Structural Correlates of the Temperature Sensitive Phenotype Derived from Saturation Mutagenesis Studies of CcdB[†]

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ABSTRACT: Temperature sensitive (ts) mutants are widely used to reversibly modulate protein function in vivo and to understand functions of essential genes. Despite this, little is known about the protein structural features and mechanisms responsible for generating a ts phenotype. Also, such mutants are often difficult to isolate, limiting their use. In this study, a library consisting of 75% of all possible single-site mutants of the 101-residue, homodimeric *Escherichia coli* toxin CcdB was constructed. Mutants were characterized in terms of their activity at two different temperatures and at six different expression levels. Of the total of 1430 single-site mutants that were screened, 231 (16%) mutants showed a ts phenotype. The bulk of these consisted of 120 ts mutants found at all 22 buried sites and 34 ts mutants at all seven active site residues involved in binding DNA gyrase. Of the remaining ts mutants, 16 were found at residues in van der Waals contact with active site residues, 36 were at partially buried residues, and 30 resulted from introduction of Pro. Thus virtually all ts mutants could be rationalized in terms of the structure of the native protein and without knowledge of folding pathways. Data were analyzed to obtain insights into molecular features responsible for the ts phenotype and to outline structure- and sequence-based criteria for designing ts mutants of any globular protein. The criteria were validated by successful prediction of ts mutants of three other unrelated proteins, TBP, T4 lysozyme, and Gal4.

Temperature sensitive (ts)[1] mutants of a gene are ones in which there is a marked drop in the level or activity of the gene product when the gene is expressed above a certain temperature (restrictive temperature). Below this temperature (permissive temperature), the phenotype of the mutant is very similar to that of the wild type (WT). ts mutants provide an extremely powerful genetic and molecular tool for studying protein function and assembly in vivo and in cell culture. These mutants provide a reversible mechanism for lowering the level of a specific gene product at any stage in the growth of the organism simply by changing the temperature of growth (*1, 2*). However, the rarity of temperature sensitive alleles and the difficulty in isolating them through random mutagenesis have limited their use (*3, 4*). Alternative strategies for construction of ts mutants include use of ts degron fusions (*5*−*7*) or ts inteins (*8*). In another approach, temperature sensitive mutants of Gal4 and Gal80 were generated in yeast and *Drosophila* using ts splicing inteins of the *Saccharomyces cerevisiae VMA1* gene (*8*). While both of the methods described above work well in yeast, their applicability to generation of ts mutants in diverse organisms remains to be tested. Therefore, it is desirable to develop strategies for sequence- and/or structure-based design of temperature sensitive mutants of proteins. Strategies for designing temperature sensitive mutants solely from amino acid sequence have been described previously (*9, 10*). These were derived from a relatively small set of ts mutants generated at four buried sites in the bacterial toxin CcdB as well as on other known ts mutants in T4 lysozyme and gene V (*11, 12*). In this study, we attempted to construct all possible single-site mutants of the protein CcdB, a 101-residue, homodimeric protein of the F plasmid of *Escherichia coli*. The protein is a poison of DNA gyrase and is a potent cytotoxin. A total of 1430 single-site mutants of CcdB (which represents 75% of all possible single-site mutants) were obtained by replacing each of the 101 residues in CcdB with 19 other amino acids using a high-throughput site-directed mutagenesis protocol, described previously (*13*). Mutants were screened for their phenotypic activity at six different expression levels and at two different temperatures (30 and 37 °C). Expression level was modulated by varying the arabinose inducer concentration as described previously (*14*). The 231 isolated ts mutants were mapped onto the crystal structure of CcdB. The expression and solubility of ts mutants were analyzed to gain insight into molecular determinants of the ts phenotype and to derive strategies for the rational design of ts mutants.

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[1] Abbreviations: ACC, percent side chain accessibility of a residue in a protein; ts, temperature sensitive.

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MATERIALS AND METHODS

Plasmids, Host Strains, Mutagenesis, and Sequencing. The ccdb gene was expressed under control of the arabinose P_{BAD} promoter in plasmid pBAD24CcdB (*10*). AraE, a low-affinity, high-capacity transporter of arabinose, was expressed under the control of a constitutive promoter P_{CP8} in pJAT8araE. It was provided by J. D. Keasling (University of California, Berkeley, CA). Three *E. coli* host strains were used: Top10, XL1Blue, and CSH501. Top10 is sensitive to the action of CcdB and was used for screening the phenotype after transformation with pJAT8araE. XL1Blue is able to tolerate low levels of CcdB protein expression because of the presence of the antidote CcdA, which is encoded by the resident F plasmid. This strain was used for plasmid propagation. CSH501 is completely resistant to the action of CcdB because the strain harbors the GyrA462 mutation in its chromosomal DNA and prevents gyrase from binding to CcdB. CSH501 was kindly provided by M. Couturier (Universite Libre de Bruxelles, Brussels, Belgium) and was used for monitoring expression levels of mutant proteins. Mutagenesis and sequencing were carried out as described previously (*13*). In all cases, the entire coding region of CcdB was sequenced. The GyrA14 fragment (residues 363−494 of *E. coli* GyrA) was expressed under control of the T7 promoter in strain BL21(DE3)pLysS. The plasmid was kindly provided by R. Loris (Vrije Universiteit Brussel, Brussels, Belgium).

Induction Studies of Top10 and Top10/pJAT8araE as a Function of Arabinose Concentration. Induction studies with arabinose were performed in LB medium. Antibiotics were added to the following concentrations: 100 μg/mL ampicillin and 20 μg/mL gentamicin. *E. coli* Top10 and Top10/pJAT8araE were grown overnight at 37 °C in an air shaker without arabinose. For induction in solid medium, LB-agar plates, with the antibiotics at the required concentration and with the inducer at various concentrations (0, 4×10^{-5}, 8×10^{-5}, 2×10^{-4}, 5×10^{-4}, 2×10^{-3}, 5×10^{-3}, 2×10^{-2}, and 2×10^{-1}%), were prepared. Five microliters of 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} dilutions of the overnight culture was spotted on the plates and incubated overnight at 37 °C. The next day, individual colonies from 10^{-4} and 10^{-5} dilutions were picked and suspended in 0.8% saline at an OD of 0.2−0.4. The culture OD_{600} was measured in a Varian Cary 100 Bio spectrophotometer, and fluorescence was measured in a Fluorolog Tau-3 fluorimeter (Horiba) with 360 nm excitation (40 nm) and 507 nm emission (10 nm) filters. Flow cytometry was performed on a Beckman-Coulter flow cytometer (Beckman Instruments) equipped with an argon laser (emission at 488 nm and 15 mW) and a 525 nm band-pass filter. For each sample, 30000 events were collected at a rate between 500 and 1000 events per second.

Screening of the Phenotype of CcdB Mutants. Mutant CcdB plasmids were transformed in Top10 *E. coli* cells containing plasmid pJAT8araE in 96-well format using half-skirted 96-well plates (from Axygen), and activity was assayed by plating 5 μL of the transformation mix on square LB-ampicillin plates (120 mm × 120 mm) placed on 96-well grids in the absence of arabinose at 37 °C. Since active CcdB is toxic to *E. coli*, only cells transformed with inactive mutants will survive. The phenotype of all mutants that were inactive at 37 °C in 0% arabinose was also examined at 2×10^{-5}, 7×10^{-5}, 2×10^{-4}, 7×10^{-4}, 2×10^{-3}, and 2×10^{-2}% arabinose inducer at both 30 °C (permissive temperature) and 37 °C (restrictive temperature) to screen for mutants that show a ts phenotype, as a function of expression level. Top10/pJAT8araE cells transformed with a ts mutant of CcdB will grow at the restrictive temperature (37 °C) where the mutant is inactive but not at the permissive temperature (30 °C). To examine possible artifacts that might result from (a) a high density of cells in each spot in the 96-well format of screening and (b) differential transformation efficiencies with different mutant plasmids, approximately 25% of the ts mutants obtained after 96-well screens were rescreened on six-well plates. After transformation, 5 μL of transformed cells was inoculated in LB-ampicillin plates and grown overnight to near-identical cell densities. For each mutant, 50 μL of a 10^{-4} dilution was subsequently spread on a single well of the six-well plate. The procedure was repeated at multiple arabinose concentrations and in duplicate. One plate was incubated at 30 °C (permissive temperature) and the other at 37 °C (restrictive temperature). Levels of CcdB protein expression were monitored for ts mutants in CSH501 at different arabinose concentrations by growing the mutants on plates at 37 °C, followed by harvesting of colonies by washing plates with 1 mL of LB medium. The resulting suspension was centrifuged (13400*g*, 5 min, room temperature). The supernatant was removed, and the cell pellet was lysed by sonication. The cell lysate was centrifuged at 16000*g* for 30 min. The protein solubility was monitored by subjecting supernatant and pellet after lysis to 15% SDS−PAGE as described previously (*13*).

Data Analysis. The phenotypes of the various mutants were determined as a function of inducer concentration at 30 and 37 °C as described above. The data were analyzed using Windows MS Access DBMS by generating various queries as a function of residue ACC, depth, substitution, secondary structure, and inducer concentration.

RESULTS AND DISCUSSION

Dose-Dependent, Homogeneous Expression under the Control of the P_{BAD} Promoter. Gene expression from plasmids containing the P_{BAD} promoter has been reported to result in a mixture of fully induced and uninduced cells at subsaturating arabinose concentrations (*15*). However, co-expression of the high-capacity, low-affinity L-arabinose transporter araE from a constitutive promoter using plasmid pJAT8araE allows homogeneous expression from P_{BAD} even at subsaturating arabinose concentrations (*16*). To confirm this, *E. coli* Top10 cells with and without the pJAT8araE plasmid were transformed with pCSAK50 which expresses GFP under control of the P_{BAD} promoter (*17*). After overnight incubation at 37 °C on LB-agar plates in the presence of varying concentrations of arabinose, the extent of GFP expression was monitored by FACS (Figure 1). In the presence of pJAT8araE, cells exhibited homogeneous dose-dependent GFP expression in the arabinose concentration range of 4×10^{-5} to 5×10^{-4}% (Figure 1). In contrast, Top10 cells lacking pJAT8araE formed a bimodal distribution of GFP-expressing and nonexpressing cells at a single arabinose concentration of 2×10^{-4}%. However, at higher arabinose concentrations, Top10 cells also exhibited a homogeneous dose-dependent induction. For subsequent

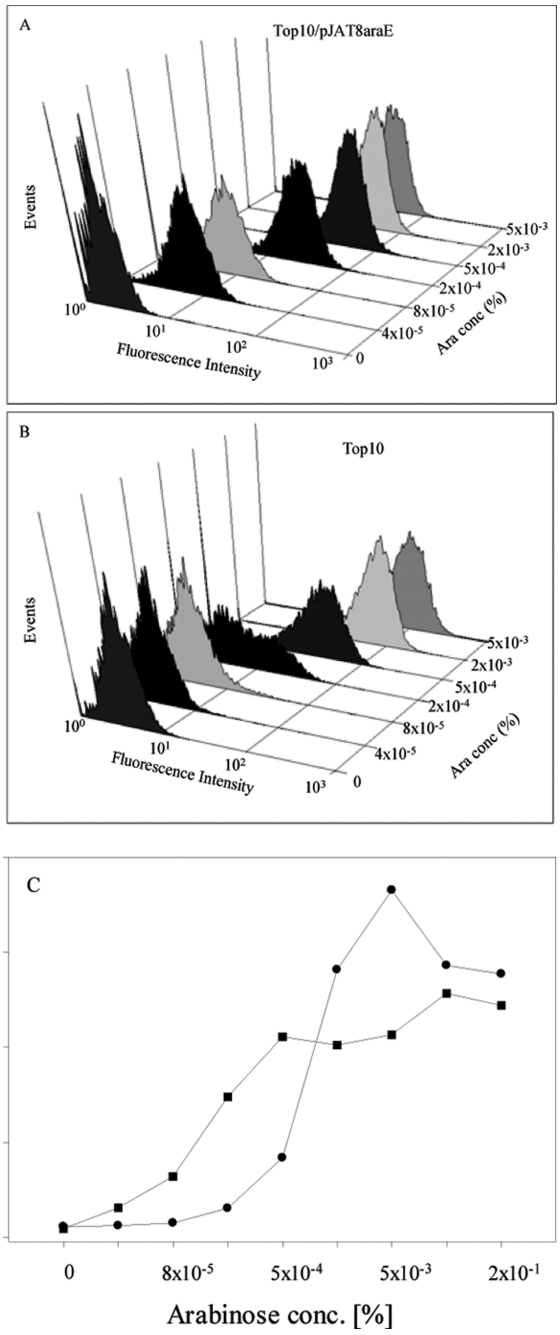

FIGURE 1: Dose-dependent and homogeneous induction of GFP under the control of the P_{BAD} promoter. Cells were induced overnight on LB plates, and colonies were resuspended in saline prior to analysis. The fluorescence intensity of individual Top10 cells harboring pCSAK50 (pBAD-gfp) with (A) and without (B) pJAT8araE as a function of arabinose concentration was measured by FACS. (A) Top10/pJAT8araE cells exhibited homogeneous, dose-dependent induction at all arabinose concentrations. (B) Top10 cells without pJAT8araE exhibited nonhomogenous expression at an arabinose concentration of 2×10^{-4}% and homogeneous expression thereafter. (C) Culture-averaged fluorescence (fluorescence/OD_{600}) of Top10 cells harboring pCSAK50 (pBAD-gfp) with (■) and without (●) pJAT8araE as a function of arabinose concentration.

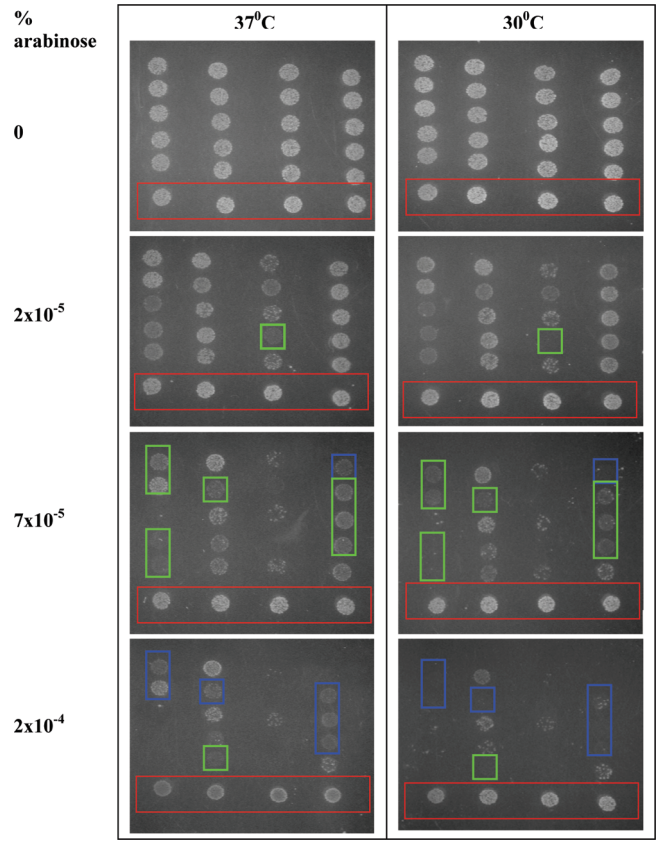

FIGURE 2: Phenotypic screening of temperature sensitive mutants of CcdB at 30 and 37 °C. Mutants were transformed in *E. coli* strain Top10/pJAT8araE, and transformation mix was spotted at seven different arabinose concentrations in duplicates. One plate was incubated at 30 °C and the other at 37 °C. Arabinose concentrations shown here are 0, 2×10^{-5}, 7×10^{-5}, and 2×10^{-4}%. Red blocks indicate inactive mutants, and blue and green blocks indicate strong and weak ts mutants, respectively. Since active CcdB is toxic to *E. coli*, cell growth is observed only under conditions where mutants display an inactive phenotype. Several ts mutants exhibit a weak ts phenotype (at least 5-fold slower growth at 30 °C relative to that at 37 °C) at low expression levels and a strong ts phenotype (at least 20-fold slower growth at 30 °C relative to that at 37 °C) at high expression levels.

studies of CcdB mutants, we therefore used the Top10/pJAT8araE cells.

Correlation between ts Phenotype and Expression Level. A total of 1430 (75%) of the 1900 possible site-directed mutants were isolated at all 101 positions of CcdB. We attempted mutagenesis reactions for generation of each mutant a maximum of three times. For some mutants, the mutagenesis reaction did not work, and for some, after sequencing of multiple clones, no mutant was obtained. Since such cases represented only a small fraction of the total mutants, we did not make further attempts to obtain these mutants. These mutants were distributed more or less randomly throughout the protein. All mutants at position 1 (the initiator Met) were inactive and are not considered in the subsequent analysis. Since active CcdB is toxic to *E. coli*, only cells transformed with inactive mutants will survive. Cells transformed with a ts mutant of CcdB will grow at the restrictive temperature (37 °C) where the mutant is inactive but not at the permissive temperature (30 °C). Cells were transformed with each of the mutant plasmids. Phenotypic screening of a representative set of ts mutants is shown in Figure 2; 231 mutants (16% of the total) exhibited a ts phenotype at some expression level. ts mutants and the range of arabinose concentrations at which they exhibit the ts phenotype are listed in Table 1 of the Supporting Information. To confirm the absence of artifacts in the 96-

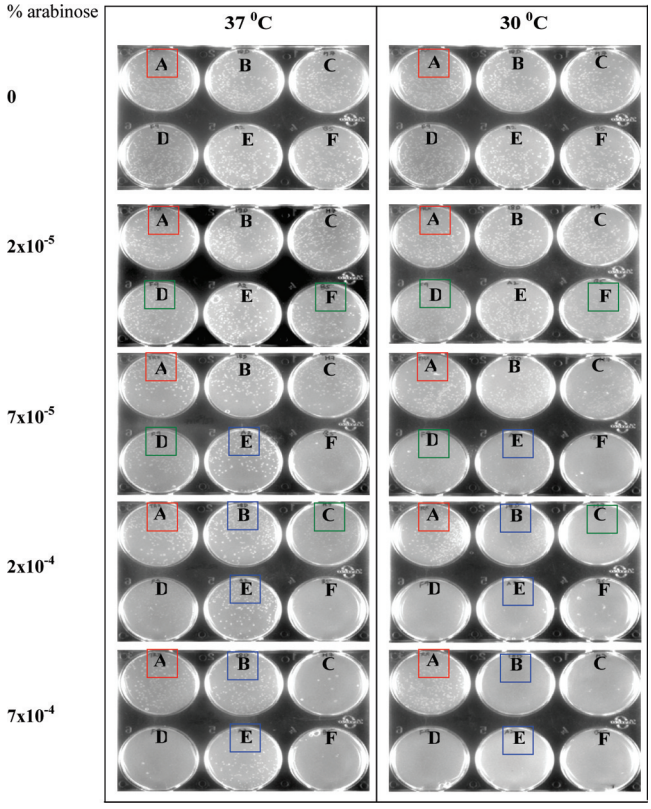

FIGURE 3: Reconfirmation of ts phenotypes of CcdB ts mutants at 30 and 37 °C. CcdB mutants were transformed in *E. coli* strain Top10/pJAT8araE. Cells were plated on six-well plates in duplicate as a function of arabinose concentration ($0-7 \times 10^{-4}$%) (see Materials and Methods). One plate was incubated at 30 °C and the other at 37 °C at each arabinose concentration. Cells transformed with pBADTRX (A) expressing the nontoxic protein thioredoxin served as a positive control and grew equally well at all arabinose concentrations. Mutants at positions B and E of the six-well plate showed a strong ts phenotype in the arabinose concentration ranges of $2-7 \times 10^{-4}$% and 7×10^{-5} to 7×10^{-4}%, respectively. Mutants at positions C, D, and F exhibited a weak ts phenotype at arabinose concentrations of 2×10^{-4}, 2×10^{-5}, 7×10^{-5}, and 2×10^{-5}%, respectively. Red blocks indicate Trx, and blue and green blocks indicate strong and weak ts mutants, respectively.

well screening, approximately 25% of the ts mutants were rescreened on six-well plates (Figure 3). Results from the more elaborate six-well screen were identical to those obtained from the 96-well screen. Consistent with the GFP data, CcdB expression driven by the P_{BAD} promoter in Top10/pJAT8araE cells plateaus at an arabinose concentration between 5 and 7×10^{-4}. At higher concentrations, little or no change is observed in phenotypes of virtually all CcdB mutants.

Correlation between ts Phenotype and Residue Location. All ts mutants were mapped onto the crystal structure of CcdB (*18*) (Protein Data Bank entry 3vub) (Table 1); 52% (120 of 231) ts mutants were observed to be at buried sites, i.e., at residues with total side chain accessibility (ACC) of <5%. The justification for using this burial cutoff has been described previously (*13*). All 22 buried sites in the protein exhibited at least one ts mutant (Table 1) with an average of 5.5 ts mutants per site; 71 (59%) mutants which resulted in a ts phenotype at these buried sites involved polar or charged amino acids. This is expected, as a change from a hydrophobic to a polar/charged amino acid in the buried core is likely to cause destabilization of the protein. Many of these substitutions are not tolerated and will lead to complete inactivation of the protein. However, a fraction of these substitutions destabilize the protein to a moderate extent and hence result in a ts phenotype; 34 (15%) ts mutants were found at each of the seven active site residues (Ile24, Ile25, Asn95, Phe98, Trp99, Gly100, and Ile101) with an average of five ts mutants per site (*13*) (Table 1). Active site residues are defined as those residues of CcdB which are involved in interaction with DNA gyrase as determined by Ala and Asp scanning mutagenesis (*13*) and confirmed by X-ray crystallography of the CcdB−GyrA14 fragment complex (*19*). There are a total of eight exposed residues whose side chains are within 4 Å of active site residues in CcdB. Sixteen (7%) ts mutants were found at seven such exposed residues, i.e., Gln2, Ser22, Arg31, Leu50, Leu96, Asp23, and Lys91, with an average of 1.1 mutants per site in this category. These residues are probably important in maintaining the conformations of active site residues. Thirty Pro and eight Gly substitutions of the WT residue also resulted in a ts phenotype. Pro and Gly are the most rigid and flexible amino acids, respectively. The value of the ϕ dihedral angle for Pro in polypeptides is constrained to values close to $-65 \pm 15°$ (*20*). Fifty-two ts mutants were obtained at sites which do not belong to any of the above-mentioned categories. Thirty-six such mutants were at partially exposed residues (5−20% ACC). These consist of five mutants at Phe3, seven at Tyr6, one at Phe8, 11 at Pro35, five at Ile56, and seven at Ser70. Sixteen ts mutants (four at Lys4, one at Leu41, one at Met64, four at Asp67, four at Pro72, one at Val80, and one at Glu87) cannot be rationalized with the currently available structures as they are at surface-exposed sites, which are not in the proximity of any active site residues. A large fraction of all sites which have at least one inactive mutant have a ts mutant. When averaged over all expression levels, this fraction is 100% for both buried and active site residues.

Correlation between ts Phenotype and Secondary Structure. ts mutants were divided into six structural categories based on their residue location. The number of ts mutants in each category was examined as a function of expression level and inducer concentration. In each category, the numbers of ts mutants as a function of arabinose concentration showed a bell-shaped curve with the peak of the curve generally at 7×10^{-5}% arabinose (Table 1). Mutations at residues present in secondary structural elements yield ts mutants with higher frequency than residues present in nonsecondary structural elements (Table 1). Substitutions at 11 of 13 residues in the C-terminal α-helix and 26 of 38 residues in β-strands yield ts mutants (Table 1), whereas only 21 of 50 residues in nonsecondary structural elements show a ts phenotype upon mutation (Table 1). This is probably because mutations in secondary structural elements are likely to destabilize the protein to a larger extent than mutations in nonsecondary structural elements. It was suggested earlier on the basis of ts mutants of p53 (*21*) that mutations in β-strands are more likely to result in a ts phenotype than in α-helices and loops. The high frequency of ts mutants observed in the single α-helix of CcdB exists because several active site residues are present at the C-terminal region of this helix.

Table 1: Number of ts Mutants as a Function of Arabinose Concentration and Structural Category

property	total no. of positions	% ts[a]	total no. of ts mutants	no. of ts mutants					
				2×10^{-5}% arabinose	7×10^{-5}% arabinose	2×10^{-4}% arabinose	7×10^{-4}% arabinose	2×10^{-3}% arabinose	2×10^{-2}% arabinose
buried[b]	22	100	120	50	61	47	28	27	22
active site[c]	7	100	34	19	16	9	9	9	7
close to active site[d]	8	88	16	8	9	10	5	4	4
helix[e]	13	85	37	21	15	13	8	6	5
β-strands[f]	38	68	120	47	73	55	34	32	27
non-sec structure[g]	50	42	80	31	50	30	19	20	18
total[h]	101	58	231	99	138	98	61	58	50

[a] Fraction of residue positions (%) with the indicated property where at least one substitution showed a ts phenotype. [b] ts mutants at buried sites, i.e., with a <5% total side chain ACC. [c] ts mutants at active site residues (see the text for details) which also includes two buried residues (98 and 100) which are adjacent to residues contacting DNA gyrase. [d] Exposed residues whose side chain is within 4 Å of active site residues. [e] Residues present in a single, C-terminal α-helix of CcdB. [f] Residues present in β-strands of CcdB. [g] Residues present in nonsecondary structural elements of CcdB. [h] Total over all categories.

Suggested Molecular Mechanisms Responsible for ts Phenotype. To gain insight into possible mechanistic differences responsible for ts phenotypes at buried and active sites, the solubilities of two buried site ts mutants, 17E and 34P, and two active site ts mutants, 24S and 99T, were examined. WT CcdB was taken as a positive control, and the inactive mutant 5D as a negative control (Figure 1 of the Supporting Information). These studies were carried out in CcdB resistant strain CSH501. For the inactive mutant (5D), virtually all the protein was targeted to inclusion bodies. For the buried site ts mutants also, an appreciable fraction is targeted to inclusion bodies, whereas for WT and active site ts mutants, virtually all the protein is soluble. The data suggest that for active site mutants, the specific activity is lower than the WT activity because the binding affinity for gyrase is lowered. However, the level of soluble protein is similar to the WT level. In contrast, for ts mutants at buried positions, the level of soluble protein is lower than the WT level and the specific activity may also be lowered because of aggregation or misfolding of protein in the soluble fraction. ts mutants at buried sites typically appear to have substitutions that are expected to destabilize the protein (Table 1 of the Supporting Information). Preliminary studies with purified mutants show that active site mutants have a stability similar to that of WT (B. Barua and R. Varadarajan, unpublished results). Hence, there is a lack of activity because the interaction with GyrA is affected. In contrast, buried site mutants are highly unstable, aggregation prone, and difficult to purify. Thus, both classes of ts mutants show reduced total activity relative to that of WT, but for different reasons. Protein aggregation and misfolding are typically enhanced at higher temperatures (*22, 23*). This may be the primary contributing factor for the phenotypes of buried site ts mutants. At the restrictive temperature for all ts mutants, the activity is below the threshold required to show an active phenotype, and at the lower, permissive temperature, the activity is above this threshold. Increasing the total protein expression level by increasing the arabinose concentration increases the total amount of protein in the soluble form and hence results in an active phenotype at high arabinose concentrations even at 37 °C for most ts mutants of CcdB. A similar rescue of ts phenotype upon overexpression has been observed previously (*24, 25*). Future studies involving detailed biophysical characterization of multiple ts mutants at both buried and active site residues will be carried out to further validate the proposed mechanism. Interestingly, not a single mutant showed a cold sensitive phenotype. Understanding this requires further study. However, it may be relevant that the interaction between WT CcdB and WT gyrase is clearly not cold sensitive; i.e., the maximal stability temperature for this interaction is clearly below 37 °C (B. Barua and R. Varadarajan, unpublished results). It is possible that for processes or pathways that are inherently cold sensitive, a larger fraction of mutants may show a cold sensitive phenotype (*26*).

Correlation between ts Phenotype and Residue Type before and after Mutation. Amino acids in CcdB were classified as aliphatic (A, C, V, I, L, and M), aromatic (F, W, and Y), polar (H, N, Q, S, and T), charged (D, E, K, and R), Gly (G), and Pro (P). There are 37 aliphatic, nine aromatic, 20 polar, 27 charged, four Gly, and four Pro residues in CcdB. ts mutants were obtained at significant frequencies at all buried sites irrespective of the nature of the WT residue with the exception of M which yielded ts mutants at low frequency (Table 2, column 2). At exposed non-active site residues, highest ts frequencies occur when the WT residue is Pro (Table 2, column 3). There is a high frequency for ts mutants where the WT residue is Gln because there is only one buried and one exposed Gln and both are close to the active site. Very low frequencies of ts mutants are obtained at exposed, non-active site residues that are charged or aliphatic. We next analyzed the frequencies of ts mutants in terms of the identity and/or class of the mutant amino acid at buried and exposed sites. All amino acids were represented at approximately equal frequency in the saturation mutagenesis library. The frequency of ts mutants was highest for His, Asn, charged, Ala, Gly, and Pro substitutions at buried sites (Table 2, column 4). Other amino acids (aliphatic, aromatic, Ser, Thr, and Gln) yielded ts mutants with similar frequencies. No strong bias was observed toward any type of substitution at exposed residues except Pro (Table 2, column 5). At active site residues (Table 2, column 6) with the exception of F, W, Q, and R, all other substitutions yielded ts mutants with reasonable frequencies.

Mutations at the Dimer Interface. CcdB is a homodimeric protein. It is therefore of interest to examine if residues buried in the core of each monomer exhibit a different probability of showing a ts phenotype than residues buried at the dimer interface. This analysis is summarized in Table 2 of the Supporting Information. If all types of residues are considered, those at the dimer interface show a marginally lower average frequency of ts mutants than those buried in the monomer cores (36 ± 7 and 41 ± 4%, respectively). However, as shown in Table 2 (column 2), positions with

Table 2: Correlation between ts Phenotype and Residue Type before and after Substitution

	frequency of ts mutants (%)				
	WT = X		mutant = X		
residue (X)	buried[a]	exposed[a]	buried[b]	exposed[b]	active[c]
C	*d*	*d*	17	10	40
M	21	*e*	20	7	33
A	*e*	5	41	8	40
I	33	22	7	6	33
L	52	11	20	12	20
V	47	2	27	8	17
F	32	*e*	22	3	0
W	*d*	23	21	7	0
Y	*d*	20	29	6	33
H	*d*	3	50	7	50
N	*d*	11	50	11	50
Q	*e*	*e*	27	6	0
S	*d*	6	27	8	17
T	*e*	3	31	7	17
D	*e*	7	65	14	50
E	*d*	2	45	10	57
K	*d*	8	67	20	83
R	*d*	3	58	13	0
G	*e*	4	39	6	17
P	*e*	43	65	27	43

[a] Frequency = (the number of ts mutants at buried/exposed sites for which the WT residue was X) × 100/(the total number of mutants at buried/exposed sites for which the WT residue was X). [b] Frequency = (the number of ts mutants at buried/exposed sites for which the mutant residue was X) × 100/(the total number of mutants at buried/exposed sites for which the mutant residue was X). [c] Frequency = (the number of ts mutants at active site residues for which the mutant residue was X) × 100/(the total number of mutants at active site residues for which the mutant residue was X). [d] No WT residue in this class. [e] Only one WT residue in this class; hence, frequency is not meaningful.

WT Met residues exhibit particularly lower frequencies of ts mutants. If such positions are excluded from the analysis, even this small difference disappears. The average frequencies of obtaining ts mutants at the dimer interface and monomer cores are now 42 ± 8 and 42 ± 4%, respectively. This demonstrates that both types of buried residues show virtually identical propensities to form ts mutants. Hence, it is not necessary to consider these two categories of buried residues separately. CcdB is an obligate homodimer at physiological pH. No folded monomer can be detected at neutral pH, and chemical and thermal denaturation studies at pH 7.0 fit well to a two-state transition between the folded dimer and unfolded monomers. Folding is thus strongly coupled to dimerization. In addition, both the monomer core and dimer interface are essentially hydrophobic. We therefore do not expect large differences in sensitivity to mutation at these two locations, in agreement with the experimental results.

Hot Spots for ts Mutations in CcdB. We define hot spots as sites at which >25% of all observed substitutions resulted in a ts phenotype. A total of 29 sites were identified as hot spots for generation of ts mutants in CcdB (Figure 4 and Table 3 of the Supporting Information). These include four active site residues (71.4% of total active site residues), 18 buried (82% of total buried sites), and one active site proximal (13% of total, exposed, active site proximal). Of the remaining, five existed at partially buried residues (primarily aromatic or Pro) with an ACC between 5 and 15%. Mutations which resulted in a ts phenotype at partially buried sites were similar to those found at completely buried sites and included mostly polar, charged, and proline substitutions. Three sites (4, 67, and 72) were identified as hot spots, but they do not fall into any of the above-mentioned categories. One of them (position 72) had a proline as a wild-type residue. Position 4 was among a contiguous stretch of hot spot sites (2−6). However, there is no obvious rationale for position 67 as a hot spot site since it is a surface-exposed site that is not in the proximity of any other active site residue.

Analysis of Previous Large-Scale Mutagenesis Studies To Isolate ts Mutants. Large-scale mutagenesis studies combined with screens for temperature sensitive mutants have been previously carried out for TATA binding protein (TBP) using a regional codon-based mutagenesis strategy and T4 lysozyme using suppressor strains (*27, 28*). None of these studies involved saturation mutagenesis of the protein, and only a single expression level was characterized. While the T4 lysozyme study yielded much valuable data, there are two limitations with use of suppressor strains. Not all substitutions can be examined, and suppressor efficiencies are strain- and context-dependent. Hence, all mutants will not be expressed at similar levels with such an approach. This study overcomes these limitations and also characterizes ts phenotypes at multiple expression levels. However, in each of the cases described above, a significant fraction of ts mutants occurred at buried sites which was observed with CcdB. For T4 lysozyme, using suppressor strains, plaque-forming phenotypes of 13 different substitutions were examined at all 163 positions at two different temperatures, 37 and 25 °C; 96 mutants exhibited a classic temperature sensitive phenotype, i.e., plaque formation at the permissive temperature and no plaque formation at the restrictive temperature. Upon analysis of the published data, we observed that 69% of these ts mutants occurred at buried sites and 31 of the 34 buried sites showed at least one ts mutant. At exposed sites, ts mutants were obtained either at residues involved in salt bridges or cation−π interactions or at residues lining the substrate binding site.

In the case of yeast TATA binding protein (TBP), the library was generated using a regional codon-based randomized mutagenesis strategy that covered residues 57−71, 117−168, and 217−240. Mutant libraries were introduced into yeast cells, and individual mutants were screened for their ability to support cell growth at 23, 30, and 37 °C. TBP is required for transcription by all three nuclear RNA polymerases; 1% of the transformants were ts. Plasmids were rescued from 262 of the clones and sequenced; 140 unique single-site ts mutants were finally identified that permitted growth at 23 and 30 °C but not at 37 °C. In this case, it is likely that not all possible ts mutants available in the library were sequenced, since for several mutants only a single clone was isolated. The library was biased as it covered only 88 sites out of a total of 240 sites in the protein that were located in regions expected to be involved in binding to RNA polymerase. However, even with these caveats, 29% of ts mutants were obtained at buried sites and 22% at active site residues; 95% of buried sites examined showed at least one ts mutant in TBP.

Another system in which several ts mutants have been characterized is P22 tailspike protein. ts mutants were reported at 41 sites, which were folding defective. These "tsf" mutants retain the activity at the restrictive temperature if synthesized at the permissive temperature but undergo

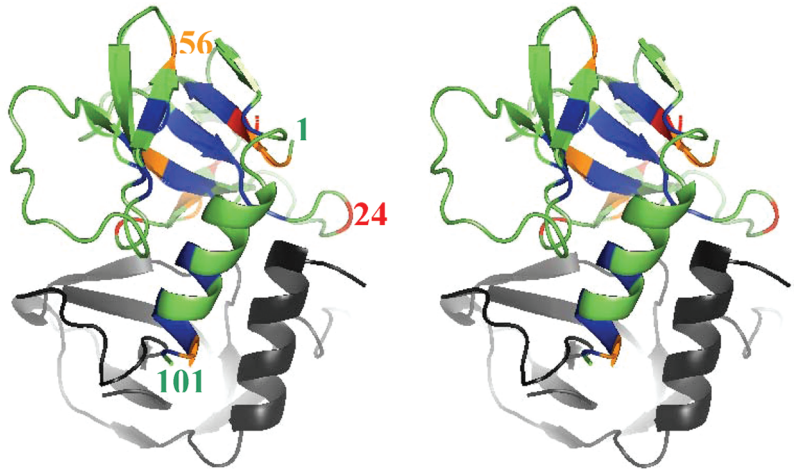

FIGURE 4: Locations of hot spot sites for ts mutants in CcdB in the context of the overall three-dimensional structure of the protein. In the stereoview, one of the monomers is shown in gray and the other in color. Hot spots (sites where >25% of the mutants were ts) were observed at buried sites (5, 17−21, 33, 34, 36, 52, 54, 63, 83, 93, 94, 97, 98, and 100), partially buried sites (2, 3, 6, 35, 56, 70, and 99), and exposed sites (4, 24, 67, and 72). These three categories are colored blue, orange, and red, respectively.

aggregation or inclusion body formation and fail to reach the mature conformation when synthesized at the restrictive temperature (*29, 30*). P22 tailspike is a large (666 amino acid residues), trimeric protein with a parallel β-coil structure and a complex folding mechanism. Many tsf folding mutations were found in exposed turn regions of the β-helix. The β-helix structure differs from typical globular protein folds in being a more extended repetitive structure and lacking a single well-defined core. It is possibly because of this that surface residues appear to play such an important role in folding. In contrast, for more typical compact folds such as those of CcdB and T4 lysozyme, mutations at surface residues appear to have little effect on stability and folding at multiple temperatures. In these cases, the ts phenotypes can easily be rationalized on the basis of the structure of the WT protein. No knowledge of the folding pathway is required.

Strategies for Designing ts Mutants of a Globular Protein. We had previously hypothesized (*10*) that ts mutants at buried sites could be generated at high frequencies by either of two strategies: (a) introduction of a stereochemically diverse set of four residues (aliphatic, aromatic, polar, and charged) at three or four buried sites and (b) substitution of each of two buried residues with the 18 remaining amino acids. Those suggestions were based on a relatively small data set of 18 mutants at each of the four buried sites of CcdB. In this study, we have generated an unbiased saturation mutagenesis library of CcdB and screened it at multiple expression levels to refine and extend our previous hypothesis. An examination of the data summarized in Table 2 suggests that the optimal choice of aliphatic, aromatic, polar, and charged residue substitutions at buried positions would be A, Y, N/H, and D/K, respectively. Since Y and H have both polar and aromatic character, to maximize diversity we chose A, F/W, N, and D, respectively, for the four substitutions. In addition, since P has unique stereochemical constraints (*31*) and also is found in a large fraction of ts mutants, we have added this to the list of suggested substitutions at both buried and exposed positions. To determine the optimal number of buried sites to be mutated, we calculated the probability of obtaining at least one ts mutant using either strategy a or strategy b as a function of the number of sites mutated (Table 3). F and W when substituted at buried sites show almost equal frequencies of generation of ts mutants. However, W in comparison to F showed a slightly higher probability of yielding at least one ts mutant using strategy a (compare rows 5 and 13 of Table 3). Thus, we restricted the allowed substitutions at buried sites to A, W, N, D, and P. The data demonstrate that to obtain a ts mutant with a >80% probability, on average three sites (15 mutants) or one site (19 mutants) is sufficient for strategy a or b, respectively. Making and characterizing such a small number of mutants is experimentally tractable in most systems. For large multidomain proteins, strategy a is preferable because it allows one to pick sites located in different regions of the protein. If only a single site is mutated, it is possible that this may be in a region or domain that is nonessential for the function of interest. In systems where screening of tens to hundreds of mutants is feasible, then strategy b may be preferable since codon randomization at a few predicted, buried sites is technically straightforward (*10*). We also examined the sensitivity of the results for strategy a to the exact choice of mutant substitutions. The results were insensitive to the exact choice of aromatic, aliphatic, charged, and polar residues (compare rows 5 and 13, 5 and 14, 5 and 15, and 5 and 16 of Table 3). However, having a stereochemically diverse set of residue substitutions was clearly superior to having similar types of residue substitutions [e.g., all aromatic, all hydrophobic residues or all polar (rows 10−12 of Table 3)]. When only charged residues (K, R, E, and D) were used, ts frequencies were comparable to that obtained with stereochemically diverse residues for the CcdB data set except at low expression levels (compare rows 5 and 9 of Table 3). We did not attempt an independent analysis of ts substitutions at exposed active site residues because this data set is relatively small and consists of a total of 34 ts mutants at seven active site residues of CcdB. Instead, we evaluated the probability of obtaining ts mutants at active residues using the same strategies and substitutions described above for buried residues. As shown in Table 3, these yielded ts mutants at a high frequency at active site residues also. The strategies decribed above were evaluated on the ts mutant

Table 3: Evaluation of Strategies for ts Mutant Design at Buried Residues (B) and Exposed, Active Site Residues (E) Using the CcdB ts Mutant Data Set

row no.	residue substitutions[b]	no. of sites mutated	probability of obtaining a ts mutant (%)[a]									
			2×10^{-5}% arabinose		7×10^{-5}% arabinose		2×10^{-4}% arabinose		7×10^{-4}% arabinose		average	
			B	E	B	E	B	E	B	E	B	E
1	AWNDP	1	73	86	68	71	41	57	46	43	57	64
2	randomization[c]	1	91	86	95	86	91	71	73	57	88	75
3	AWNDP	2	93	98	90	92	65	63	70	68	80	80
4	randomization	2	99	98	99.8	98	99	92	93	63	98	88
5	AWNDP[c]	3	98	99.7	97	98	80	92	84	81	90	93
6	randomization	3	99.9	99.7	99.9	99.7	99.9	97.7	98	92	99.4	97
7	AWNDP	4	99.6	99.9	99	99	88	97	91	89	94	96
8	randomization	4	99.9	99.9	99.9	99.9	99.9	99.3	99.5	97	99.8	99
9	KRED	3	68	98	97	99.7	95	81	88	92	87	93
10	FWY	3	36	37	45	0	45	0	36	0	41	9
11	AILV	3	54	64	68	37	62	0	36	37	55	34
12	HNQST	3	93	92	80	64	68	64	54	37	74	61
13	AFNDP	3	98	99.7	93	98	80	92	74	81	86	93
14	VWNDP	3	99	99.7	95	98	84	92	84	81	91	93
15	AWNKP	3	93	81	98	98	91	81	62	37	86	74
16	AWHDP	3	97	99.7	95	92	79	81	84	81	89	88

[a] Probability of obtaining a ts mutant $= 1 - [(y - x)/y]^n$, where y is the total number of sites in the protein with a structural property specified in the respective strategy, x is the number of such sites where at least one ts mutant is obtained using the suggested strategy, and n is the number of such sites where the strategy is employed (column 3). [b] Suggested amino acid substitutions in single-letter code at each site. [c] Final recommended strategies.

Table 4: Probabilities of Obtaining ts Mutants at Buried Residues (B) and Exposed, Active Site Residues (E) in the CcdB, T4 Lysozyme, and TBP ts Mutant Data Sets[a]

strategy	probability of obtaining a ts mutant (%)[b]					
	CcdB		T4 lysozyme		TBP	
	B	E	B	E	B	E
a	90	93	94.5	78.4	78.4	75.6
b	88	75	91	80	95	100

[a] Strategies a and b were derived from CcdB data set analysis and are described in the text and summarized in rows 5 and 2 of Table 3. [b] The probability of obtaining a ts mutant $= 1 - [(y - x)/y]^n$, where y is the total number of sites in the protein with the structural property specified in the respective strategy, x is the number of such sites where at least one ts mutant is obtained, and n is the number of such sites where the strategy is employed.

data sets of buried residues of TBP and T4 lysozyme (Table 4). Strategy a when applied to three sites with A, W, N, D, or P substituted at each site yielded similar results when evaluated on the T4 lysozyme and TBP data sets (probabilities of 94.5 and 78.4%, respectively). In the case of T4 lysozyme, Gln and Glu were used in place of Asn and Asp, respectively, to check the accuracy of strategy a since Asn and Asp mutations were not introduced experimentally for this protein (*28*). Strategy a with charged residue substitutions (K, R, E, and D) yielded probabilities of obtaining ts mutants of 93 and 27% for T4 lysozyme and TBP, respectively, suggesting that using the stereochemically diverse sets is better than using only charged residues. Single-residue randomization at buried sites (strategy b) evaluated on the T4 lysozyme and TBP data sets resulted in 91.2 and 95% probabilities of obtaining ts mutants, respectively. We have shown previously (*9*) that buried residues can be accurately predicted from protein sequence and experimentally confirmed by Asp scanning mutagenesis (*13*). Hence, ts mutant generation at buried sites does not require NMR or crystallographic structures. While such structural information is useful for identification of exposed active site residues, it is not essential as we have also shown (*13*) that active site residues can be identified from Ala and Asp scanning mutagenesis. In the case of T4 lysozyme and TBP, since such scanning mutagenesis data were not available, active site residues were identified from the crystal structures of a T4 lysozyme substrate complex [Protein Data Bank (PDB) entry 148L (*32*)] and a TBP−RNA polymerase BrfI subunit complex [PDB entry 1ngm (*33*)], respectively. It is well-known that in a macromolecular complex, not all residues at the interface (active site residues) contribute equally to the stability of the complex. Hence, in the two structures mentioned above, we only considered those residues which were exposed in the free protein (ACC > 5%), were completely buried (<5% ACC) in the complex, and exhibited a change in ACC of >5% upon complex formation. This consisted of residues 26, 105, 106, 114, and 136 in T4 lysozyme and residues 89, 92, 96, 106, 129, 137, 138, 140, 142, 152, 155, and 157 in TBP. No mutants were made for residues 89, 92, 96, and 106 in TBP, so these residues were excluded from the analysis. We reasoned that use of stringent criteria might yield a subset of residues that made important contributions to the stability of the complex and hence would be enriched in ts mutants. Strategies a and b, when evaluated on the active site residues of the TBP data set mentioned above, resulted in 75.6 and 100% success rates of obtaining ts mutants, respectively, despite the absence of an exhaustive ts mutant data set for TBP. Strategies a and b, when evaluated on the active site residues of the T4 lysozyme data set mentioned above, resulted in 78.4 and 80% success rates of obtaining ts mutants, respectively. ts mutants at active site residues have the advantage of affecting activity directly without affecting protein levels and hence may show induction kinetics more rapid than those at buried sites. In some situations where simple screens are available, instead of introducing specific mutations at buried and active side residues, we can individually randomize a few such residues. Using this strategy, we have recently isolated several ts mutants of yeast Gal4 at both buried (*10*) and exposed DNA binding residues (*34*) and have shown that ts mutants of Gal4

at DNA binding residues isolated in yeast also show a ts phenotype in *Drosophila*. The substitutions observed to result in ts phenotypes in Gal4 were consistent with predictions based on the CcdB data. At three predicted buried sites (residues 68−70) in Gal4, mutants F68P, F68D, L69P, and L70P showed ts phenotypes (*34*). At two exposed DNA binding residues, substitutions R15W, K23P, and K23W showed ts phenotypes. In cases of biological interest, one is often interested in making ts mutants of a protein of unknown stability and copy number and typically at a fixed but unknown transcriptional level. An important feature of this study is that the proposed criteria yielded ts mutants of CcdB at many different expression levels. The criteria also successfully predicted ts mutants of three other unrelated proteins, TBP, T4 lysozyme, and Gal4. This analysis indicates that these strategies for generating ts mutants are quite robust and should be applicable to most globular proteins in diverse organisms.

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SUPPORTING INFORMATION AVAILABLE

A list of all the temperature sensitive mutants of CcdB as a function of their position and ACC (Table 1), the observed frequencies of ts mutants at sites buried at the dimer interface with corresponding values for residues buried in the core of each monomer (Table 2), details of sites in CcdB where more than 25% of mutants showed a ts phenotype (hot spot sites) (Table 3), and relative solubilities of ts mutants at buried and active sites depicted in a SDS−PAGE gel (Figure 1). This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Novick, P., and Schekman, R. (1979) Secretion and cell-surface growth are blocked in a temperature-sensitive mutant of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U.S.A. 76*, 1858–1862.
2. Novick, P., and Botstein, D. (1985) Phenotypic analysis of temperature-sensitive yeast actin mutants. *Cell 40*, 405–416.
3. Suzuki, D. T., Grigliatti, T., and Williamson, R. (1971) Temperature-sensitive mutations in *Drosophila melanogaster*. VII. A mutation (para-ts) causing reversible adult paralysis. *Proc. Natl. Acad. Sci. U.S.A. 68*, 68–93.
4. Hashimoto, T., and Sekiguchi, M. (1976) Isolation of temperature-sensitive mutants of R plasmid by in vitro mutagenesis with hydroxylamine. *J. Bacteriol. 127*, 1561–1563.
5. Dohmen, R. J., Wu, P., and Varshavsky, A. (1994) Heat-inducible degron: A method for constructing temperature-sensitive mutants. *Science 263*, 1273–1276.
6. Rajagopalan, S., Liling, Z., Liu, J., and Balasubramanian, M. (2004) The N-degron approach to create temperature-sensitive mutants in *Schizosaccharomyces pombe*. *Methods 33*, 206–212.
7. Kanemaki, M., Sanchez-Diaz, A., Gambus, A., and Labib, K. (2003) Functional proteomic identification of DNA replication proteins by induced proteolysis in vivo. *Nature 423*, 720–724.
8. Zeidler, M. P., Tan, C., Bellaiche, Y., Cherry, S., Hader, S., Gayko, U., and Perrimon, N. (2004) Temperature-sensitive control of protein activity by conditionally splicing inteins. *Nat. Biotechnol. 22*, 871–876.
9. Varadarajan, R., Nagarajaram, H. A., and Ramakrishnan, C. (1996) A procedure for the prediction of temperature-sensitive mutants of a globular protein based solely on the amino acid sequence. *Proc. Natl. Acad. Sci. U.S.A. 93*, 13908–13913.
10. Chakshusmathi, G., Mondal, K., Lakshmi, G. S., Singh, G., Roy, A., Ch, R. B., Madhusudhanan, S., and Varadarajan, R. (2004) Design of temperature-sensitive mutants solely from amino acid sequence. *Proc. Natl. Acad. Sci. U.S.A. 101*, 7925–7930.
11. Alber, T., Sun, D. P., Nye, J. A., Muchmore, D. C., and Matthews, B. W. (1987) Temperature-sensitive mutations of bacteriophage T4 lysozyme occur at sites with low mobility and low solvent accessibility in the folded protein. *Biochemistry 26*, 3754–3758.
12. Sandberg, W. S., Schlunk, P. M., Zabin, H. B., and Terwilliger, T. C. (1995) Relationship between in vivo activity and in vitro measures of function and stability of a protein. *Biochemistry 34*, 11970–11978.
13. Bajaj, K., Chakrabarti, P., and Varadarajan, R. (2005) Mutagenesis-based definitions and probes of residue burial in proteins. *Proc. Natl. Acad. Sci. U.S.A. 102*, 16221–16226.
14. Guzman, L. M., Belin, D., Carson, M. J., and Beckwith, J. (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J. Bacteriol. 177*, 4121–4130.
15. Siegele, D. A., and Hu, J. C. (1997) Gene expression from plasmids containing the araBAD promoter at subsaturating inducer concentrations represents mixed populations. *Proc. Natl. Acad. Sci. U.S.A. 94*, 8168–8172.
16. Khlebnikov, A., Datsenko, K. A., Skaug, T., Wanner, B. L., and Keasling, J. D. (2001) Homogeneous expression of the P(BAD) promoter in *Escherichia coli* by constitutive expression of the low-affinity high-capacity AraE transporter. *Microbiology 147*, 3241–3247.
17. Khlebnikov, A., Risa, O., Skaug, T., Carrier, T. A., and Keasling, J. D. (2000) Regulatable arabinose-inducible gene expression system with consistent control in all cells of a culture. *J. Bacteriol. 182*, 7029–7034.
18. Loris, R., Dao-Thi, M. H., Bahassi, E. M., Van Melderen, L., Poortmans, F., Liddington, R., Couturier, M., and Wyns, L. (1999) Crystal structure of CcdB, a topoisomerase poison from *E. coli*. *J. Mol. Biol. 285*, 1667–1677.
19. Dao-Thi, M. H., Van Melderen, L., De Genst, E., Afif, H., Buts, L., Wyns, L., and Loris, R. (2005) Molecular basis of gyrase poisoning by the addiction toxin CcdB. *J. Mol. Biol. 348*, 1091–1102.
20. Schimmel, P. R., and Flory, P. J. (1968) Conformational energies and configurational statistics of copolypeptides containing L-proline. *J. Mol. Biol. 34*, 105–120.
21. Shiraishi, K., Kato, S., Han, S. Y., Liu, W., Otsuka, K., Sakayori, M., Ishida, T., Takeda, M., Kanamaru, R., Ohuchi, N., and Ishioka, C. (2004) Isolation of temperature-sensitive p53 mutations from a comprehensive missense mutation library. *J. Biol. Chem. 279*, 348–355.
22. Ganesh, C., Zaidi, F. N., Udgaonkar, J. B., and Varadarajan, R. (2001) Reversible formation of on-pathway macroscopic aggregates during the folding of maltose binding protein. *Protein Sci. 10*, 1635–1644.
23. Qing, G., Ma, L. C., Khorchid, A., Swapna, G. V., Mal, T. K., Takayama, M. M., Xia, B., Phadtare, S., Ke, H., Acton, T., Montelione, G. T., Ikura, M., and Inouye, M. (2004) Cold-shock induced high-yield protein production in *Escherichia coli*. *Nat. Biotechnol. 22*, 877–882.
24. Cruz-Vera, L. R., Toledo, I., Hernandez-Sanchez, J., and Guarneros, G. (2000) Molecular basis for the temperature sensitivity of *Escherichia coli* pth(Ts). *J. Bacteriol. 182*, 1523–1528.
25. Ohya, Y., and Botstein, D. (1994) Structure-based systematic isolation of conditional-lethal mutations in the single yeast calmodulin gene. *Genetics 138*, 1041–1054.
26. Pogliano, K. J., and Beckwith, J. (1994) Genetic and molecular characterization of the *Escherichia coli* secD operon and its products. *J. Bacteriol. 176*, 804–814.
27. Cormack, B. P., and Struhl, K. (1993) Regional codon randomization: Defining a TATA-binding protein surface required for RNA polymerase III transcription. *Science 262*, 244–248.
28. Rennell, D., Bouvier, S. E., Hardy, L. W., and Poteete, A. R. (1991) Systematic mutation of bacteriophage T4 lysozyme. *J. Mol. Biol. 222*, 67–88.
29. Goldenberg, D. P., Berget, P. B., and King, J. (1982) Maturation of the tail spike endorhamnosidase of *Salmonella* phage P22. *J. Biol. Chem. 257*, 7864–7871.
30. Sturtevant, J. M., Yu, M. H., Haase-Pettingell, C., and King, J. (1989) Thermostability of temperature-sensitive folding mutants of the P22 tailspike protein. *J. Biol. Chem. 264*, 10693–10698.
31. Bajaj, K., Madhusudhan, M. S., Adkar, B. V., Chakrabarti, P., Ramakrishnan, C., Sali, A., and Varadarajan, R. (2007) Stereo-

chemical Criteria for Prediction of the Effects of Proline Mutations on Protein Stability. *PLoS Comput. Biol. 3*, 2465–2475.
32. Kuroki, R., Weaver, L. H., and Matthews, B. W. (1993) A covalent enzyme-substrate intermediate with saccharide distortion in a mutant T4 lysozyme. *Science 262*, 2030–2033.
33. Juo, Z. S., Kassavetis, G. A., Wang, J., Geiduschek, E. P., and Sigler, P. B. (2003) Crystal structure of a transcription factor IIIB core interface ternary complex. *Nature 422*, 534–539.
34. Mondal, K., Dastidar, A. G., Singh, G., Madhusudhanan, S., Gande, S. L., VijayRaghavan, K., and Varadarajan, R. (2007) Design and isolation of temperature-sensitive mutants of Gal4 in yeast and *Drosophila*. *J. Mol. Biol. 370*, 939–950.

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