Toward development of a screen to identify randomly encoded, foldable sequences

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The ability to identify sequences in a randomly encoded polypeptide library that are capable of acquiring unique and stably folded structures would be valuable in the examination of protein-folding issues. The quality control system of the yeast secretory pathway prevents the release of incompletely folded polypeptides. Earlier work has shown that this feature can be used in a screen to identify mutations that increase the stability of a protein. We sought to extend this strategy for use with random sequence libraries by combining a quality-control system-based screen with generic tag-based immunodetection that can be applied to any sequence. To test this method, we screened a library encoding random mutations in a bovine pancreatic trypsin inhibitor variant containing a small generic tag. Initial on-plate screening resulted in a large number of false positives: sequences that were secreted but not foldable. These false positives were excluded successfully in additional screening steps that used a liquid-culture secretion screen and a gel electrophoresis assay. Three positive clones were obtained that showed midpoint thermal denaturation temperatures 10–16°C higher than the original bovine pancreatic trypsin inhibitor variant. Thus, this multistep screening method may be useful for finding novel, foldable sequences.

Protein sequences with the ability to form a unique, tightly packed, and well defined conformation (i.e., native structure) are referred to as "foldable sequences." The determinants required to define a foldable sequence are unknown. It would be interesting to identify foldable sequences from a randomly encoded polypeptide library, but satisfactory methods for identifying such sequences in large libraries are lacking. Available screening and selection methods typically are based on specific biochemical or structural features of the foldable sequence such as enzymatic activity, substrate specificity, or oligomeric state.

Work by Kranz, Wittrup, and coworkers utilizes a screening method based on the quality-control system of the yeast secretory pathway that has been shown to prevent the release of misfolded or incompletely folded proteins (1–4). The quality-control system uses resident chaperones in the endoplasmic reticulum such as Bip, GRP94, calnexin, and protein disulfide isomerase to discriminate between native and incompletely folded structures in a broad range of proteins (5–8). After posttranslational events, native or correctly folded proteins are secreted, whereas misfolded proteins are retained and degraded within the cells. Because secretion efficiency correlates with protein stability, this screening strategy can be used also to identify stable protein mutants (9–11).

The screening method used by Kranz, Wittrup, and coworkers relies on sequence-specific visualization with an antibody that recognizes the native structure of the target protein. To extend the utility of their screening method it would be useful to have a small, generic tag for detection, which could be applied to any sequence, that would allow for the screening of sequences without prior knowledge of the target sequence.

In this work we combined the aforementioned screening method based on the cellular quality-control system with a small, generic tag for detection and screened a library encoding random mutations in an unstable bovine pancreatic trypsin inhibitor (BPTI) variant. Although we were able to identify mutations that stabilized the unstable BPTI variant, our screen uncovered many false positives (i.e., mutations that passed the genetic screen but did not confer protein stability). Through subsequent use of a liquid-culture secretion screen and a gel electrophoresis assay, we were able to eliminate these false-positive mutants. Our results suggest that it may be feasible to use such a multistep screening method for identifying foldable sequences in random sequence libraries.

Materials and Methods

Construction of the Expression Plasmid and Random Mutants Library. *Saccharomyces cerevisiae* strain RY810556–20A (*MATa ura3-1 leu2-3,112 his 3-11,15 can1-100*) was a gift from G. Fink at the Whitehead Institute (Cambridge, MA). All transformations were performed with an E. coli Pulser (Bio-Rad). Signal sequences, prosequences, a multicloning site, and a Flag-tag sequence all were inserted sequentially into p415GALS to create p415GSSE (12, 13). A series of mutant BPTIs varying in thermal stability were cloned into this vector and transformed into *S. cerevisiae*. PCR-based random mutagenesis was carried out by using *Taq* polymerase (Hoffman–La Roche) in the presence of Mn^{2+} (14–16). To introduce a high number of mutations, multiple rounds of 20-cycle PCRs were carried out in 10 mM Tris·HCl (pH 9.0)/50 mM KCl/0.1% Triton X-100/0.5 mM $MnSO_4$/0.2 mM dNTP. Three pools of mutants were generated by using 7, 9, and 11 rounds of the 20-cycle PCRs and transformed into *Escherichia coli* XL-1 Blue (Stratagene). The total number of colonies exceeded 1.3×10^5. Approximately 60% of the colonies contained successfully ligated plasmids, and some of those plasmids were sequenced. We found that ≈70–80% of the sequenced plasmids had at least one mutation in the target sequence. Thus, the library was expected to have $\approx 6 \times 10^4$ clones with mutated 1-SS BPTI variant sequences. The sequences of randomly picked clones from the library showed that A-to-G and T-to-C mutations comprised ≈70% of the total mutations. These mutations were known to be predominant in PCR-based mutagenesis with Mn^{2+} in the absence of Mg^{2+} (15). No clustering of mutations was observed along the sequences, and each clone had three amino acid mutations on average.

Expression and Detection of Secreted Proteins. A large-scale library was screened in three steps based on secretion efficiencies and a simple biophysical assay for comparing molecular weights. The proteins were induced on plates or in liquid medium with 2%

Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; 1-SS BPTI, BPTI containing only the 5–55 disulfide bond with the mutations C14A, C30A, C38A, and C51A; 2-SS BPTI, BPTI containing the 5–55 and 14–38 disulfide bonds with the mutations C30A and C51A.

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Table 1. Midpoint temperature of thermal unfolding values (Tm) of wild-type BPTI and its variants

Protein	Mutations	Number of disulfide bonds	Tm, °C	Ref.
Wild-type BPTI	None	3	>90	22
2-SS BPTI	C30A, C51A	2	66	26
1-SS BPTI	C14A, C30A, C38A, C51A	1	39	25
1-SS BPTI (Y35A)	C14A, C30A, C38A, Y35A, C51A	1	31	19
1-SS BPTI (N44A)	C14A, C30A, C38A, N44A, C51A	1	15	19
1-SS BPTI (N43A)	C14A, C30A, C38A, N43A, C51A	1	<0	19

galactose. For on-plate immunodetection, plates of transformants were covered with a nitrocellulose membrane to transfer the secreted proteins after 3–4 days of induction. Cells stuck to the membrane were washed off by water without cell disruption. In this experiment, only secreted proteins were bound to the membrane. Proteins were detected by an anti-Flag M2 monoclonal antibody (Kodak) and chemiluminescence (Amersham Pharmacia). Obtained candidate clones were cultured in both synthetic complete and drop-out (SC) and natural liquid (YEP) media with 2% galactose instead of glucose as a carbon source for 2 days (17). The cultured synthetic and natural media were subjected to slot-blot analysis and 15% nonreducing SDS/PAGE, respectively. The M2 antibody assay was used also for the slot blot and Western blotting of gels.

Physical Analysis of the Mutants. The obtained candidates and original BPTI mutants were cloned into an *E. coli* expression vector, pAED4 (18). The cloned sequences were designed to have a start codon, residual amino acids from the prosequence, and an epitope tag. They were expressed in *E. coli* strain BL-21 Lys-S and purified by reverse-phase HPLC (19). The molecular weights of the purified proteins were measured by matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (PerSeptive Biosystems, Framingham, MA) and found to be correct in each case (±0.025%). Sedimentation equilibrium experiments were carried out on an XLA-90 analytical centrifuge (Beckman Coulter) at 30,000 and 38,000 rpm at 20°C. Circular dichroism spectra were collected with a J-720 (Jasco, Tokyo) at 20 and 30°C. Thermal unfolding was monitored by the change in ellipticity at 222 nm at 8 μM protein concentration. The buffers for both sedimentation equilibrium and circular dichroism contained 10 mM sodium-phosphate (pH 7.0)/150 mM NaCl/1 mM EDTA. A one-dimensional NMR experiment was performed on a DRX 500 (Bruker) at 20 and 30°C in 50 mM sodium-phosphate (pH 7.2) with 10% ^2H_2O. The concentration of protein in stock solution was determined by absorbance at 280 nm (20).

Results and Discussion

Secretion Efficiencies of Known BPTI Mutants. To determine whether the yeast quality-control system recognizes the foldability of a protein in our experimental system, we examined the secretion efficiencies of known BPTI mutants with different stabilities (see also refs. 10 and 11). BPTI, widely used to study the mechanism of protein folding (21), consists of 58 amino acids with three disulfide bonds at the 5–55, 14–38, and 30–51 positions. These bonds have been shown to be important for the stability of the native structure of BPTI (22–26). Here, the variant proteins are defined as follows: 1-SS BPTI, BPTI containing only the 5–55 disulfide bond with the mutations C14A, C30A, C38A, and C51A; 2-SS BPTI, BPTI containing the 5–55 and 14–38 disulfide bonds with the mutations C30A and C51A. In addition, we measured the secretion of 1-SS BPTI variants with point mutations at noncysteine residues. Yeast colonies expressing each of these variants grew to the same size. The amount of secreted protein, however, was highly variable and correlated with the thermal stability of the proteins (Table 1 and Fig. 1). This result confirmed the ability of the yeast quality-control system to recognize the foldability of proteins (9–11). We incorporated this system into our screening method to identify mutations in a large, randomized library (see *Materials and Methods*) that stabilize 1-SS BPTI.

1-SS BPTI is not secreted efficiently in this screen. Although 1-SS BPTI folds into a native structure at 20°C, at the optimum temperature for yeast growth (30°C) ≈10% of 1-SS BPTI is unfolded (25). This unfolded fraction, under equilibrium with the native state, may be continuously trapped and degraded by the quality-control system, resulting in the gradual disappearance of 1-SS BPTI in the cell.

Screening Strategy for a Large Library of Randomized 1-SS BPTI Mutants. We sought to identify mutations that stabilize 1-SS BPTI and therefore would be expected to result in more efficient

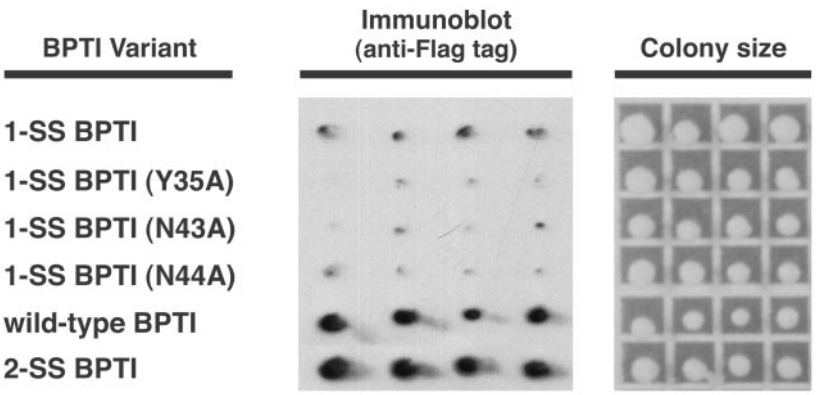

Fig. 1. Colony size and the amount of secreted BPTI mutants. Wild-type BPTI has all three natural disulfide bonds; 2-SS BPTI mutants have the 14–38 and 5–55 disulfide bonds; and 1-SS BPTI mutants have only the 5–55 disulfide bond. Point mutations such as N44A are shown. Although the size of each colony was almost identical, the amount of secreted protein detected by the M2 antibody differed.

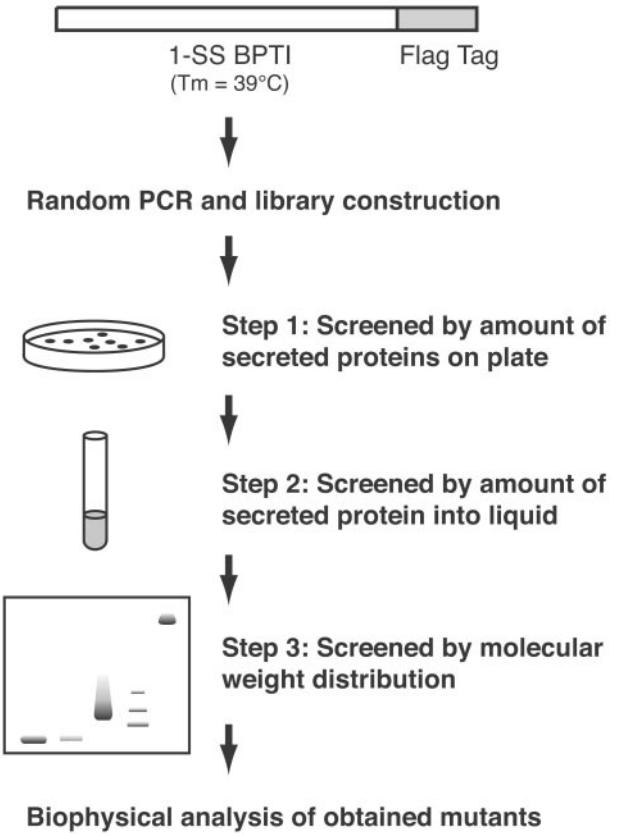

Fig. 2. A screening method for obtaining foldable sequences. The library of mutant sequences was made by PCR-based random mutagenesis of 1-SS BPTI. (Step 1) Secretion efficiencies of the mutants first were examined on plates. The colonies with the most intense signals were recovered. (Step 2) Obtained candidates were cultured in liquid, and the secreted proteins were assayed by slot blot. Clones with high secretion efficiencies in liquid culture, comparable with 2-SS BPTI, were selected. (Step 3) These positive clones were subjected to nonreducing SDS/PAGE analysis. Clones showing a single, dense, unsmeared band were selected.

secretion. We created a library containing randomized 1-SS BPTI variant sequences for this purpose. Because we found that the initial on-plate screening resulted in a large number of false positives, we introduced a multistep screening method to eliminate them (Fig. 2). Clones that secreted a large amount of protein onto plates and into liquid were selected at the first and second screenings. These selected clones were screened further by SDS/PAGE to identify proteins with a defined oligomeric state.

The total number of independent mutants in the library was estimated to be $\approx 6 \times 10^4$, calculated from efficiencies of mutation and ligation (see *Materials and Methods*). On average, each sequence contained three mutations. Transformed yeast were incubated on plates with galactose (Fig. 3). The growth of each colony was usually identical, with no discernible size differences. Approximately 1,000 colonies were observed on each plate, and colonies with the most intense signals were recovered ($\approx$10). Approximately 250 positive clones were obtained from $\approx$30,000 colonies in the first screening.

After the first screening, over 90% of the positive clones had a mutation at Lys-41. Although substitutions of this Lys with Glu, Arg, Asn, or Met were observed, the K41E mutation predominated and was observed in $\approx$70% of the positive clones, which can be explained by the observation that A-to-G and T-to-C mutations were favored over other mutations in PCR-based random mutagenesis by using Mn^{2+} in the absence of Mg^{2+} (15). Indeed, these mutations were dominant ($\approx$70%) in our library. A mutation from A to G at the first codon results in a Lys-to-Glu substitution. Substitution with Arg was the second most abundant mutation ($\approx$20% of the positive clones), in which the second codon of Lys was mutated from A to G. Mutations at Lys-41 were not found when nine negative clones were picked randomly from plates and sequenced. K41E and K41R were chosen for further experimentation, because K41E was the most abundant mutation and K41R was the least perturbed mutation. These mutants were expressed in *E. coli* and purified for determination of their thermal stabilities in circular dichroism studies. The stabilities of these mutants were not increased (unpublished data). We conclude that these mutations resulted in false positives at this stage of screening. The degradation of

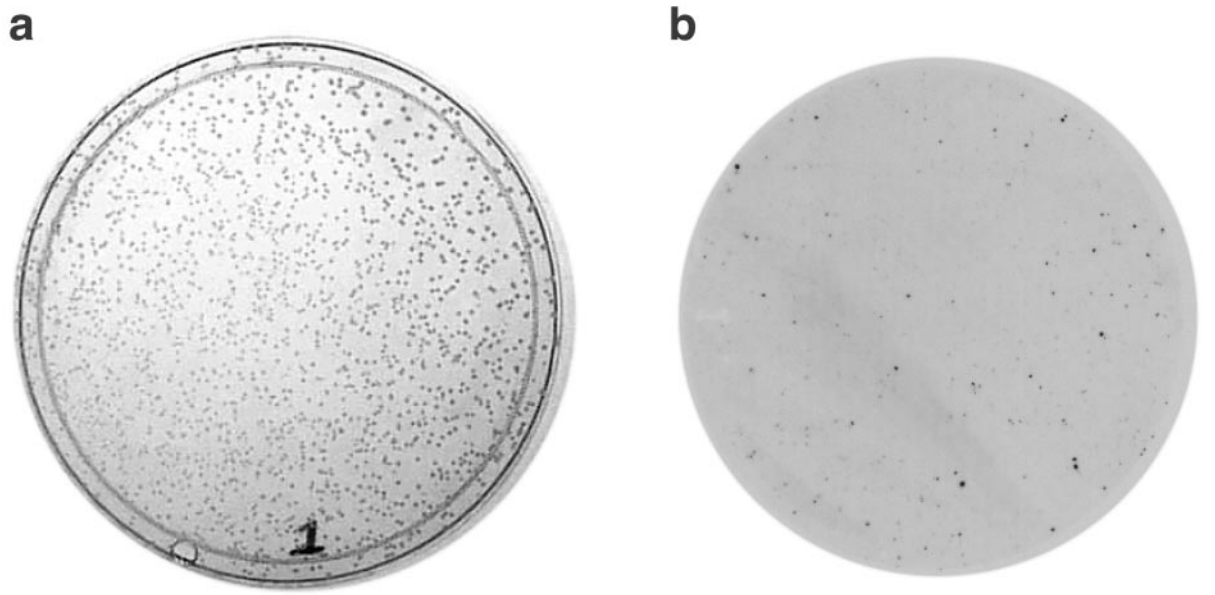

Fig. 3. First screening step of the randomly mutated 1-SS BPTI library. (*a*) Colony size was relatively uniform. (*b*) Result of immunodetection of the secreted BPTI mutants on the same plate. The expression of protein was induced directly on a plate with galactose. Approximately 10 colonies from each plate exhibiting the most intense signals were recovered for the next screening step.

misfolded proteins by the ubiquitin–proteasome system has been reported to be important for the quality-control system in the endoplasmic reticulum (27, 28). Thus, one possible explanation for the aberrant secretion of Lys-41 mutants would be the removal of an ubiquitination site, although it is unknown whether Lys-41 is ubiquitinated.

In an effort to overcome the problem of false positives, we screened positive clones from the first screen with two additional assays. Because an important overall goal for this work was to develop a method that permits screening of polypeptides with any sequence, the anti-Flag tag M2 antibody used for immunodetection in the first screening was used also in the second and third screenings. To evaluate the amount of protein more precisely, clones selected in the first screening were cultured in liquid induction medium, and samples of supernatant were transferred to either poly(vinylidene difluoride) or nitrocellulose membranes. Secretion levels of individual clones were assayed by immunodetection and compared. Most of the Lys-41 mutants showed lower signal intensities than the positive control (2-SS BPTI) and therefore could be identified as false positives. It is unclear why most of the Lys-41 false positives show low secretion levels in liquid culture. It is possible that the more quantitative assay at this step emphasizes differences in secretion efficiencies between the Lys-41 mutants and positive clones.

For the final step of the assay, we introduced a quick screening method based on a generic biophysical feature of the proteins. SDS/PAGE in nonreducing conditions was used to exclude species with abnormal molecular weight distributions caused by factors such as aggregation and/or truncation of the polypeptide sequence. In this screen, the clones showing a single, dense, unsmeared band were selected.

Screening for More Stable 1-SS BPTI Mutants. Following the screening strategy above, we obtained three positive clones (denoted C13, 148, and 199) from the randomized 1-SS BPTI library. In the first screening on plates, several strong signals were detected by immunoassay, and all the corresponding clones (≈250) were selected (Fig. 4*a*). The selected clones then were cultured, and the mutant proteins were expressed in liquid media. Supernatants of these media were subjected to slot-blot analysis. Fifty clones were chosen at this stage of screening based on the amount of secreted protein (Fig. 4*b*). These clones were analyzed by SDS/PAGE in nonreducing conditions and immunoblotted. In Fig. 4*c*, the band corresponding to clone 148 (solid arrow) was as dense as the positive standard, 2-SS BPTI (dotted arrow). Other than clone 148, clones C13 and 199 were the only clones with dense bands.

In the third screening, we found that ≈15% of the clones showed large and dense bands that were smeared (Fig. 4*c*, lane 6). Therefore, aggregation was also a source of false positives, which is interesting because aggregates generally are not secreted but rather accumulate within cells. The Z-allele mutation of α1-antitrypsin, which causes liver disease often leading to a fatal childhood cirrhosis, is a well known example of such a case, whereby large amounts of protein accumulate in the hepatocyte endoplasmic reticulum (29).

Characterization of Obtained Mutants. Mutant proteins that passed all three screens were expressed in *E. coli* and purified. The midpoint temperature of thermal unfolding values for C13, 148, and 199 were 49, 54, and 50°C, respectively, compared with 39°C for 1-SS BPTI (Fig. 5*a*). A one-dimensional NMR spectrum was measured for clone 148 (Fig. 5*b*). In the amide region the peaks are dispersed and qualitatively similar to those seen with wild-type BPTI, suggesting that clone 148 is folded correctly and has a structure similar to that of wild-type BPTI. All three mutants and 1-SS BPTI were confirmed to be monomeric by analytical ultracentrifugation (see the Fig. 5 legend).

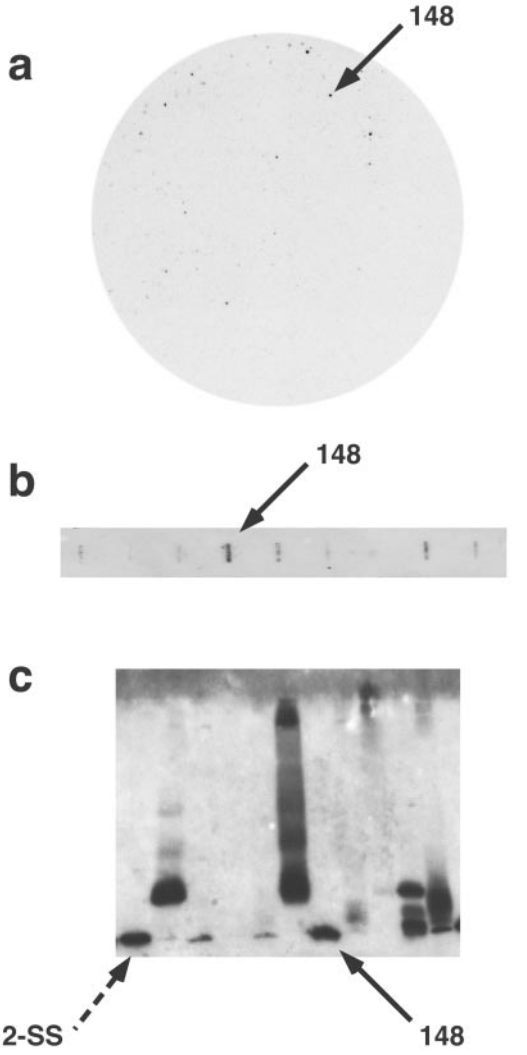

Fig. 4. Three-step screening method for selection of clones more stable than 1-SS BPTI. As a representative example, each step is shown for one positive clone: 148. The solid arrows in *a–c* point to signals from clone 148. (*a*) The first screening step was carried out in the manner shown in Fig. 3. (*b*) Selected clones were cultured and induced in liquid media. The amount of secreted protein from these candidate clones was evaluated by the slot-blot method. (*c*) Fifty clones were selected based on the intensity of signals from the secreted protein and assayed by SDS/PAGE and Western blotting. The supernatant of the culture from 2-SS BPTI, indicated by a dotted arrow, was used as a positive control. Positive clones were selected by their molecular weight and the amount of protein secreted. In all screens, the secreted protein, bound to nitrocellulose or poly(vinylidene difluoride) membrane, was detected by an M2 antibody against generic Flag tag.

C13, 148, and 199 all share a common mutation at position A14G (Fig. 5*c*). Residue 14 in wild-type BPTI is Cys but was mutated to Ala to remove the disulfide bond to make 1-SS BPTI. Because the only mutation in 199 is A14G, this mutation likely also contributes to the increased stability observed in C13 and 148. More work is needed to understand the origin of this increased stability.

Efforts to estimate the fraction of foldable sequences in large sequence libraries have met with mixed results. When sequences were biased to form a four-helix bundle and screening methods based on the expression efficiency of proteins in *E. coli* were used, it was estimated that 35% of the sequences formed a folded structure (30, 31). Yomo *et al.* (32, 33) found that 20% of random sequences consisting of 20 amino acid residues expressed in *E. coli* were soluble. However, these sequences formed oligomeric

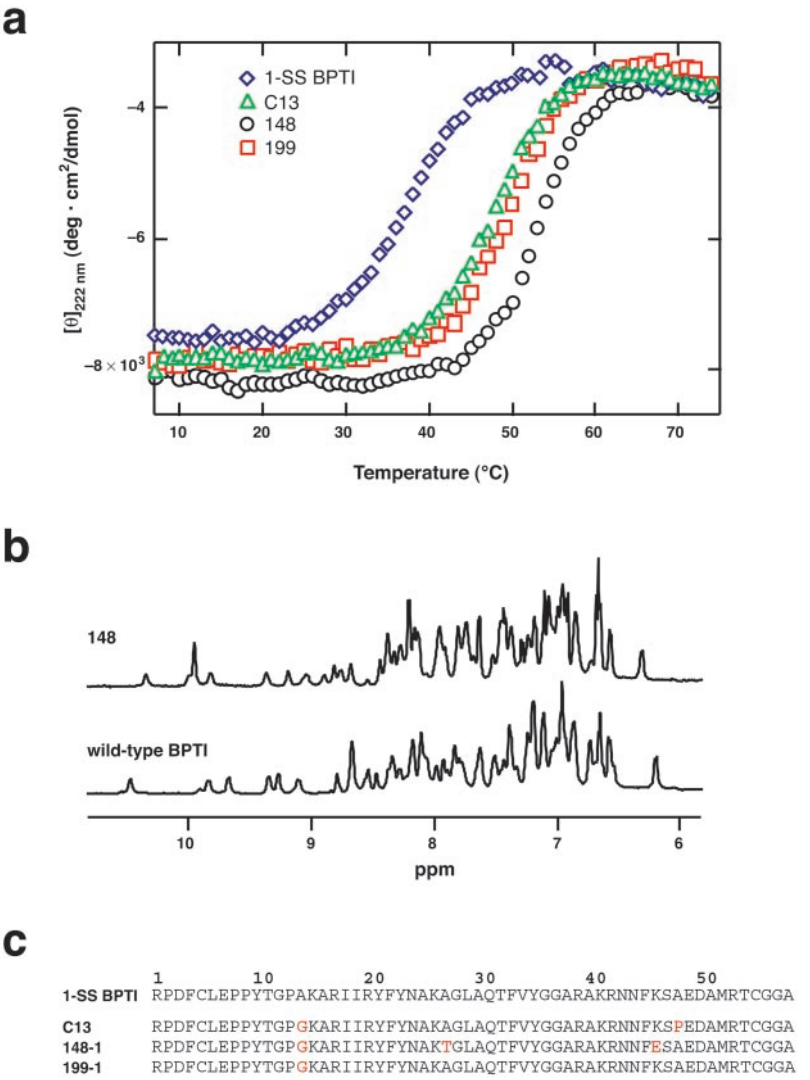

Fig. 5. (*a*) Thermal unfolding transitions of 1-SS BPTI and obtained mutants measured by circular dichroism (ellipticity at 222 nm) in 10 mM sodium-phosphate (pH 7.0)/150 mM NaCl/1 mM EDTA. All three mutants were >10°C more stable than 1-SS BPTI. (*b*) The amide region of a one-dimensional NMR spectrum of clone 148 and wild-type BPTI at 20°C in 50 mM sodium-phosphate buffer (pH 7.2). (*c*) The amino acid sequences of 1-SS BPTI and obtained mutants C13, 148, and 199. Mutated residues are shown in red. The ratios of molecular weight measured by ultracentrifugation compared with those expected for C13, 148, 199, and 1-SS BPTI were 0.97, 1.02, 0.88, and 1.05, respectively (M. Sakai and Y.H., unpublished results).

structures and did not have marked secondary structures. Screening of a library composed mainly of random combinations of Gln, Leu, and Arg revealed that 3–5% of the in-frame sequences were folded by the criteria of proteinase resistance, helicity, and resistance to chemical and thermal denaturation, although most of the obtained sequences were soluble only in the presence of denaturants (34, 35).

The multistep screening method we describe has the potential to identify foldable sequences without using specific biochemical characteristics of the target protein. The screening method described here also has other potential applications. For example, it offers an alternative to protein dissection for the identification of small, stable domains in large proteins. The conventional protein-dissection technique, which combines limited proteolysis with peptide mapping, is labor-intensive and requires significant amounts of protein. The screening method presented here could allow for quick identification of stable domains from libraries made by partial truncation of target genes.

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