Global Suppression of Protein Folding Defects and Inclusion Body Formation

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Amino acid substitutions at a site in the center of the bacteriophage protein P22 tailspike polypeptide chain suppress temperature-sensitive folding mutations at many sites throughout the chain. Characterization of the intracellular folding and chain assembly process reveals that the suppressors act in the folding pathway, inhibiting the aggregation of an early folding intermediate into the kinetically trapped inclusion body state. The suppressors alone increase the folding efficiency of the otherwise wild-type polypeptide chain without altering the stability or activity of the native state. These amino acid substitutions identify an unexpected aspect of the protein folding grammar—sequences within the chain that carry information inhibiting unproductive off-pathway conformations. Such mutations may serve to increase the recovery of protein products of cloned genes.

THE FOLDING OF POLYPEPTIDE CHAINS INTO THEIR NATIVE states requires the selection of one conformation out of an enormous ensemble of sterically available but incorrect conformations (*1*). During the in vitro refolding of polypeptide chains formation of incorrect aggregated states frequently competes with folding into the native conformation (*2–4*). Polypeptide chains synthesized within both prokaryotic and eukaryotic cells are also lost to aggregation, particularly at higher temperatures (*5–7*). One important function of the heat shock proteins is probably interaction with folding intermediates to chaperone them through the productive folding pathway by preventing aggregation (*8–10*). The expression of the protein product of cloned genes in foreign hosts often results in accumulation of the newly synthesized polypeptide chains in an aggregated nonnative state or inclusion body (*11, 12*). The few systematic studies on this subject suggest that aggregates are off-pathway polymeric structures derived, both in vitro and in vivo, from folding intermediates in the productive pathway (*13, 14*). An unanswered question is to what extent these aggregation processes reflect the folding instructions encoded in the amino acid sequence.

Mutational analysis of intracellular chain folding. Mutational and comparative sequence studies suggest that the residues determining chain conformation are dispersed throughout the sequence (*15–17*). At least two kinds of information are contained in these critical sequences. One kind includes residues stabilizing the native state (*18–21*). A second kind of sequence information probably ensures that the newly synthesized polypeptide chain forms and passes through the correct intermediate conformations (*22–25*). This includes both auxiliary sequences—such as propeptides, registration peptides, and signal sequences (*26*)—and residues within the mature chain (*27–29*).

We have been engaged in efforts to identify residues and sequences controlling the conformation of intermediates and perhaps also off-pathway steps, initially through the isolation and characterization of temperature-sensitive folding (*tsf*) mutations in the P22 tailspike protein (*6, 17, 27, 30*). The tailspike protein of *Salmonella* phage P22 is a homotrimer of three 666 amino acid chains (*31, 32*) whose secondary structure is dominated by β sheet, as revealed by Raman spectroscopy (*33, 34*). Although normally bound to the mature virion, native tailspikes accumulate as soluble structural proteins when capsid assembly is blocked by mutation.

As a result of a number of properties of the thermostable tailspike and of phage-infected cells, the cytoplasmic folding and aggregation pathway of tailspike polypeptide chains can be studied in vivo (*23, 27*). The pathway involves only conformational transitions with no known covalent modifications. Single chain and triple chain partially folded intermediates have been characterized in lysates of infected cells. Although the native tailspike is thermostable, the single chain intermediate is thermolabile and partitions between the productive pathway and nonnative, aggregated forms at elevated temperatures (*6, 27, 35*).

Chains synthesized at high temperatures can reenter the productive pathway if shifted to permissive temperature early enough (*6, 27, 30*). However, the aggregated chains are kinetically trapped and are not recoverable.

After denaturation of purified tailspikes with acid urea, the polypeptide chains can be refolded in vitro by dilution into physiological buffer (*36*). The in vitro refolding reaction is also sensitive to temperature with off-pathway aggregation dominating above room temperature.

Temperature-sensitive folding mutations prevent the newly synthesized chain within infected cells from reaching the native state at high temperatures (*30*). They interfere with the folding of the single chain intermediate, preventing it from attaining the conformation necessary for association into the protrimer intermediate (Fig. 1) (*27*). The mutant chains are not degraded, but aggregate into inclusion bodies (*6*).

The failure of the *tsf* mutations to reach their native state at high temperatures is not due to lowered stability or activity of the native state. Once the native state is attained at permissive temperature, the mutant proteins are as stable as the wild type when the temperatures are raised, with melting temperatures near 88°C (*34, 35, 37*). The physiological functions of the native forms of the mutant proteins,

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such as binding to phage heads, and adsorption to bacterial cells are not distinguishable from wild type (*35*).

Characterization of the native forms of the mutant proteins suggested that most *tsf* mutations are in sequences resembling those reported for turns and located at the protein surface in the native conformation (*17, 38*). These mutations may prevent correct β-turn and β-sheet formation in the critical folding intermediates at restrictive temperature (*38, 39*). Thus the *tsf* sites identify positions in the chain important for the folding pathway, but making only minor contributions to the stability of the native state.

Mutants defective in tailspike stability and head binding to the phage head have also been described by Berget and co-workers (*40*). They isolated a second site conformational suppressor that corrects such a defect in stability and head binding. This suppressor appears to alleviate the loss of a critical salt bridge in the NH_2-terminal region of the native protein.

Fane and King (*41*) isolated second-site suppressors which alleviated the folding defects conferred by missense and *tsf* substitutions. Fane *et al.* (*42*) found that two second-site mutations were repeatedly isolated; they are V331→A and A334→V (*43*). These two mutations suppressed absolute folding defects and temperature-sensitive folding defects at a number of sites in the chain, indicating that the suppressors operate through some global or general mechanism (*42*).

A well-characterized tailspike *tsf* mutant suppressed by the amino acid substitutions V331→A and A334→V in the tailspike protein is Tsf G244→R (formerly *tsfH304*; (*30, 44*). Synthetic peptides with the wild-type sequence form β turns in solution, while the mutant substitution decreases this tendency fourfold (*39*). To investigate the mechanism of the suppression we examined the intracellular maturation and the properties of the native state for Su A334→V:Tsf G244→R, Tsf G244→R, and Su A334→V chains.

Stability and activity of mature protein, carrying global suppressor substitutions. The growth curves of phages carrying suppressor mutations in their genomes were not distinguishable from growth curves of wild type, an indication that the amino acid substitutions did not interfere with the various functions of the native state of the tailspike. To examine these mutant proteins more carefully, we purified the native forms of the Tsf G244→R, Su A334→V: Tsf G244→R, Su V331→A and Su A334→V proteins (*38*). A native gel separation of the purified mature forms of the mutant proteins is shown in Fig. 2. Since the proteins carrying the Tsf G244→R substitution have an extra positive charge, they migrate more slowly through the gel (*38*). The presence of the suppressor mutation had no effect on the mobility of the native forms of the tailspike.

Table 1. Thermostability of the purified native forms of mutant and suppressor tailspike proteins. Melting temperatures were determined by differential scanning calorimetry in a DASM-4 microcalorimeter (*36*). Proteins were prepared in 20 mM potassium phosphate buffer, pH 7.4. A scan rate of 1 K per minute was used. The estimated uncertainty of the T_m measurement is ±0.3°C.

Protein	Concentration (mg/ml)	T_m/ °C
Wild type	3.5	88.0
Tsf G244→R	5.2	88.3
	1.04	88.7
Su A334→V: Tsf G244 →R	4.23	87.9
	4.23	88.2
	3.65	87.8
	2.19	87.7
Su A334→V	4.13	89.2
	4.13	88.2
Su V331→A	6.30	88.7
	4.42	88.75

The melting temperatures (T_m) were determined by scanning calorimetry as described earlier for the native trimeric forms of the wild-type and *tsf* mutant proteins (*37*); the *tsf* mutations by themselves had little effect on the T_m. The suppressor mutations have very little effect on the melting temperatures of the proteins, alone or in combination with Tsf G244→R (Table 1). These mutations alter neither the ability of the proteins to bind to phage heads nor their infectivity properties. Thus, the suppressor mutations do not have detrimental effects on the function and stability of the native product.

Effect of suppressor substitutions on polypeptide chain folding and aggregation. To determine if the suppression was occurring during polypeptide chain folding and association, we examined the intracellular synthesis, folding, and assembly of tailspike chains into the native SDS-resistant tailspike. For these experiments we constructed phage strains that carried amber mutations in gene *5* encoding the major coat protein and in gene *13* controlling lysis. In the restrictive *Salmonella* host cells infected with these strains the tailspike is one of the major polypeptide chains synthesized (*30, 45*).

We exposed infected cells to a 90-second pulse of ^{14}C-labeled amino acids in order to label newly synthesized polypeptide chains. The folding and assembly of the chains were stopped by rapid

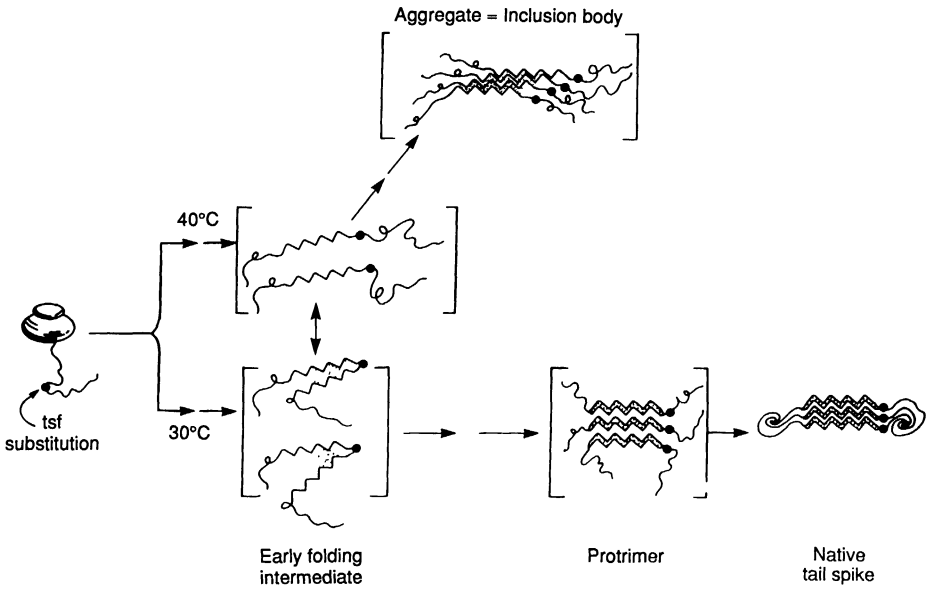

Fig. 1. Pathway of intracellular folding and association of the P22 tailspike protein carrying Tsf substitutions (*6, 23, 27*). Newly synthesized chains form a partially folded single chain intermediate which at permissive temperature proceeds to a conformation competent for specific chain-chain recognition. A "protrimer" species is formed, in which the polypeptide chains are associated but still incompletely folded. This folds further to the mature tailspike with concomitant acquisition of the resistance to SDS, proteases, and heat. The single-chain intermediates are thermolabile even for the wild-type chain, with only a fraction of the synthesized chains passing through the productive pathway at restrictive temperature (*31*). The *tsf* mutations, which probably affect turn formation, interfere with chain folding at high temperature before the chain association step. At permissive temperature, however, a large fraction of the mutant chains pass through the productive pathway to the biologically active native state. Once formed at the permissive temperature, the native states of the mutant proteins show similar activity and stability to those of the wild-type protein (*34–36*).

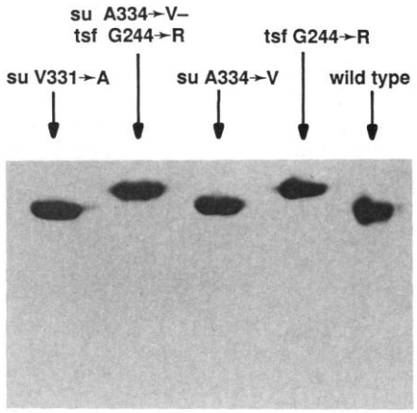

Fig. 2. Electrophoretic mobility of purified native Tsf, suppressor Tsf, and suppressor proteins. The purified native forms of the proteins produced at permissive temperature were subjected to electrophoresis, through a native gel. The protein concentration of all the applied samples was 1.1 mg/ml. The gels were stained with Coomassie blue. The suppressor:Tsf mutant protein (A334→V: G244→R) retains the electrophoretic mobility of the Tsf G244→R mutant. The suppressor proteins V331→A and A334→V display the wild-type electrophoretic mobility.

chilling, and the concentrated cells were frozen and thawed at 4°C to promote lysis. The resulting lysates were fractionated by centrifugation. The sediment and supernatant fractions were assayed for tailspike chains by SDS–gel electrophoresis without prior heating of the samples. Under these conditions, the mature trimeric tailspike remained native in SDS gels, whereas the ensemble of folding and assembly intermediates dissociated into complexes of SDS and polypeptide chains (*31*). The tailspike chains that sedimented at low speed and were dissociable in SDS without heating are defined as the aggregated inclusion body state (*6*). These procedures distinguished three classes of newly synthesized tailspike species, namely, soluble partially folded intermediates, aggregated inclusion body forms, and SDS resistant native trimers.

The rates of synthesis of mutant and wild-type chains were similar in the lysates. The wild-type chains (Fig. 3, lower right) formed soluble partially folded intermediates, which partitioned between native tailspike and aggregated chains. At 39°C, the temperature of this experiment, about 35 percent of the chains reached the native state, while the remaining 65 percent ended up in the aggregated inclusion body state.

In the infected cells with the *tsf* mutant (Fig. 3, upper left) at restrictive temperature, a soluble partially folded species formed, but this species failed to mature to tailspike trimers. All of the chains aggregated into the inclusion body state (*6*).

The presence of the Su A334→V substitution altered the behavior of the Tsf chains. Thirty five percent of the double mutant chains matured to the native tailspike, approaching what was found with the wild type. The aggregated fraction was correspondingly less (Fig. 3, upper right).

Chains carrying the suppressor alone (Su A334→V) matured more efficiently than wild type, yielding 60 percent of the chains in the native conformation compared with 35 percent for wild-type chains. Again, this increased efficiency appears to result from the decreased fraction of aggregated chains (Fig. 3, lower left). The kinetics of the disappearance of the soluble folding intermediates was similar for the wild type, suppressor, *tsf*, and suppressor:*tsf* infected cells. The suppressor mutations seem to act by altering the partition of the soluble intermediate between the aggregated state and the productive pathway. At the permissive 30°C temperature, 60 percent of the Tsf chains matured to native tailspikes, while more than 90 percent of the wild-type, suppressor, and suppressor:Tsf chains matured to the native state. Qualitatively similar results were found with the Su V331→A suppressor and Tsf G244→R, and with both suppressors combined with a different well-characterized *tsf* mutation, Tsf E309→V (*38*).

The *tsf* mutant chains do not reach the protrimer species at restrictive temperature and thus appear to be blocked before chain-chain association (*27*). We examined the ability of chains carrying only the suppressor substitution to rescue other chains carrying the Tsf substitution. Cells were coinfected with phage strains carrying separately the Tsf G244→R and Su A334→V substitutions. The altered electrophoretic mobility of Tsf G244→R and the wild-type mobility of Su A334→V made it possible to distinguish the formation of mutant, suppressor, and hybrid trimers in a mixed infection experiment (*31, 38*). At permissive temperature, both strains formed native homotrimers, as well as hybrid trimers (Fig. 4).

At restrictive temperature only suppressor trimer formation was observed, indicating that chains carrying suppressors could not rescue other chains carrying Tsf substitutions. The simplest explanation for this is that suppression occurred through the monomeric folding intermediate, prior to chain-chain association.

Mechanisms of inclusion body formation. Although aggregation has often been considered a nonspecific process, the experimental evidence from both in vivo and in vitro studies indicate otherwise; aggregates are formed from specific partially folded intermediates (*2, 3, 13, 14, 46*). Thus, the interactions in the aggregate are likely to resemble intrachain interactions between units of secondary structure found in the folded proteins. For example, in gelatin the bonds holding the chains together are locally triple helical as in native collagen, but the chains are out of register (*47*). Our data establish that a single, relatively subtle amino acid substitution can inhibit the intracellular aggregation of the tailspike folding intermediates. These data confirm that the steps leading to inclusion body formation involve specific regions of the amino acid sequence.

Aggregation of the tailspike chains may well involve interactions between specific sites on the folding intermediates. In that case the suppressor substitution may be disrupting these interactions directly, thus identifying the aggregation site on the folding intermediate. Alternatively, the substitutions may be influencing the aggregation process through less localized effects on the conformation of the folding intermediates. Lee, Koh, and Yu (*48*) have substituted different amino acids at the 331 and 334 sites and found that the ability to suppress *tsf* mutants is found only with a limited set of substitutions.

The local sequence of the suppressors

(suAla) (suVal)
↑ ↑
Ser - Tyr - Gly - Ser - **Val** - Ser - Ser - **Ala** - Gln - Phe - Leu - Arg

reveals no singular features. High serine content is associated with β strands and with flexible regions. The location of the *tsf* mutation, S333→N, suggests that the local conformation in the native state might be a surface β turn (*17*).

If *tsf* mutations decrease the thermal stability of a monomeric folding intermediate, the suppressors may confer increased stability to this critical intermediate species. Alternatively, they could kinetically influence the partition of the critical intermediates between intramolecular and intermolecular fates.

The purified Tsf, suppressor, and suppressor:Tsf proteins have also been refolded from the fully denatured state according to the procedures described by Seckler *et al.* (*36*). At 36°C the presence of the 334 substitution significantly increased the recovery of the native trimeric tailspikes, compared to the very low yield of the Tsf substitution alone (*49*); although it is not known if the efficacy of the suppressor in vitro corresponds to the in vivo efficiency, the result does show that the in vitro maturation of tailspikes is directly affected by the amino acid substitution, in the absence of any cellular factors.

The in vitro results do not exclude the possibility that the in vivo aggregation of the wild-type chains at high temperature could be due to failure of, or poor interaction of, folding intermediates with a molecular chaperone. This would explain the thermolability of the single chain folding intermediate in the wild-type pathway. The suppressor mutation would create, improve, or restore a site of chaperonin binding or recognition. Van Dyk, Gatenby, and La Rossa (*50*) have reported evidence that some tailspike *tsf* mutants can be rescued by overproduction of GroE chaperonin. The role of host cell chaperonins is under investigation (*51*).

Observations from Raman spectroscopy (*33*), x-ray diffraction, and electron microscopy indicate that the chains are in a cross-β conformation, with short lengths of β strand running orthogonal to the long axis of the tailspike. If the correct intrachain interactions are disturbed past a turn site, the intermediate may transiently accumulate as partially formed β-strand structures. Aggregation of those intermediate structures would subsequently occur through "out-of-register" interchain strand interactions.

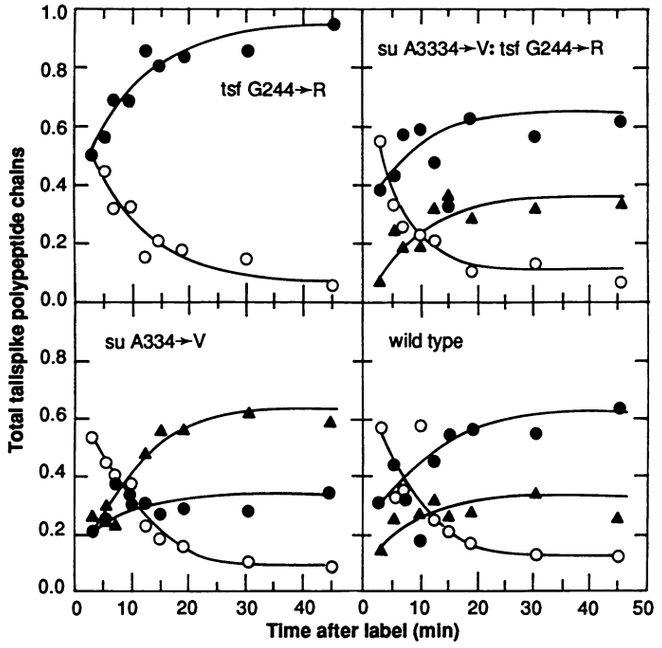

Fig. 3. Folding and intracellular aggregation for wild type, mutant, and suppressor tailspike chains at restrictive temperature (39°C). (○) Soluble partially folded species; (●) aggregated species; (△) native tailspike. (Upper left panel) Tsf G244→R mutant. Upper right panel: Double mutant (su A334→V: Tsf G244→R). Lower left: su A334→V. Lower right: Wild type. A culture of *Salmonella typhimurium* was infected at 39°C with P22 phage strains, carrying, in addition to the *tsf*, *su*:*tsf*, or *su* mutations of interest, a nonsense mutation in gene *5*, to block capsid assembly, and in gene *13*, to delay lysis. Ten minutes after the culture was infected, a portion was shifted to 30°C, to serve as the permissive control. Forty minutes after the infection, ^{14}C-labeled amino acids were added to a concentration of 2 μCi/ml in the 39°C culture, and incorporation was terminated by addition of unlabeled excess amino acids at 41.5 minutes. The same protocol was followed at 60 minutes for the permissive culture. Samples were taken at intervals after the addition of the excess amino acids and rapidly chilled, concentrated tenfold, and frozen. After being thawed to promote lysis, the lysed cells were centrifuged in a microfuge. The pellet was washed by resuspending and was centrifuged again. The supernatants were pooled, and the pellet was resuspended to the volume of the pooled supernatants. The resulting samples were mixed with SDS sample buffer without heating and subjected to electrophoresis through a 7.5 percent SDS gel. After fixing and drying, autoradiograms were prepared and scanned with an LKB 2202 Ultroscan densitometer, and band intensity was integrated with an LKB 2220 integrator. Each value represents the percentage of total ^{14}C-labeled tailspike polypeptide chains present in the sample. At the 30°C permissive temperature, more than 90 percent of the wild-type, suppressor, and suppressor:Tsf chains matured into native tailspikes. The Tsf chains alone were less efficient, with 60 percent maturing into native tailspikes.

Whatever the mechanism, these single amino acid substitutions direct the polypeptide chain away from aggregation traps, without altering the activity and stability of the mature protein. This suggests that it is possible to optimize folding pathways without affecting the desired properties of the final native state. The isolation of such mutations in other proteins may permit the engineering of scientifically or industrially important proteins to improve their recovery from heterologous hosts or in downstream processing (*52*).

Implications for the protein folding grammar. As a result of the flexibility of polypeptide chains, a large ensemble of conformations is sterically available during the folding process (*1*). Successful negotiating of the folding pathway to reach the native conformation requires avoiding the many conformations that would lead to incorrect chain interactions. Many of the aggregated states are liable to be kinetic traps corresponding to local energetic minima.

We presume that the sites or sequences generated by these suppressor mutations identify a class that is well represented in the wild-type sequence of the tailspike and in other polypeptide chains. Mutations isolated by Wetzel *et al.* that altered inclusion body formation of human interferon may be of this character (*52*). Such sequences may have only a limited role in stabilizing the native state. Indeed, their main role could be kinetically blocking off-pathway interactions or destabilizing incorrect conformations. Such residues represent a third kind of sequence information, in addition to those described above.

In comparing globin amino acid sequences from more than 260 species, Bashford, Chothia, and Lesk (*16*) identified more than 30 solvent-exposed positions associated with the protein surface at which hydrophilic residues were conserved. Some of these sites may represent the kinds of residues reported here, whose role is not stabilization of the native state, but destabilization of incorrect states—for example, helices packed through the wrong faces. The concept of local sequences that destabilize incorrect structures has also emerged out of efforts to design a chain that would fold into a specific conformation where residues were incorporated to block incorrect interactions (*53*).

The existence of sequences whose function is to block incorrect structures makes it easier to understand why it may not be possible to deduce the folding grammar simply by the comparison of the native state and the primary sequence.

Note added in proof: While this paper was in press Tsai *et al.* (*54*) reported the isolation of temperature-sensitive mutations of the human receptor–like protein tyrosine phosphate, as well as isolation of suppressors of the temperature-sensitive mutations. The suppres-

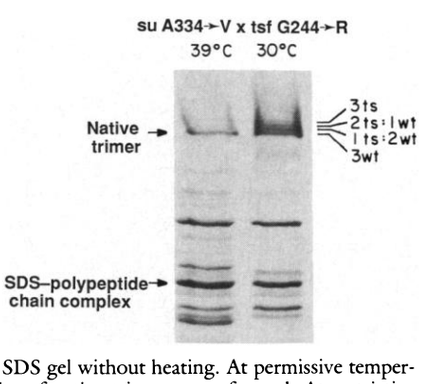

Fig. 4. The suppressors only alleviate mutations in the same chain. Cells were coinfected with *su* A334→V and *tsf* G244→R phage, in *5*-amber:*13*-amber background and pulse-labeled with ^{14}C-labeled amino acids as previously described. Thirty minutes after the addition of the unlabeled amino acids, samples were harvested and subjected to electrophoresis through an SDS gel without heating. At permissive temperature (30°C), all four species of native trimers were formed. At restrictive temperature, however, only the suppressor homotrimer was formed.

sor increased the yield of correctly foled protein in *Escherichia coli* and may be functioning like the suppressors described here.

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43. Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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