Selecting proteins with improved stability by a phage-based method

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We describe a method for the stabilization of proteins that links the protease resistance of stabilized variants of a protein with the infectivity of a filamentous phage. A repertoire of variants of the protein to be stabilized is inserted between two domains (N2 and CT) of the gene-3-protein of the fd phage. The infectivity of fd phage is lost when the three domains are disconnected by the proteolytic cleavage of unstable protein inserts. Rounds of in vitro proteolysis, infection, and propagation can thus be performed to enrich those phage containing the most stable variants of the protein insert. This strategy discriminates between variants of a model protein (ribonuclease T1) differing in conformational stability and selects from a large repertoire variants that are only marginally more stable than others. Because fd phage are exceptionally stable and the proteolysis in the selection step takes place in vitro a wide range of solvent conditions can be used, tailored for the protein to be stabilized.

Keywords: directed evolution, phage display, protein stabilization, proteolysis

For many applications proteins must be stable for a long time and under a wide range of conditions—in particular, at high temperature. These criteria are rarely met by the naturally occurring proteins from mesophilic organisms, and therefore general methods for increasing protein stability are of great interest. Efforts to stabilize proteins by site-directed mutagenesis have been successful only in a few cases because the principles of protein stability are not yet well enough understood, and because current prediction methods are not accurate enough to evaluate the consequences of mutations.

Evolutionary approaches provide an attractive alternative to search for stabilized variants of a protein in a large repertoire of sequences[1–3]. Direct selection methods have been successful in cases where the function of an essential protein could be linked to the survival of a microorganism (e.g., to the growth of a thermophilic bacterium in the presence of an antibiotic)[4]. In other cases, screening of single clones was required[3].

Proteins with specific binding functions can be displayed on the surface of a filamentous phage[5] as a fusion to the minor coat protein (gene-3-protein, g3p). In the presence of wild-type g3p, the display frequency of the fusion protein can depend on the folding properties of the displayed fusion partner[6], as an aggregation-prone protein is only poorly incorporated in the phage coat[6,7]. Consequently, selection for stability and folding yield via specific binding is possible[6,8–11].

We present a general method for protein stabilization by directed evolution that does not rely on specific properties of the displayed protein, such as binding to a ligand or enzymatic activity. This method links the proteoloysis resistance of the protein to be stabilized with the infectivity of a filamentous phage, which is a selectable property. In the native, folded state a protein is normally much more resistant to cleavage by a protease than in an unfolded state, and therefore conformational stability and proteolytic sensitivity are often inversely correlated[12–15]. The protein to be stabilized (the guest) is inserted covalently between the N2 and CT domains of g3p of the fd phage (the host protein) (Fig. 1A). g3p consists of three domains, N1 (68 aa), N2 (131 aa), and CT (150 aa), which are connected by glycine-rich linkers of 18 and 39 residues, respectively. The N1 and N2 domains are required for infectivity[16,17]. In the absence of N2, phage still infect cells, albeit with very low efficiency. Without N1, infection is impossible[18]. Therefore, the presence of N1 on the phage is a strong selection tool for propagation of the phage DNA.

This idea has been exploited previously in the selectively infective phage (SIP) technology[19] in which either N1 or N1–N2 are disconnected from the phage and fused to a ligand whose receptor is in turn fused to CT. Only upon cognate interaction does the phage become infectious.

In selecting for stabilized protein variants, we exploit the fact that infection requires a tight linkage between the domains of g3p, which can also be provided by inserted foreign proteins, as shown for β-lactamase[18]. Therefore, we introduce a repertoire of protein variants into g3p and subject the resulting pool of phage to an in vitro proteolysis step. The phage that host the most stable variants should escape proteolysis most frequently and thus remain infectious, leading to a simple selection procedure. By combining several cycles of in vitro proteolysis, infection and phage propagation phage hosting the most stable variants of a protein should become strongly enriched (Fig. 1B). We applied this procedure to select for stabilized variants of ribonuclease T1 (RNase T1) from a repertoire with partially randomized sequences.

Results

Stability of a guest protein and proteolytic inactivation of the host phage. The phage fd and M13 show a high resistance toward proteolytic inactivation by site-specific proteases such as trypsin, chymotrypsin, and GluC[20–22], a requirement for this selection procedure. After an incubation for 1 h with 2.5 μM trypsin or chymotrypsin (at 37°C, pH 7.0) or with 1 μM pepsin (at 15°C, pH 4.0) fd phage remained fully infectious.

Starting from a phage that hosted TEM1 β-lactamase (263 aa) between the N2 and CT domains of g3p[18] two other phage were

constructed that contained either CspB or RNaseT1(4A) at this position. CspB (67 aa) is a cold shock protein of *Bacillus subtilis*[23]. RNaseT1(4A) (104 aa) is an unstable variant of ribonuclease T1 that has all four cysteine residues converted to alanine and therefore lacks the two disulfide bonds. CspB (ΔG_{stab} = -12 kJ/mol, at 25°C, pH 7)[24] and TEM1 β-lactamase (ΔG_{stab} = -23 kJ/mol, at 25°C, pH 7)[25] are both in a folded state at 15°C, pH 7, whereas RNaseT1(4A) is unfolded (ΔG_{stab} = +8 kJ, at 25°C, pH 7).

Phage of all three variants as well as phage without guest protein were incubated with several proteases under different conditions (Table 1). For all three target proteins, trypsin with its narrow sequence specificity showed the lowest and proteinase K the highest proteolytic activity. Proteinase K also inactivates wild-type fd phage without a protein insert, but at a very low rate. These data show that various proteases can specifically degrade proteins that are inserted into g3p on the phage head. Proteolytic degradation is slow when the guest protein is in a folded state, but rapid when it is unfolded. The type and concentration of the protease, temperature, solvent conditions, and the duration of the proteolysis step can be varied in these experiments to obtain an optimal discrimination beween folded and unfolded forms of an inserted protein.

Table 1. Sensitivity of phage with different host proteins to different proteases.

Protease	Incubation	fd0	fdbla	fdcs	fd4a
0.8 µM trypsin	37°C, pH 7, 60 min	100%	30%	40%	0.1%
1.0 µM chymotrypsin	15°C, pH 7, 30 min	100%	8%	50%	0.001%
1.0 µM pepsin	15°C, pH 4, 20 min	100%	40%	60%	10%
1.0 µM proteinaseK	15°C, pH 10, 20 min	20%	0.5%	0%	0%

fd0: fd-phage without guest protein, fdbla: fd-phage with β-lactamase as guest protein, fdcs: fd-phage with CspB as guest protein, fd4a: fd-phage with RNase T1(4A) as guest protein

Single stabilizing amino acid substitutions in the guest protein usually do not change its overall conformational state from fully unfolded to fully folded. Rather, they lead to small increases in stability and thus to a slightly higher population of the folded state. We therefore modulated the stability of the guest protein by mild changes in the solvent conditions and by amino acid substitutions and examined whether the extent of proteolysis and thus the infectivity of the phage correlates with these changes in stability.

The stability of RNaseT1 can be fine-tuned by NaCl[26–28]. RNaseT1(4A) is almost completely unfolded in the absence of NaCl (at 15°C, pH 8.0), but a folding transition is induced when NaCl is added (Fig. 2A). The increase in the conformational stability of free RNaseT1(4A) is paralleled by an increase (400-fold between 0.3–1.1 M NaCl) in the fraction of phage with RNaseT1(4A) at the guest position that remained infectious after a 30 min incubation with 1 µM chymotrypsin (Fig. 2B). The extent of proteolysis of a guest protein in the fd phage and thus the infectivity seem to be linked with the conformational stability of this protein. The enzymatic activity of chymotrypsin is not affected by 0–2 M NaCl (data not shown).

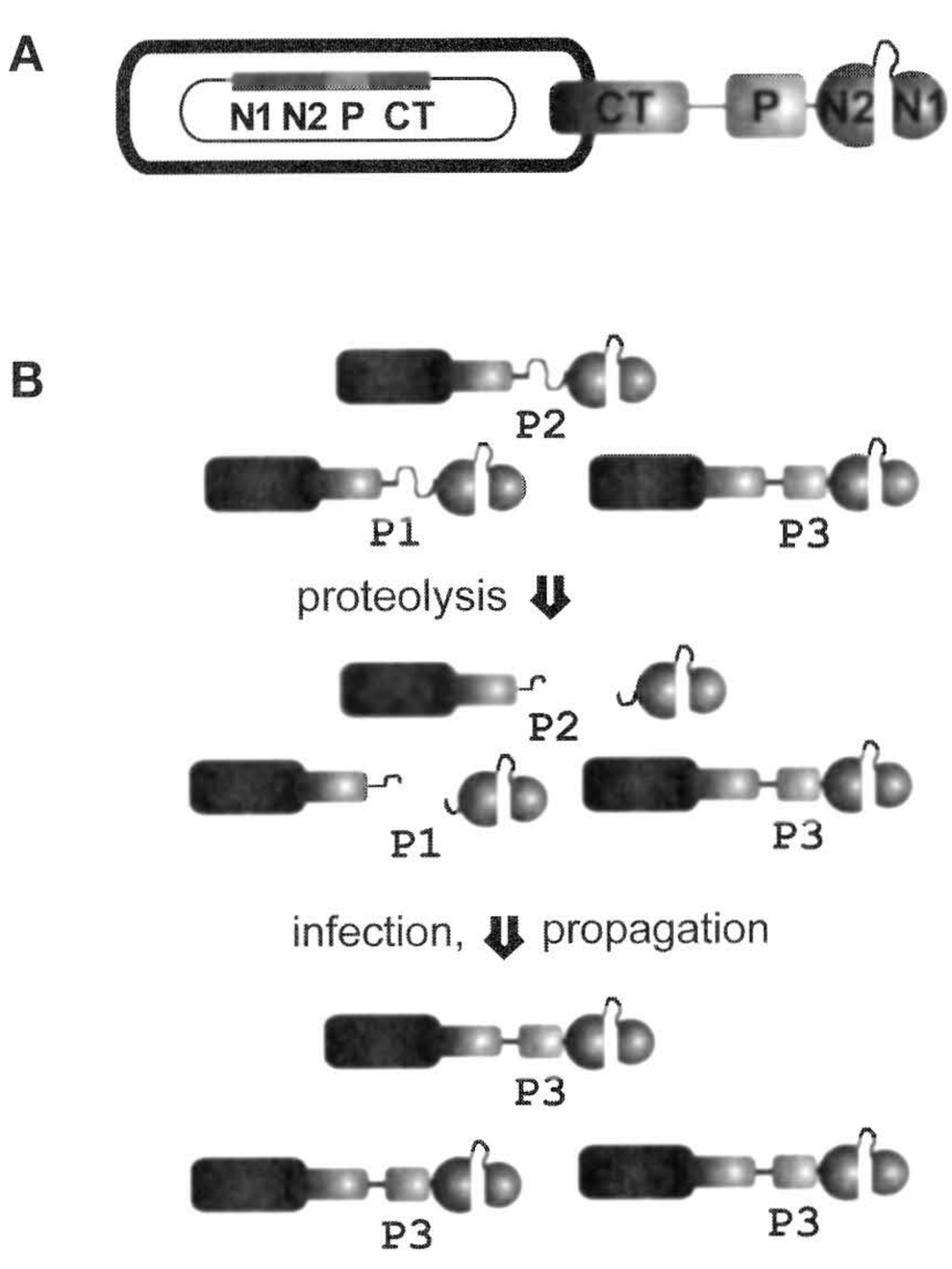

Figure 1. (A) Schematic drawing of a phage hosting a protein (P) between the C-terminal (CT) and the two N-terminal (N1, N2) domains of the gene-3-protein. (B) Outline of the selection procedure. Phage hosting different variants of an inserted protein (P1, P2, and P3) are subjected to a proteolysis step. Phage containing less stable variants (P1 and P2) lose their infectivity due to cleavage of the connection between CT and N2. Phage that host more stable variants (such as P3) remain infectious, can propagate, and are enriched.

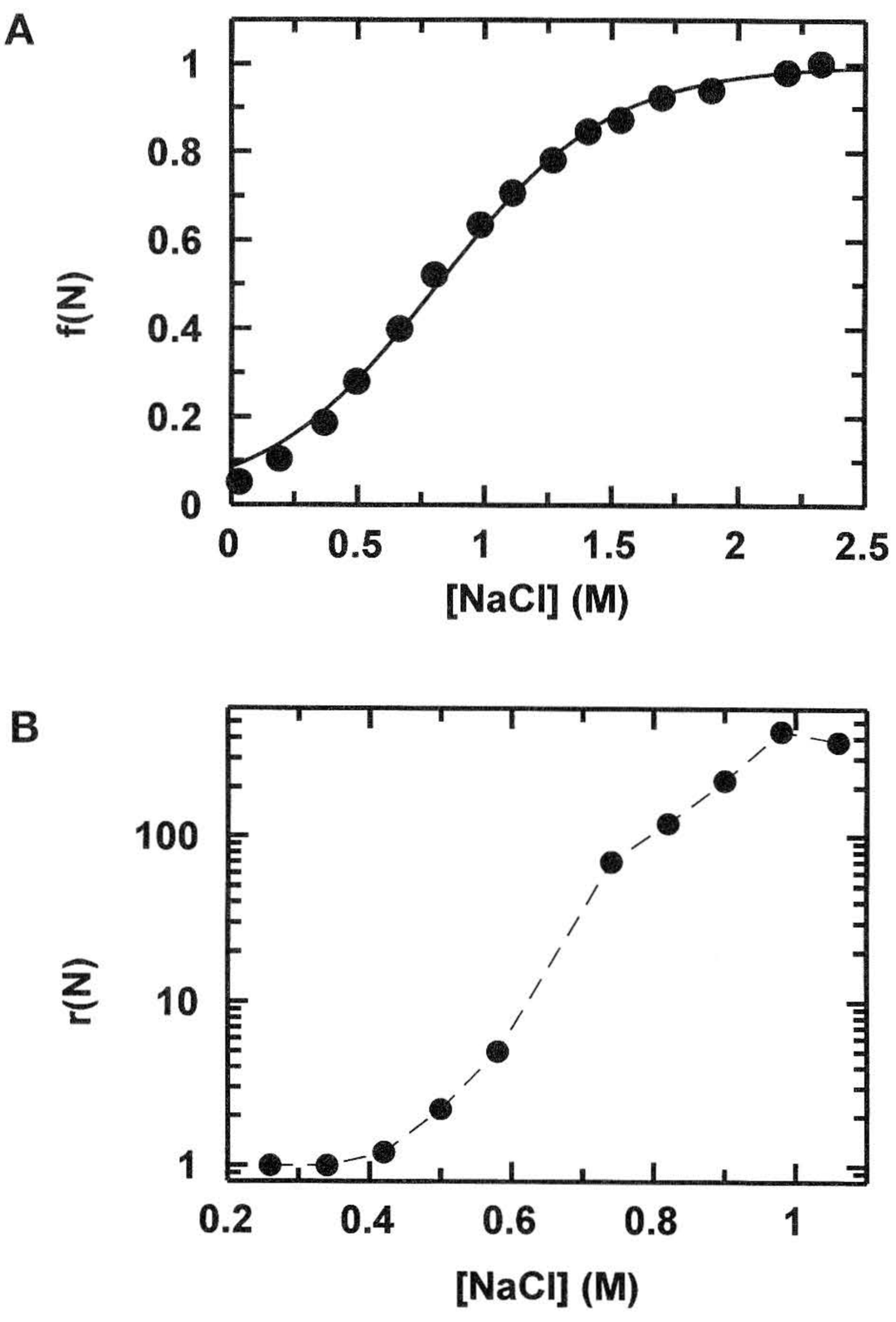

Figure 2. (A) NaCl-induced folding transition of 0.7 µM RNaseT1(4A) in 100 mM Tris/HCl, pH 8.0, 15°C. f(N): the fraction of folded molecules. The continuous line represents the fit of a two-state unfolding transition curve to the experimental data[45]. (B) Proteolysis of phage hosting RNaseT1(4A). r(N): the numbers of phage that were infectious after proteolysis.

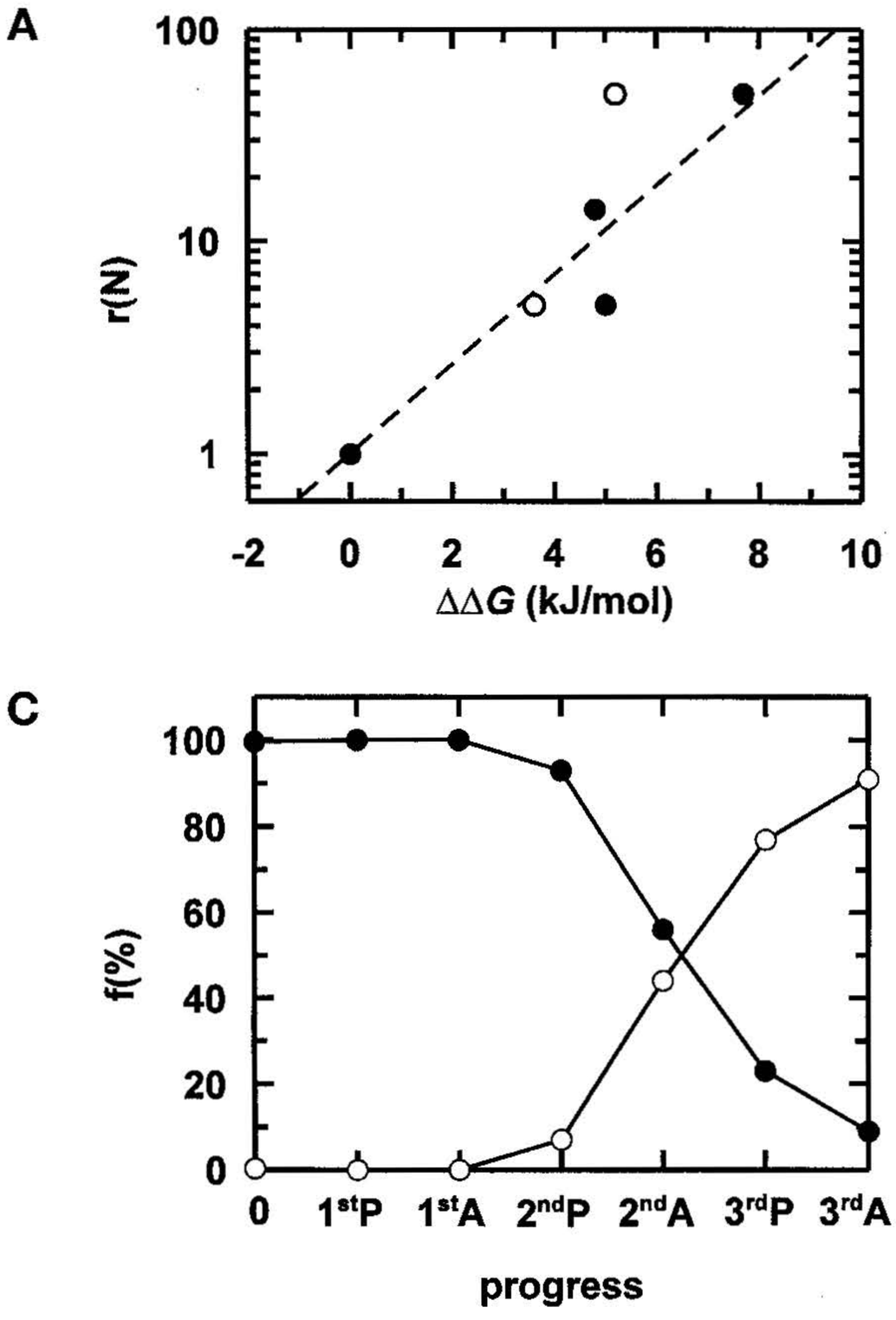

Figure 3. Proteolysis of phage hosting variants of RNaseT1(4A), with different conformational stabilities. (A) The relative number of phage that were infectious after proteolysis with chymotrypsin as a function of $\Delta\Delta G$, the difference in conformational stability between the variants and the wild-type protein in the presence (●) or in the absence (○) of the disulfide bonds. (B) The relative number of phage that were infectious after proteolysis with pepsin (▲), trypsin (△), and proteinase K (○), as a function of DDG, the difference in conformational stability between the variants and the wild-type protein in the presence of the disulfide bonds. (C) Three-round selection on a mixture of phage hosting 0.4% RNaseT1(4A)-3 (○) and 99.6% RNaseT1(4A) (●). The phage were treated with chymotrypsin, and bacterial cells were infected (first P). Infected cells were allowed to produce phage to amplify the selected DNA (first A). This cycle was repeated three times. The composition of the mixtures were analyzed by nested PCR.

Site-directed mutagenesis has identified several mutations that increase the conformational stability of RNaseT1 with intact disulfide bonds. The mutation Tyr42Phe increases the stability by 4.8 kJ/mol[29], Asp29Asn by 3.3 kJ/mol[30], and Ser17Ala by 2.6 kJ/mol[29]. Accordingly, three variants of RNaseT1(4A) were constructed: RNaseT1(4A)-1 (with the mutation Tyr42Phe), RNaseT1(4A)-2 (with Tyr42Phe and Asp29Asn), and RNaseT1(4A)-3 (containing the mutations Tyr42Phe, Asp29Asn, and Ser17Ala). RNaseT1(4A)-2 and RNaseT1(4A)-3 also contain the slightly destabilizing mutations Ser54Gly and Pro55Asn (about 3 kJ/mol)[31]. The contributions of these mutations to the conformational stability of RNaseT1(4A) are approximately additive in the presence and in the absence of the two disulfide bonds.

Phage hosting RNaseT1(4A) or the three variants were subjected to proteolysis with chymotrypsin and the numbers of phage that remained infectious were determined (Fig. 3A). These numbers correlated with the conformational stability of the respective displayed protein variant. Correlations were also observed when other proteases, such as pepsin, trypsin, or proteinase K were used (Fig. 3B). Together, these results suggest that the rates of proteolytic inactivation of the phage is reciprocal to the stability of the guest proteins.

Selection for stabilized variants of the guest protein. A mixture of 99.6% phage hosting RNaseT1(4A) and 0.4% phage hosting RNaseT1(4A)-3, which is stabilized by 7.7 kJ/mol compared with RNaseT1(4A), was subjected to proteolysis. Bacteria were then infected to propagate the resistant phage, and amplified phage were purified and subjected to the second proteolysis step. After each infection of bacteria the resulting colonies were analyzed by PCR to determine whether they contained phage hosting RNaseT1(4A) or RNaseT1(4A)-3 (Fig. 3B). The amount of phage with the more stable insert increased from 0.4% to 90% in three rounds of selection.

Phage hosting the more stable variant are enriched not only during the proteolysis step and the subsequent infection, but also during amplification of the bacteria. It is likely that the propagated phage are released into the medium during this phase and can infect new cells. Proteolysis of the inserted protein can occur by bacterial proteases in the periplasm of the cells as well as in the growth medium, which would also favor phage carrying the more stable variant.

To examine whether this procedure can find known combinations of stabilizing residues or more stable ones, a pool of phage that display a repertoire of variants of RNaseT1(4A) was created. By use of degenerate oligonucleotides and PCR we randomized positions 17, 29, and 42 of RNaseT1(4A) (only the introduction of cysteine residues and stop codons was excluded), and the repertoire of the mutated genes (with a theoretical diversity of about 2×10^4 at the DNA and 7×10^3 at the protein level) was introduced into the phage genome. The number of transformants (approximately 10^6) and the concentration of phage in subsequent steps (approximately 10^9–10^{10} cfu/ml) were high enough to ascertain a representation of more than 99% of the repertoire.

This pool of phage was used for the first round of proteolysis and infection of bacteria. To eliminate recombinant phage that were resistant to proteolysis because the guest protein had been deleted, the genes for the mutants of RNaseT1(4A) were amplified from the bacteria by PCR and reintroduced into the phage genome.

After the fourth round of proteolysis and infection 39 bacterial colonies were analyzed, of which 20 carried the guest protein. The 10 variants with the highest protease resistance were sequenced. No mutations outside the randomized positions were found (Table 2). One wild-type RNaseT1(4A) gene was found (number 1). All other sequences had a phenylalanine at position 42. The Tyr42Phe substitution is known to stabilize RNaseT1 by about 4.8 kJ/mol. At position 17, eight out of the nine recovered sequences had Ala, also a known stabilizing substitution (2.6 kJ/mol). With Leu one new

Table 2. Selection of stabilized RNaseT1(4A) variants.

#	Position 17		Position 29		Position 42	
	codon	AA	codon	AA	codon	AA
Wt	AGA	Ser	GAC	Asp	TAC	Tyr
1	AGA	Ser	GAC	Asp	TAC	Tyr
2	CTT	Leu	GCG	Ala	TTC	Phe
3	GCT	Ala	GAC	Asp	TTC	Phe
4	GCT	Ala	CGT	Arg	TTC	Phe
5	GCG	Ala	GCG	Ala	TTC	Phe
6	GCT	Ala	CTG	Leu	TTC	Phe
7	GCG	Ala	CTG	Leu	TTC	Phe
8	GCG	Ala	CTG	Leu	TTC	Phe
9	GCT	Ala	CTT	Leu	TTC	Phe
10	GCG	Ala	CTG	Leu	TTC	Phe

Codons and amino acids at positions 17, 29, and 42 of RNaseT1(4A) of the 10 phage that were analyzed after the fourth round of selection are listed. At each position a mixture of the codons (AC)(ACGT)(CGT) and T(TA)C and TGG was allowed.

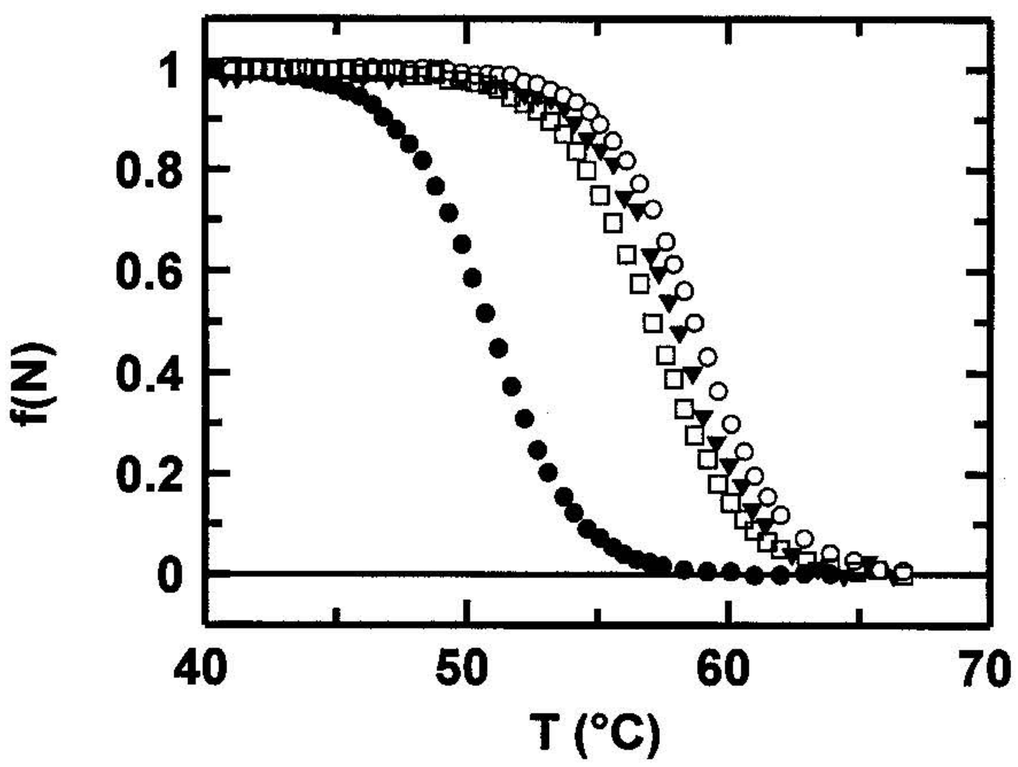

Figure 4. Stability effect on RNaseT1 (with intact disulfide bonds) of amino acid substitutions at positions 17, 29, and 42. f(N): the thermal unfolding transitions (displayed as the fractional change in signal) of 10 μM wild-type RNaseT1 (●), and the LAF (□), AAF (▼), and ALF (○) variants in 100 mM Na-cacodylate buffer, pH 7.0. Two-state analyses of the transitions[30] gave transition midpoints of 50.9°C for the wild-type protein and 57.1, 57.9, and 58.7°C for the LAF, AAF, and ALF variants, respectively. At 55°C the LAF, AAF, and ALF variants were stabilized by 9.5, 11.0, and 12.4 kJ/mol, respectively, relative to the wild-type protein.

stabilizing residue was found at position 17. Position 29 showed a higher variability. Five clones showed Leu at this position, two had Ala, one Arg, and one Asp as in the wild-type protein. The codon for this aspartate was GAC, not GAT that coded for Asp in the mixture of degenerate oligonucleotides. It is therefore likely that the GAC codon was introduced by a repair mechanism in *Escherichia coli* after transformation of the chimeric DNA. The previously known stabilizing substitution Asp29Asn was not found at all. The five variants with Ala17, Leu29, and Phe42 are encoded by three different sets of codons, which suggests that they were selected independently of each other.

To determine whether the selected sequences are stabilized variants of RNaseT1, we introduced three of them into wild-type RNaseT1. The conformational stability of the purified variants AAF (Ala17, Ala29, and Phe42), ALF (Ala17, Leu29, and Phe42), and LAF (Leu17, Ala29, and Phe42) were analyzed by reversible thermal denaturation (Fig. 4). All three mutants showed increased stability, ranging from 9.5 kJ/mol (LAF) to 12.4 kJ/mol (ALF). The most stable variant, ALF, is the one that was found most often, i.e., in five out of the nine new variants that were analyzed.

With yeast RNA and GpC as substrates all three mutants (AAF, LAF, and ALF) showed only about 50% of the activity of wild-type RNaseT1. This decrease in activity is caused by the Tyr42Phe mutation. The side chain of amino acid 42 participates in substrate binding in the active site of RNaseT1, and the mutation Tyr42Phe decreases the k_{cat} value by about 50%[32].

Stability of fd phage under various conditions. Phage were incubated for 1 h at 37°C at different pH values ranging from 1–14 and then analyzed for infectivity. Between pH 4 and 10, the phage showed little or no decrease in infectivity. At pH 2 about 40% and at pH 12 about 10% of the phage retained their infectivity. They also remained fully infectious after a 1 h incubation in 50% dimethylformamide (at pH 7, 37°C) or 5.4 M guanidinium chloride (GdmCl) at pH 7.4, 37°C. Filamentous phage also resist 6 M urea or reducing agents[33], are not susceptible to osmotic shock, and survive drying[22].

We could further increase the stability of fd phage at high pH by several rounds of mutagenesis and selection. RF-DNA of phage was transformed in XL1-Red cells[34] and phage were prepared. They were incubated for 5 h at pH 12 and used for the infection of XL1-Blue cells. Fresh RF-DNA was prepared from these cells and used for the transformation of XL1-Red cells. Six rounds of selection were thus performed. After the last round the resulting phage showed an about 10-fold higher infectivity after incubation for 3 h at pH 12, 37°C than the original phage. In all of the sequenced stabilized phage the Asn182-Gly183 sequence of the N2 domain of g3p was eliminated by an Asn182Ser mutation. All other mutations (in g3p, g7p, g8p, and g9p) were silent, leaving the amino acid sequences unchanged. Asn-Gly sequences are very sensitive to transamidation or cleavage, especially in alkaline solutions[35].

Discussion

We describe a rapid method for the selection of stabilized variants of a protein, by exploiting the relationship between the stability of a protein and its resistance to proteolysis. Proteolytic stability was linked with the infectivity of a filamentous phage. We call this method Proside, which stands for "*pro*tein *s*tability *i*ncreased by *d*irected *e*volution." Proside can select from a large repertoire variants that are only marginally more stable than others. In the selection procedure performed with RNaseT1 the variant that was found most frequently is only about 1 kJ/mol more stable than the second most stable one.

Proside is not based on specific properties of the protein to be stabilized such as ligand binding or biological activity. Thus, it should be generally applicable to globular proteins provided that a large repertoire of sequences can be generated, e.g., by a combination of error-prone PCR and gene shuffling[36–38]. The method requires that the inserted proteins do not abolish the assembly or infectivity of the phage and therefore probably is best suited for compact monomeric proteins. Large multidomain proteins or proteins with flexible, but essential regions are probably more difficult to stabilize by Proside. As the nature and concentration of the protease can be varied in the selection step, it may be possible to find suitable conditions for large proteins with many potential cleavage sites.

If the initial stability of a protein is already so high that it is a poor substrate for proteases, the inserted protein in the phage can be destabilized by raising the temperature or including a denaturant such as GdmCl in the selection step. Alternatively, destabilizing mutations can be introduced into the protein before the selection (suppressor mutation technique[39]). Effects of mutations are often approximately additive[40], and therefore the newly identified stabilizing mutations can be introduced into the original wild-type protein to obtain the stabilized final product as was accomplished with RNaseT1. The stabilizing mutations that were found in the

selection for the variant without the native disulfide bonds also stabilized the native protein with intact disulfide bonds (Fig. 4).

We believe that the sensitivity is due to the fact that the selection step (the proteolysis) mainly takes place in vitro. The conditions of this step can be varied in a controlled fashion and thus can be adjusted in an individual selection. The range of conditions is limited only by the demands of the protease used and by the stability of the phage. Apparently, the Proside method benefits also from an additional in vivo selection during phage propagation. Phage with stabilized protein inserts are probably more soluble, more resistant to intracellular proteolysis, and assemble more efficiently.

Filamentous phage are able to maintain infectivity after long periods of time outside a cell[22]. To stabilize a protein against denaturation by organic solvents, acidic or alkaline media, the selection can be performed by incubating the phage under these extreme conditions and then subjecting them to the proteolysis in a subsequent step. If the intrinsic stability of fd phage itself is still too low for a special application, it can be increased by several rounds of mutagenesis and selection of the phage alone under the respective conditions. By such a procedure we could increase the tolerance of fd phage toward alkaline pH. This alteration could be especially useful for enzymes that have to be stabilized for industrial processes. Proside may find general application in the study of proteins, and in tailoring proteins for practical applications.

Experimental protocol

Construction of phage variants. Experiments were carried out with fd phage fCKCBS[18], which contains a *cat* gene for selection and TEM1 β-lactamase as an insert between the N2 and CT domains of g3p. The genes for CspB, RNaseT1(4A), and their mutants were amplified by PCR and cloned into the SfiI restriction enzyme sites of this phage, thus replacing the gene for β-lactamase. *E. coli* XL1-Blue cells (Stratagene, La Jolla, CA) were transformed with phage DNA by electroporation and plated on dYTG^{cam} Agar (16 g/L yeast extract, 10 g/L trypton, 5 g/L NaCl, 1% glucose, 1% glycerol, 50 mM $MgCl_2$, and 25 mg/L chloramphenicol). Primers for PCR were 5′GCT CAG AGG GCC CAG CCG GCC GCC GCG GAC TAC ACT GC3′ and 5′CGA CTA GTG GCC CCC GAG GCC GTC GCT TCA ACG AAG TTG3′ for CspB (amplified from pCSPB3[23]) and 5′CGA CTA GTG GCC CCC GAG GCC GTC GCT TCA ACG AAG TTG3′ and 5′GCT CAG AGG GCC CAG CCG GCC GCC GCG GAC TAC ACT GC3′ for RNaseT1(4A) and derivatives (amplified from templates that were prepared by site-directed mutagenesis) in pA2T1[41]. All respective phage variants were verified by sequencing. Phage were prepared from *E. coli* XL1-Blue by polyethylene glycol precipitation[42] and stored in phosphate buffered saline containing 0.01% sodium azide.

Replacement of linkers. The identical stretches of 21 nucleotides immediately adjacent to the target gene insert, originally present in fCKCBS, were found to facilitate unwanted recombination, which in the case of the original β-lactamase insert could be suppressed by using antibiotic selection. We therefore replaced the linker that was located downstream of the insert. Using the Quik Change site-directed mutagenesis kit (Stratagene) and the mismatch primers 5′CCT GTC AAC GCT GGC GG3′ and 5′CCG CCA GCG TTG ACA GG3′, a new HincII restriction site was introduced at position 3715 of the phage genome. Phage-DNA was digested with HincII and EcoRI, which resulted in the removal of one of the two stretches. A new linker comprising the strands 5′AAT TCC CAG GTA CCC CGG TT3′ (plus strand) and 5′AAC CGG GGT ACC TGG G3′ (minus strand) was inserted. The correct sequence was verified by sequencing phage DNA.

Proteolysis of phage and titer analysis. In analytical proteolysis reactions the concentration of phage was generally adjusted to 10^9 cfu/ml. Proteolysis of different protein variants was performed with 0.8 μM trypsin at 37°C in 0.1 M sodium phosphate buffer, pH 7, for 60 min; with 1 μM chymotrypsin at 15°C in 0.1 M sodium phosphate buffer, pH 7, for 30 min; with 1 μM pepsin at 15°C in 0.1 M sodium citrate/phosphate/borate buffer, pH 4 for 20 min and with 1 μM proteinase K at 15°C in 0.1 M sodium citrate/phosphate/borate buffer, pH 10 for 20 min. Proteolysis of variants of RNase T1(4A) was performed with 0.25 μM chymotrypsin at 15°C in 0.66 M NaCl, 0.1 M potassium phosphate buffer, pH 7.5 0.4 M $CaCl_2$ for 30 min; with 10 μM pepsin at 15°C, in 0.1 M sodium citrate/phosphate/borate buffer, pH 4.0, for 15 min; with 8 μM trypsin at 15°C in 0.3 M NaCl, 0.1 M potassium phosphate buffer, pH 7.5 for 15 min, and with 0.01 μM proteinase K at 15°C in 0.4 M NaCl, 0.1 M sodium citrate/phosphate/borate buffer, pH 10.0 for 15 min. After the proteolysis, 10 μl of the reaction mixture were added to 490 μl *E. coli* XL1-Blue ($OD_{600nm} \approx 1.0$) in Luria-Bertani medium[42] and incubated at 37°C for 25 min for infection. The number of infected bacteria was determined by plating serial dilutions on dYTG^{cam} agar. For the selection performed with phage that hosted a mixture of variants of RNaseT1 the remaining 40 μl of the reaction mixture was added to 5 ml of *E. coli* XL1-Blue ($OD_{600nm} \approx 1.0$) in LB medium and incubated at 37°C for 30 min. Bacteria were then pelleted by centrifugation, resuspended in dYTG^{cam} medium, and grown for 6–8 h.

Test of salt dependence of the activity of chymotrypsin. Chymotrypsin was incubated at 15°C in 100 mM potassium phosphate (pH 8.0) at 0–2 M NaCl. The initial rate of hydrolysis was determined with 3 μM enzyme, 1.1 mM *p*-nitrophenylacetate by following the increase in absorbance at 404 nm and with 0.2 μM enzyme, 78 μM succinyl-Ala-Leu-Pro-Phe-4-nitroanilide following the increase in absorbance at 390 nm.

Analysis of the identities of the guest proteins RNaseT1(4A) and RNaseT1(4A)-3. *E. coli* XL1-Blue cells were infected with mixtures of phage hosting RNaseT1(4A) or RNaseT1(4A)-3 and plated on dYTG^{cam} agar. Between 32 and 48 colonies were individually resuspended and DNA was amplified by PCR using the primer 5′GCT TCA ACG AAG TTG TTA CC3′ and 5′CAC TGC CGG TTC TAA CG3′. Two microliters of each PCR mixture were used as template for a nested PCR with the primer 5′GCT TCA ACG AAG TTG TTA CC3′ and 5′CTC TGT GAG CTC TCC3′. The latter binds only to the gene of RNaseT1(4A), not to the gene of RNaseT1(4A)-3.

Random mutagenesis. Random mutations were introduced at the codon positions 17, 29, and 42 of the gene for RNaseT1(4A) by the method of overlap extension PCR (*Genesoeing*)[43] using the following oligonucleotides: n17a: 5′CTT CAG ACG TTX XXA CTG CTC AAG3′; n17b: 5′CTT GAG CAG TZZ ZAA CGT CTG AAG3′; n29a: 5′CTT CAC GAA XXX GGT GAA ACT G3′; n29b: 5′CAG TTT CAC CZZ ZTT CGT GAA G3′; n42a: 5′CCA CAC AAA XXX AAC AAC TAC G3′; n42b: 5′CGT AGT TGT TZZ ZTT TGT GTG G3′; rrandr: 5′GCT TCA ACG AAG TTG TTA CC3′; and rrandl: 5′CAC TGC CGG TTC TAA CG3′, where XXX represents a mixture of the degenerated codons VNK (coding for all amino acids except for Cys, Tyr, Phe, and Trp), TWC (coding for Tyr and Phe), and the codon TGG (coding for Trp). ZZZ represents a mixture of the respective opposite strand codons (MNB, GWA, and CCA). The primer rrandl was phosphorylated at the 5′ end. DNA-fragments were amplified by PCR using the gene of RNaseT1(4A) as template together with the primer pairs rrandl + n17b, n17a + n29b, n29a + n42b, and n42a + rrandr. After purification on agarose gels, about 1 pmol of each fragment was combined and used as template and primer to assemble the complete mutated genes of RNaseT1(4A) by PCR. About 2.5 fmol of the resulting product was amplified by PCR with the primer rrandl and rrandr.

Introduction of the mutated genes of RNaseT1(4A) into the phage genome. The PCR-amplified gene fragments (about 100 ng) of RNaseT1(4A) containing the random mutations were hybridized to the (+)-strand of phage hosting RNaseT1(4A) (80 ng), serving as a primer for the synthesis of the (-)-strand by Pfu-polymerase. The nicks were ligated, and remaining ss-DNA was digested by Mung Bean nuclease. *E. coli* XL1-Blue cells were transformed with the obtained ds-DNA chimeras (nonmutated and mutated RNaseT1[4A]) by electroporation and plated on dYTG^{cam} agar. About 10^6 transformants were obtained. After each selection round the RF-DNA of the mixture of phage that survived proteolysis was prepared and used as the template to amplify the genes of the selected variants of RNaseT1(4A) by PCR with the primers rrandl und rrandr. The product was reintroduced into the phage genome.

Production, purification, and characterization of RNaseT1 variants. By using the primers 5′GGC TCT TCC ATG GCA TGC GAC TAC ACT TGC GGT TCT AAC TGC TAC TCT TCT3′ and 5′GCG TGC GGA GAT CTC GGA GAC CCC ATT GTA CAT TCA ACG AAG TTG TTA CCA GAA GC3′ and single-strand DNA of phage hosting RNaseT1(4A)-AAF, -ALF, and -LAF as templates the genes for these three variants were amplified by PCR. These primers are coding for cysteine residues at positions 2, 6, 10, and 103 of wild-type RNaseT1. The PCR products were digested with PaeI and Bsp1407I, inserted into the expression vector pA2T1[41] by ligation and used to transform *E. coli* DH5α cells. Overexpression and purification of the proteins were performed as described[44]. The enzymatic activities and the conformational stabilities of the variants of RNaseT1 were analyzed by reversible thermal and NaCl-induced denaturation as described[30]. NaCl-induced unfolding was followed by fluorescence at 320 nm after excitation at 278 nm, thermal unfolding was followed by the change in differential absorption between 286 nm and 274 nm.

Test of phage stability. Phage without a guest protein (10^9 cfu/ml) were incubated for 1 h at 37°C in the following solvents. pH dependence: 100 mM CBP buffer (sodium citrate, borate, and phosphate) between pH 1 and pH 13 in intervals of 1 pH unit. DMF: mixtures of 100 mM potassium-phosphate, pH 7.5, 0.4 mM $CaCl_2$, and 0–70% DMF in 10% steps. GdmCl: 100 mM Tris/HCl, pH 7.4, and 0–5.4 M GdmCl in 0.9 M increments. With 10 µl of the incubation mixtures 490 µl *E. coli* XL1-Blue cells ($OD_{600nm} \approx 1$) in phosphate buffered LB (100 mM potassium-phosphate, 5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl) were infected.

Stabilization of phage against inactivation at alkaline pH. To increase the stability of phage in alkaline solutions mutagenesis of the whole phage genome combined with a selection procedure was performed. *E.coli* XL1-Red cells[34] (Stratagene) were transformed with about 150 ng of phage RF-DNA. XL1-Red cells are F^- and therefore transformation and not infection had to be used. Phage were prepared from these cells. Forty microliters phage suspension (about 10^{11} cfu/ml) were added to 360 µl 100 mM CBP buffer, pH 12, and incubated for 5 h at 37°C. *E. coli* XL1-Blue cells (5 ml, $OD_{600nm} \approx 1$) in phosphate buffered LB medium were infected with 250 µl of this phage suspension and incubated at 37°C for 30 min. Cells were then pelleted, resuspended in $dYTG^{cam}$ medium, and grown overnight. Phage RF-DNA was prepared and used to again transform *E. coli* XL1-Red cells. Six rounds of transformation, incubation, and infection were performed. After the sixth round phage of all steps were analyzed for their stability by an incubation at 37°C, pH 12 for 3 h. Of four of the stabilized phage the genes 3, 7, 8, and 9 were sequenced.

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