7 α_1AT (Table 2). Although this method cannot be applied to proteins whose stabilities are comparable to M13 coat protein, a large number of proteins (with free energies of unfolding in the range 5 to 15 kcal/mol) have been shown to unfold in urea or GdmCl at ≤6 M (Creighton, 1992). This suggests that the three proteins used in the present study were suitable as marker proteins and that our method widely applicable to real proteins. In addition, this method is generally suitable for selecting stable variants

stability that is high enough to survive an unfolding treatment, this method is generally suitable for selecting stable variants

with an altered folding property, and for which activity-based screening systems or conformation-specific monoclonal antibodies are unavailable, *e.g.* the latent form 366A/M7 α_1AT.

In summary, we have developed a phage display strategy, based on the differential resistance of proteins to denaturant-induced unfolding, to select stable protein variants with improved conformational stability. Our phage display method is expected to be generally useful for the high-throughput screening of stable protein variants obtained from randomly mutated libraries. The method should be particularly useful in systems not amenable to activity-based screening.

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