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Quantitative Correlation between the Protein Expression Level in *Escherichia Coli* and Thermodynamic Stability of Protein In Vitro

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Protein expression using *Escherichia coli* is a common and important method for recombinant protein production. Herein, we quantitatively analyzed the correlation between protein expression in vivo and thermodynamic structure stability in vitro using the tetramerization domain of tumor suppressor protein p53. We found a strong positive relationship between the expression level and the thermodynamic stability. Our study suggests that a minimum thermodynamic stability of a protein is required for substantial protein expression in bacterial cells.

Protein expression using *Escherichia coli* is one of the most powerful and widely used methods for the production of recombinant proteins. *E. coli* has the ability to grow rapidly on inexpensive substrates and to produce recombinant proteins. To date, many researchers have improved the performance of the bacterial expression system to obtain various types of recombinant proteins. *E. coli* expression systems are most commonly used for industrial and pharmaceutical protein production. However, with the bacterial expression system there are still difficulties with expressing and obtaining recombinant proteins, because of the poor growth of the host cell, inclusion bodies formation, protein inactivity, and sometimes not obtaining any protein at all.[1–6] Several studies have reported that the protein expression level of recombinant proteins in *E. coli* depends on protein synthesis and degradation, which are regulated by various factors such as mRNA stability, differences in codon usage between prokaryotes and eukaryotes, and the protein folding state.[7–12] In general, it is thought that the expression of proteins with low structural stability is often difficult. However, quantitative analysis of the correlation between the protein expression level and the structural stability of the protein is still unclear.

Tumor suppressor protein p53 induces cell cycle arrest and apoptosis in response to genotoxic stress, and it functions in a tetrameric form. The tetramerization domain of p53 (p53TD) itself forms a unique oligomeric structure with a size of 20 kDa (Figure 1).[13,14] The tetramer formation of p53TD can be simply described as an equilibrium between unfolded monomers and folded tetramers. The structural features of p53TD have been well characterized in many studies, including comprehensive Ala-scanning and intensive mutational analyses.[15,16] Thermodynamic analysis has revealed that missense mutations of p53TD in tumors destabilized the tetramer formation.[16] The destabilization effects of the mutations ranged from minimal to severe. p53TD missense mutations have the least effects on mRNA stability, because of a point mutation and a simple protein folding pathway other than thermodynamic stability, which are important factors for protein expression in bacterial cells. Therefore, the use of p53TD variants is highly suitable for analyzing the correlation between the structural stability and the expression level. In this study, we quantitatively analyzed the amount of p53TD expressed in *E. coli*, and clearly showed that the amount of the p53TD mutant expressed in *E. coli* was in proportion to the thermodynamic stability of the p53 oligomer formation.

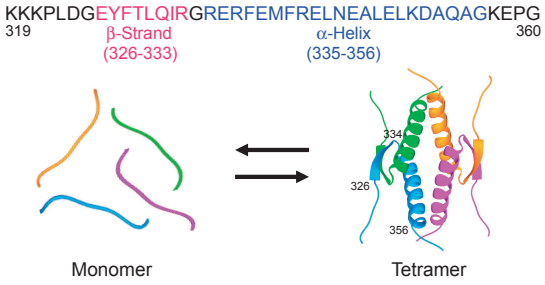

Figure 1. Sequence and structure of the p53 tetramerization domain. The tetramerization domain consists of a β-strand (Glu326–Arg333), a tight turn (Gly334) and an α-helix (Arg335–Gly356). Two monomers form a dimer through their antiparallel β-sheets and α-helices, and two dimers form a tetramer through the formation of a four-helix bundle (Protein Data Bank code 3SAK).

To reveal whether there is a correlation between the expression level in *E. coli* and the thermodynamic stability of p53TD, we expressed a wild-type p53TD (WT) and a variety of tumor-associated mutants in *E. coli* at 37 °C, and quantified their expressed amount in bacterial cells (Table 1).[17–19] The WT showed a high expression level in the cells. Twenty-two mutants (Entries 2–23 in Table 1) were expressed from 23.4% to a similar amount to WT. The expression level of six mutants (Entries 24–29) was very low, and that of the other eight mutants (Entries 30–37) was less than 5% of WT. The relative expression levels of the p53TD mutants compared with WT were analyzed using the ΔG_u values at 37 °C, which were calculated from the in vitro thermal denaturation analysis data.[16] As shown in Figure 2, the expression level of the p53TD mutants strongly correlated with the thermodynamic stability of their tetramer. In the case of p53TD mutants with minimal to moderate destabilization, the correlation between expression levels and their ΔG_u values was approximately proportional. These mutants had ΔG_u values of more than ca. 100 kJ mol^{-1}. On the other hand, the mutants with significant destabilization with lower ΔG_u values were barely expressed in *E. coli*. The mutants with extremely low ΔG_u values and monomer mutants (R342P, R337P, L330R, and L344P) showed virtually no expression in *E. coli*. These results suggest that a substantial p53TD expression level requires thermodynamic structure stability with a certain level of ΔG_u value.

To examine the effect of temperature, we expressed the p53TD mutants at a lower temperature. At 25 °C, the expression levels of the p53TD mutants also showed a positive correlation

Table 1. Parameters of p53TD variants in vivo and in vitro[a]

Entry No.	Mutant	Relative expression level (%WT) at 37 °C	$\Delta G_u^{37°C}$ /kJ mol^{-1}	T_m/°C[b]
1	WT	100 ± 0	144.1	68.4
2	G356A	101.8 ± 2.8	140.9	70.3
3	Y327H	78.8 ± 4.6	125.6	61.2
4	I332V	78.6 ± 10.3	138.0	67.9
5	A353T	72.4 ± 4.5	125.1	63.0
6	E346A	70.0 ± 10.7	132.2	64.6
7	Q354R	67.1 ± 3.5	124.2	66.7
8	R342Q	66.1 ± 6.9	126.3	62.1
9	Q331H	65.0 ± 3.9	138.0	68.6
10	Y327S	64.2 ± 2.5	112.1	56.4
11	E326G	61.4 ± 4.1	130.0	66.3
12	A347G	59.9 ± 5.8	110.5	55.3
13	R342L	57.7 ± 9.7	127.3	62.4
14	G334W	56.2 ± 9.0	110.6	53.0
15	D352H	54.1 ± 7.0	124.0	60.5
16	G334V	54.0 ± 8.7	110.6	49.9
17	E349D	44.4 ± 20.0	105.8	54.3
18	E343G	43.2 ± 10.6	117.9	57.9
19	G356W	38.9 ± 6.4	136.2	68.5
20	F328L	34.1 ± 7.8	108.5	54.5
21	L348F	29.6 ± 6.8	116.9	55.0
22	Q331P	27.4 ± 3.0	124.0	60.2
23	R335G	23.4 ± 4.2	102.3	46.4
24	A347T[c]	9.2 ± 2.9	35.4	44.3
25	R335H	9.2 ± 4.2	117.3	57.8
26	F328V	8.2 ± 2.0	95.0	39.7
27	R337H	6.0 ± 2.1	90.8	36.9
28	F338I	5.6 ± 4.6	90.6	36.8
29	R337L	5.1 ± 5.3	91.5	37.6
30	L330H	4.5 ± 5.4	75.6	27.2
31	L344R[c]	3.5 ± 2.0	31.5	39.0
32	R342P	3.1 ± 6.0	N.D.[d]	Monomer
33	R337C	2.9 ± 4.2	67.9	21.6
34	R337P	2.8 ± 5.0	N.D.[d]	Monomer
35	L330R	2.4 ± 4.5	N.D.[d]	Monomer
36	L344P	2.3 ± 4.5	N.D.[d]	Monomer
37	F341C[c]	0.8 ± 3.8	16.3	23.8

[a]In the second column, the first letter, number, and the last letter indicate the wild-type residue, position of mutation, and mutated residue, respectively, e.g. "G356A" means Gly at position 356 in WT is replaced by Ala in the mutant. The mutants' ΔG_u values of Gibbs free energy during dissociation of tetramer to monomer at 37 °C were calculated using previous experimental data in ref 16. [b]The T_m (melting temperature) values are cited from ref 16. [c]A dimer mutant that only forms a dimeric structure. [d]N.D. indicates ΔG_u value not determine.

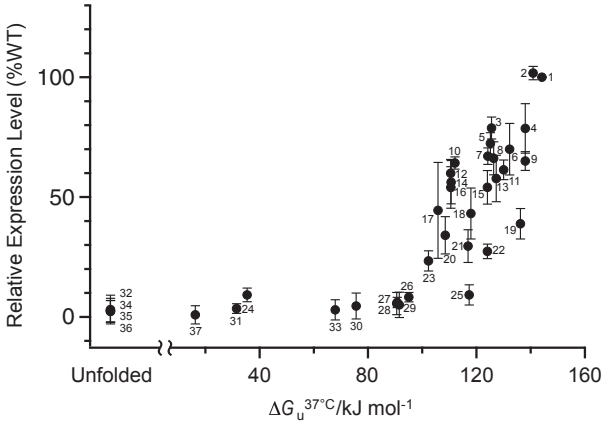

Figure 2. Correlation between thermodynamic stability and expression level of p53TD. The relative expression level to WT is plotted as a function of the ΔG_u value at 37 °C, which was calculated using data of a previous study.[16] Each point is shown by Entry No. as listed in Table 1. "Unfolded" indicates a monomer mutant. The standard errors ($n = 3$) are indicated.

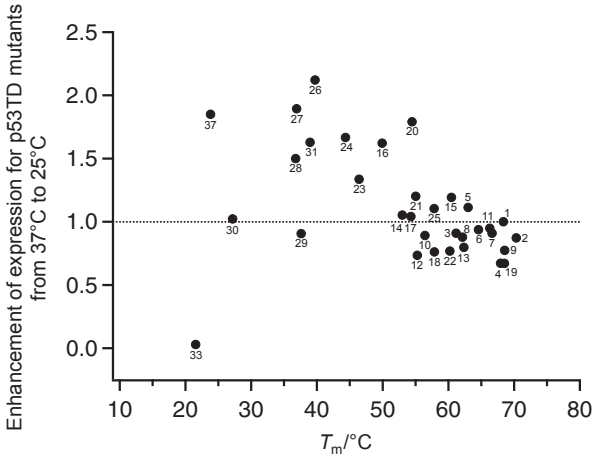

Figure 3. Enhancement of expression by lowering the temperature from 37 to 25 °C. The ratio of mutant expression level at 25 °C to mutant expression level at 37 °C was normalized to that of WT. The ratio is plotted as a function of the T_m value. Each point is shown by Entry No. as listed in Table 1. Unfolded monomer mutants were excluded because of their extremely low expression.

with the thermodynamic stability (Table S1 and Figure S1). Figure 3 shows enhanced expression level of each p53TD mutant between 25 and 37 °C compared with that of the WT. Interestingly, the moderately destabilized mutants, including the mutants with T_m values of 25–55 °C (F328V, G334V, R335G, R337H, and F338I) and dimer mutants (F341C, L344R, and A347T), showed a significant enhanced expression level between 25 and 37 °C compared with the WT (Figure 3). Conversely, the mutants with thermodynamic stability comparable to the WT showed almost the same ratio of expression at 25 and 37 °C as that of the WT. Also, the monomer mutants did not show any increase in their expression levels. These results suggest that the enhancement effect may be due to stabilization of the folded tetramer form by lowering the temperature, and that the content of the tetramer form is important for the bacterial expression. Thus, the enhancement was observed in the moderately destabilized mutants.

In this study, we clearly showed that there is a positive relationship between the protein expression level and the thermodynamic stability of p53TD variants in *E. coli*. Our study suggests that the stability of the folded structures is directly related to the expression level. We think that this is one of the reasons for the fact that expression at a low temperature is often effective for unstable proteins. It has been reported that the thermodynamic stability of the folded structures of protein domains correlates with the rate of intracellular degradation.[20,21] In *E. coli*, there are two major ATP-dependent proteases, Lon and ClpAP, which degrade abnormal proteins.

These proteases bind unfolded regions in the target proteins and degrade them.[22–26] This suggests that destabilized p53TD mutants may be degraded by proteases, because they exist as an unfolded monomer in the cells. However, further studies are required to clarify the detailed mechanism of regulating degradation and synthesis in vivo.

In summary, we demonstrated by quantitative analysis that the expression level of proteins highly correlated with the thermodynamic stability of the folded structure, and that a minimal thermodynamic stability of the folded structure is required for substantial protein expression in *E. coli*.

Supporting Information is available on http://dx.doi.org/10.1246/cl.151019.

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17 Cloning of the p53TD expression plasmid. The *TP53* gene fragment encoding the p53 tetramerization domain (residues 319–360) was amplified by PCR using a plasmid containing wild-type p53 or its mutant using below primers.
p53TD-forward_primer:
5′-GATCTCATATGAAGAAGAAACCACTAGATG-3′
p53TD-reverse_primer:
5′-CCGGATCCGTCGACCTACCCGGGCTCCTTC-3′
The amplification products were digested by *NdeI* and *BamHI*, and ligated with an *NdeI*/*BamHI*-digested pET-3a vector.
18 p53TD expression (37 or 25 °C).
The pET-3a (p53TD) vector was introduced into BL21(DE3) pLysS (TOYOBO) and selected on LB plates containing ampicillin (100 μg mL^{-1}). A single colony was inoculated into LB medium containing ampicillin (100 μg mL^{-1}) and grown overnight at 37 °C. Then, 100 μL of the medium were added into the same LB medium. After 2 h incubation, p53TD expression was induced by addition of IPTG (isopropyl β-D-1-thiogalactopyranoside) to 0.5 mM final concentration for 7 h. The lysate was prepared in 300 μL of SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 6% 2-mercaptoethanol, 10% glycerol, 0.005% bromophenol blue).
19 Quantification of the p53TD expression level.
Each sample was diluted by SDS sample buffer, standardized by OD_{600} and separated on 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After staining with Colloidal Coomassie^{R} G-250 (staining for 5 h, destaining for 17 h), the band intensity of a non-specific endogenous protein at 36 kDa was quantified by Image J. Then, the amount of protein in the samples was standardized again accurately. The final standardized samples were quantified again by the same protocol. The results are shown as a relative value of p53TD intensity, based on the endogenous 36-kDa protein. Relative expression means mutant intensity normalized against wild-type intensity.
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