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Allosteric Switching by Mutually Exclusive Folding of Protein Domains

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Many proteins are built from structurally and functionally distinct domains. A major goal is to understand how conformational change transmits information between domains in order to achieve biological activity. A two-domain, bi-functional fusion protein has been designed so that the mechanical stress imposed by the folded structure of one subunit causes the other subunit to unfold, and *vice versa*. The construct consists of ubiquitin inserted into a surface loop of barnase. The distance between the amino and carboxyl ends of ubiquitin is much greater than the distance between the termini of the barnase loop. This topological constraint causes the two domains to engage in a thermodynamic tug-of-war in which only one can exist in its folded state at any given time. This conformational equilibrium, which is cooperative, reversible, and controllable by ligand binding, serves as a model for the coupled binding and folding mechanism widely used to mediate protein–protein interactions and cellular signaling processes. The position of the equilibrium can be adjusted by temperature or ligand binding and is monitored *in vivo* by cell death. This design forms the basis for a new class of cytotoxic proteins that can be activated by cell-specific effector molecules, and can thus target particular cell types for destruction.

Keywords: molecular switch; unfolding; natively unfolded; allostery

Proteins often display modular architecture that combines protein or small molecule interaction domains with catalytic domains. In such cases, the domains must be coupled, both functionally and structurally, for the protein to attain overall biological activity. For example, ligand binding or phosphorylation can induce structural changes within a regulatory domain that then trigger activity in a catalytic domain. A related type of switching mechanism is illustrated by the recent discovery of proteins that are unstructured in physiological conditions but fold upon binding to their cellular targets.[1,2] Examples include elongin C[3,4] and the GTPase-binding domain of the Wiskott–Aldrich syndrome protein.[5] In these instances, the folding/unfolding of a regulatory domain modulates function of the intact protein *via* propagation of structural changes. Protein folding makes a particularly effective functional switch because it is reversible and inherently cooperative. Understanding the molecular basis for this type of mechanism is important because it is widely used to regulate protein–protein interactions and in signaling pathways that control cellular behavior.[6]

The goal of this study was to develop a two-domain, bi-functional protein model for an allosteric switch governed by protein folding and unfolding. In this system, the mechanical stress imposed by the folded structure of one subunit forces the other subunit to unfold, and *vice versa*. The two domains cannot exist in their native states simultaneously. The position of the conformational equilibrium, and hence the overall function of the protein, is determined by the relative stabilities of the two domains, which in turn can be controlled by external factors such as ligand binding, temperature, or pH. By choosing domains with important biological functions, we further show

T.L.R. & A.I.M. contributed equally to this work.

Abbreviations used: Bn, barnase; Bs, barstar; BU, barnase–ubiquitin fusion protein; GdnHCl, guanidine hydrochloride; GpUp, guanylyl(3′–5′)uridine 3′-monophosphate; Ub, ubiquitin.

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that this design can be exploited to generate a variety of novel applications.

The system consists of a fusion protein in which human ubiquitin (Ub) is inserted into a surface loop of the ribonuclease barnase (Bn) from *Bacillus amyloliquefaciens*. These proteins were chosen for the following reasons. First, Bn is extremely lethal to both prokaryotic and eukaryotic cells. It is able to be synthesized in *B. amyloliquefaciens* only because it is co-expressed with its intracellular inhibitor barstar (Bs).[7] This cytotoxic property allows the enzymatic activity of the fusion protein to be assayed *in vivo* by cell death. Second, existing structural and folding studies of both proteins provide a biophysical framework to construct the fusion protein and interpret the results. The third and most important reason is that they satisfy the primary design consideration, which demands that the distance between the amino and carboxyl ends of the inserted protein be much greater than the distance between the termini of the surface loop in the target protein. Figure 1 shows that the N to C distance in Ub is 38.5 Å and the C^{α}–C^{α} distance between Pro64 and Thr70 of Bn is 10.4 Å. Pro64 and Thr70 represent the approximate termini of a large surface loop. Introduction of cleavage sites or mutations within this loop have minimal effects on Bn structure, stability, and activity.[8–10]

Given the large difference in distances, we predicted that if the Ub fold is more stable than the Bn fold, the Ub domain would forcibly stretch and unfold the Bn domain. If Bn is more stable, it would similarly distort and denature the Ub domain. The topology of the fusion protein should thus cause the two domains to engage in a thermodynamic tug-of-war from which only one can emerge in its folded state at any given time. To test this hypothesis, we inserted the Ub gene between the codons for Lys66 and Ser67 of Bn (at the tip of the surface loop), purified the fusion protein (BU) from *E. coli*, and characterized its structure, stability, and enzymatic function. The circular dichroism (CD) spectra are shown in Figure 2(a) as a function of temperature. Below 20 °C, BU exhibits molar ellipticities and spectral features nearly identical with those of Bn. A particularly diagnostic characteristic of Bn that is observed for BU also is a minimum at 231 nm attributed to Trp94.[11] As temperature is increased, however, the spectrum shifts to one that strongly resembles Ub.[12,13] The 231 nm minimum disappears, and the position and molar ellipticity of the new minimum are consistent with native Ub. The transition has a midpoint of ~30 °C (Figure 2(b)) and is fully reversible upon cooling (not shown). Further, it appears to be two-state: an isodichroic point near 223 nm is apparent, and plotting the transition at different wavelengths yields similar midpoints (not shown). The possibility that the Ub and Bn domains are folded simultaneously is ruled out by the observation that the molar ellipticity of BU never exceeds that of the individual proteins at any wavelength or temperature.

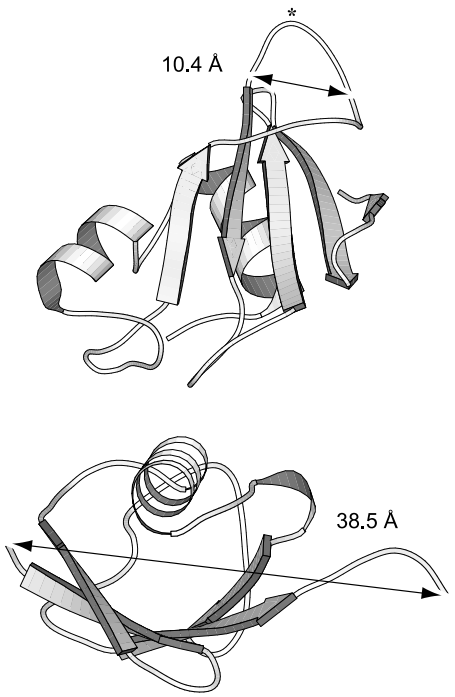


Figure 1. Ribbon diagrams[25] of Bn (top) and Ub (bottom), showing C^{α}–C^{α} distances between Pro64 and Thr70 of Bn and between N and C termini of Ub. The BU gene was constructed by inserting the Ub gene between the Lys66 and Ser67 codons of Bn. Gly-Thr and Gly-Gly-Ser linker sequences were added to the N and C termini of Ub, respectively. The BU expression plasmid pETMT was created by inserting the BU gene into the pET25b(+) plasmid (Novagen). In order to maintain plasmid stability, it was necessary to introduce the Bs gene (together with its natural promoter from *B. amyloliquefaciens*; a gift from Y. Bai, National Institutes of Health) into the same plasmid. BU was expressed in *E. coli* by co-transforming BL21(DE3) cells with a second, kanamycin-resistant plasmid containing the Bs gene under control of the phage T7 promoter. Cells were grown on Luria–Bertani medium[26] to $A_{600} = 1.0$ and induced with 0.4 mM IPTG. Cells were centrifuged three hours later and lysed in 50 mM Tris (pH 7.5), 0.1 mM EDTA. Then 8 M urea was added to dissociate bound Bs, which was subsequently removed by passing the solution through a DE52 column (Whatman). The flow-through was loaded onto a HiTrap heparin column (Pharmacia), washed with 10 mM potassium phosphate (pH 7.5), 6 M urea and eluted with a 0–0.5 M NaCl gradient. BU eluted as one of a series of poorly resolved peaks; Western blot analysis using an anti-Ub antibody revealed that the contaminants were chiefly truncated BU proteins in which the Ub was partially proteolyzed. These products presumably arise because BU is expressed in *E. coli* as the BU–Bs complex, in which the Ub domain is unfolded and susceptible to the action of protease. We were able to purify BU to >95% homogeneity by choosing fractions conservatively; however, this reduced yields to 1–2 mg per liter of starting culture. Finally, urea was removed by extensive dialysis against double-distilled water and the protein was lyophilized.

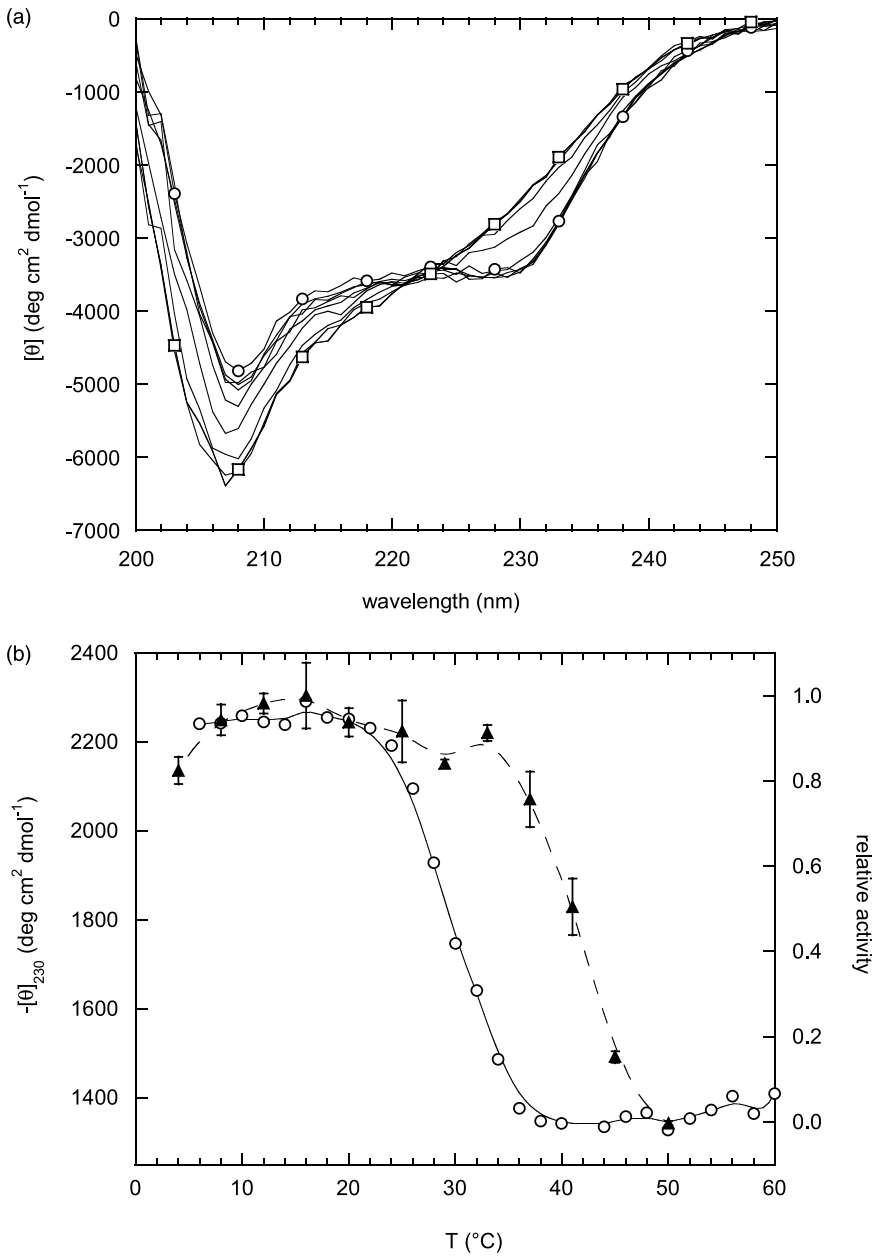


Figure 2. (a) Temperature-induced conformational change of BU demonstrated by CD. All experiments were performed with 1 μM BU in 10 mM potassium phosphate (pH 7.5), 0.1 M NaCl. Circles and squares indicate 5 °C and 50 °C, respectively; other scans were recorded at 5 deg. C increments between these two limits. Data were collected using an Aviv model 202 CD spectropolarimeter. (b) Conversion from the Bn to the Ub form of BU monitored by ellipticity at 230 nm (open circles, continuous line) and Bn enzymatic activity (filled triangles, broken line). Lines are for illustrative purpose only. A 1 ml solution of 50 μM GpUp (Sigma) was allowed to come to thermal equilibrium at the indicated temperature, whereupon 15–25 μl of BU was added (final concentration 2 μM). Initial velocities were recorded by monitoring change in absorbance at 275 nm on a Varian Cary-100 spectrophotometer. Activity is reported as the average initial velocity from three measurements, and is normalized to the highest value observed.

To further test the mutual exclusivity of Ub and Bn domain folding, we monitored urea-induced denaturation by CD and Trp fluorescence. Ub and Bn contain zero and three Trp residues, respectively. Fluorescence is therefore expected to report primarily on structural changes within the Bn domain, whereas CD reports on both domains. At 10 °C, the CD and fluorescence curves reveal a single cooperative unfolding transition (Figure 3). It appears to be two-state: thermodynamic parameters obtained by fitting both data sets to the linear extrapolation equation[14] are identical within error (ΔG= 3.1(± 0.2) kcal mol^{-1}, m=1.5(± 0.1) kcal mol^{-1} M^{-1}, C_m = 2.0(± 0.1) M). The fact that only one transition is apparent by CD confirms that only one domain is folded. The agreement between CD and fluorescence curves indicates that this domain is Bn.

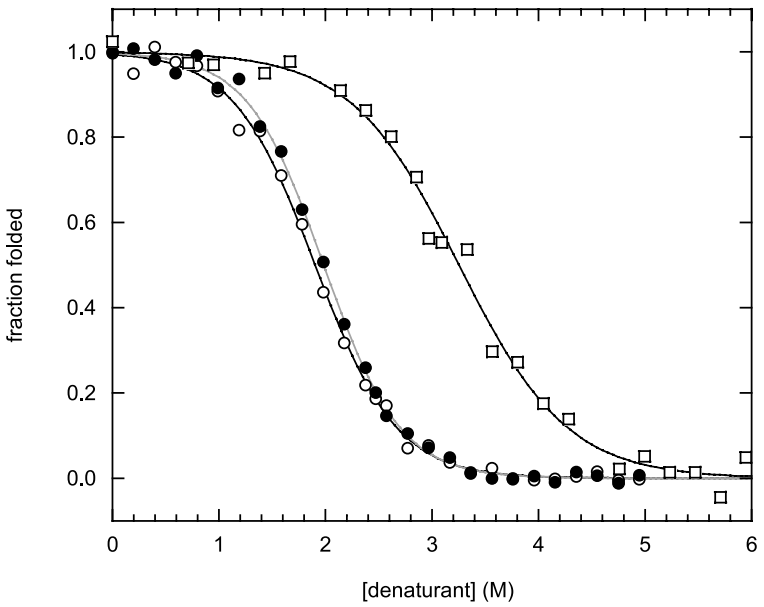
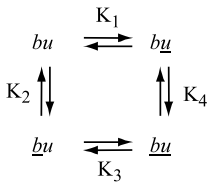


Figure 3. Denaturation of the Bn domain (10 °C, circles) and the Ub domain (50 °C, squares) induced by urea and GdnHCl, respectively. Data were collected by CD at 230 nm (open symbols, black lines) or by Trp fluorescence at 320 nm (filled symbols, grey line), using a Jobin-Yvon/SPEX Fluoromax-3. Lines represent best fits to the linear extrapolation equation, and reported errors are the standard deviations obtained from three samples prepared on different days. Samples were prepared by adding equal volumes of BU to buffer alone and to buffer containing urea (Sigma) or GdnHCl (ICN Biochemicals). The solutions were then mixed in various ratios using a Hamilton Microlab 540B dispenser to yield the final indicated concentrations of denaturant. Denaturant concentrations were determined by measuring refractive index.[27]

If the situation is reversed by raising the temperature above 30 °C, addition of denaturant at 50 °C is predicted to generate an unfolding transition by CD but no transition by fluorescence. Treatment with 6 M urea failed to produce a significant change in either spectrum (not shown). This result is consistent with the previously reported findings that the midpoint of Ub unfolding is 6 M urea at pH 5,[15] and that stability grows dramatically with increasing pH.[16] Addition of the stronger denaturant guanidine hydrochloride (GdnHCl), however, yields the expected CD unfolding transition, with the following thermodynamic parameters: $\Delta G = 4.1(\pm 0.2)$ kcal mol^{-1}, $m = 1.26(\pm 0.1)$ kcal mol^{-1} M^{-1}, $C_m = 3.25(\pm 0.1)$ M (Figure 3). In contrast to molar ellipticity, fluorescence emission at 320 nm does not change significantly as a function of [GdnHCl] (data not shown), suggesting that the Bn Trp residues are solvent-exposed at all concentrations of denaturant. This conclusion is supported by the finding that the wavelength of maximum emission remains constant at 356 nm, the value for unfolded Bn.[17] At 10 °C, this wavelength shifts from 340 nm in the absence of denaturant to 356 nm in 6 M urea (not shown). These data prove that: (i) folding of the Bn domain induces unfolding of the Ub domain; and *vice versa* (ii) temperature provides an efficient switch between the two folded conformations of BU.

The apparent temperature-induced transition from the Bn to Ub forms follows the trend expected from the thermal stabilities of the individual proteins. Figure 4(a) plots the stabilities of free Bn (pH 5.5) and free Ub (pH 4.0) as a function of temperature, using the calorimetric data reported by Privalov and co-workers.[18,19] Bn is more stable than Ub at low temperatures. The larger ΔC_p value of Bn, however, causes its ΔG to curve more sharply, so that it unfolds at a lower temperature (55 °C) than Ub (90 °C). The two proteins are equally stable at 29 °C. To compare our BU results with these data, we model the thermodynamics of BU folding using equation (1):



(1)

where underlined and non-underlined letters denote folded and unfolded domains, respectively. If $\underline{bu}$ is not populated under any conditions, as available evidence indicates, then K_3 and K_4 equal zero and the fractions of folded Bn and Ub domains reduce to equations (2) and (3):

$$f(\underline{b}u) = K_2/(1 + K_1 + K_2) \quad (2)$$

$$f(b\underline{u}) = K_1/(1 + K_1 + K_2) \quad (3)$$

K_1 and K_2, which represent the stabilities of each domain when the other is unfolded, cannot be readily determined by experiment. We therefore calculate the predicted folding/unfolding transition of the Bn and Ub domains by setting K_1 and K_2 equal to the values in Figure 4(a). The resulting curve (Figure 4(b)) shows a two-state transition from folded Bn/unfolded Ub domains to unfolded Bn/folded Ub domains with a midpoint of 29 °C. The close agreement between the experimental data and the theoretical curve (differences in experimental pH values notwithstanding) suggests that the relative stabilities of Bn and Ub are indeed similar in their free states and in the context of the

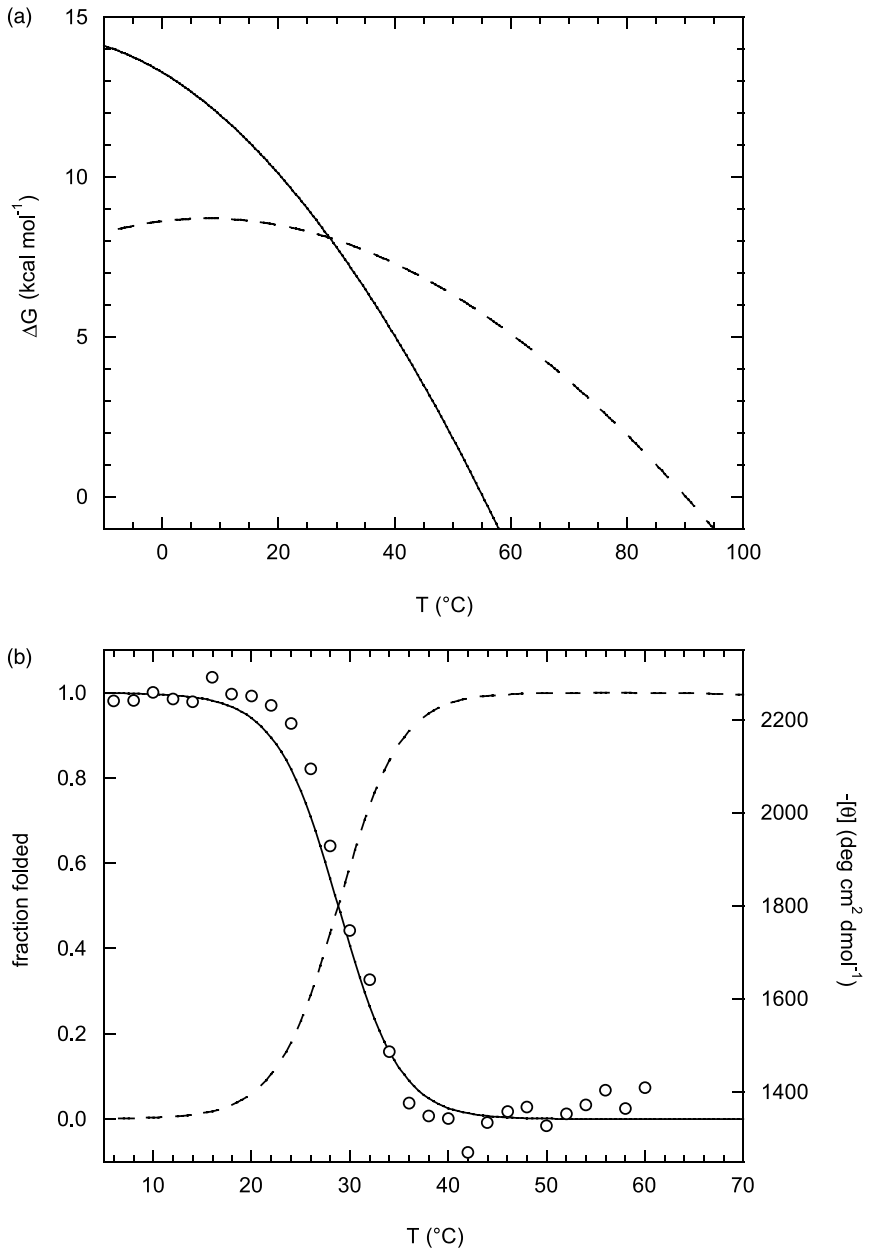


Figure 4. Thermodynamics of BU, Bn and Ub folding. (a) Temperature dependence of the free energies of free Bn (pH 5.5, continuous line) and free Ub (pH 4.0, broken line) folding. The following parameters were used to generate the curves: Bn, $t_m = 55.1$ °C, $\Delta H_m = 119.6$ kcal mol^{-1}, $\Delta C_p = 1.39$ kcal mol^{-1} K^{-1};[19] Ub, $t_m = 90.0$ °C, $\Delta H_m = 73.7$ kcal mol^{-1}, $\Delta C_p = 0.79$ kcal mol^{-1} K^{-1}.[18] ΔC_p was assumed to be independent of temperature. (b) Coupled folding/unfolding of the Bn domain (continuous line) and the Ub domain (broken line) of BU, calculated from equations (2) and (3) (see the text for details). Open circles are the experimental CD data points from Figure 2(b).

BU fusion protein, although their absolute ΔG values may have changed. Most importantly, these data indicate that folding and unfolding of the Bn and Ub domains are tightly coupled, as specified by equations (2) and (3).

To explore the functional consequences of this coupling, we measured BU enzymatic activity as a function of temperature, using the substrate guanylyl(3′–5′)uridine 3′-monophosphate (GpUp). Loss of BU activity mirrors the structural conversion from Bn to Ub (Figure 2(b)), although the apparent midpoint of the former transition occurs at a higher temperature. One explanation is that upon Ub domain folding, the unfolded Bn fragments from two different molecules can refold by intermolecular complementation, thus regenerating an active enzyme. NMR studies of the 1–68 and 69–110 Bn fragments showed that they do in fact form a highly native-like complex (at 2 mM concentration) in which structural changes are located mainly in regions close to the cleavage site.[8] We tested for fragment complementation by repeating the thermal denaturation experiments with BU concentrations from 1 to 10 μM.

Intermolecular folding would be expected to displace the apparent midpoint of the Bn to Ub transition to higher temperatures. No shift was observed, although the low-temperature CD baseline exhibited significant positive drift at 10 μM BU (data not shown). A more likely reason for the shift observed in Figure 2(b) is that substrate binding in the enzyme assays preferentially stabilizes the Bn domain. The concentration of GpUp used in the present assay is 2.5-fold greater than the K_m value of 19.9 μM reported for the Bn–GpUp complex at pH 5.8.[20]

The preceding results suggest that temperature can be used to regulate cytotoxicity of BU *in vivo*. We tested this prediction by transforming *E. coli* BL21(DE3) with a plasmid containing the BU gene under control of the IPTG-induced phage T7 promoter. IPTG was then added to increase intracellular BU levels while the cells were growing at different temperatures. Like Bn, BU is extremely toxic: basal expression levels are lethal to *E. coli* even in the absence of IPTG (data not shown). It was therefore necessary to include the Bs gene (under control of its natural promoter) in the same plasmid. Bs suppresses ribonuclease activity until it is overwhelmed by BU upon induction by IPTG.[21]

At 15 °C, BU and Bn exhibit similar toxicities, as evidenced by a comparable number of colonies at each concentration of IPTG (Table 1). The ribonuclease activity of Bn therefore does not appear to be compromised when it is in the BU fusion protein. In contrast, BU is significantly less toxic than Bn at 37 °C. IPTG at a concentration of 100 μM is sufficient to kill all bacteria bearing the Bn plasmid, whereas 150 μM IPTG is required to achieve that result with cells expressing BU. The extra IPTG required indicates that the intracellular concentration of BU must be higher than that of Bn in order to hydrolyze similar amounts of endogenous RNA. This difference in intracellular enzyme levels is likely to be substantial, because protein induction increases sharply with [IPTG], as demonstrated by the precipitous drop in observed colonies above a characteristic [IPTG]. These findings reflect the temperature-induced conformational shift from the toxic Bn-form of BU to the harmless Ub form. Further, they demonstrate that the BU/*E. coli* system is highly responsive to the relative stabilities of the two domains within the temperature range of bacterial growth.

"Natively unfolded" proteins contain subunits that are unstructured or partially structured in physiological conditions. The free energy of folding is provided by ligand-binding interactions, and the resulting conformational change is used to modulate function of distant domains.[1,2] BU captures this property as well. When Bs is added to the Ub form of BU at 40 °C, a conformational change is observed (Figure 5(a)). The CD spectrum of the BU–Bs complex, after subtracting the spectrum of free Bs, resembles that of BU at 15 °C (Figure 5(b)). The two are not identical, apparently because of minor structural differences between the free and bound states of Bn and/or Bs. However, the CD spectra of the BU–Bs complexes at 40 °C and 15 °C are virtually indistinguishable. This result signifies that Bs binding is coupled to folding of the Bn domain, which in turn drives unfolding of the Ub domain. To our knowledge, no existing, natively unfolded protein exhibits this property. Rather, it has been proposed that unstructured domains exist because their increased flexibility allows them to interact with multiple cellular partners, or that their greater capture radius increases the rate of binding.[22] Our results suggest another function for natively unfolded domains: the extensive conformational changes that accompany folding can be harnessed to force a correspondingly extreme structural change in another part of the molecule.

In summary, we have demonstrated that the folding free energy of one protein domain can be used to drive unfolding of another. Because folding is reversible and inherently cooperative, this mechanism constitutes an efficient and responsive allosteric switch for coupling conformational change to protein function. The cytotoxic activity of Bn in the present case provides the basis for several novel applications. Stability-enhanced Ub variants can be identified rapidly from combinatorial libraries by their ability to allow *E. coli* to survive in the presence of IPTG at concentrations (>150 μM at 37 °C or >50 μM at 15 °C) that would be toxic to cells expressing wild-type BU. A major advantage that this approach affords over other directed evolution techniques (such as phage display) is that the entire selection takes place inside a living bacterium, and stabilizing mutations are sorted from destabilizing mutations

Table 1. Cytotoxicity of BU and Bn as a function of temperature and [IPTG]

Variant	t (°C)	Number of colonies at indicated [IPTG]					
		0 μM	10 μM	30 μM	50 μM	100 μM	150 μM
BU	15	229 ± 71	189 ± 37	13 ± 17	0 ± 0	0 ± 0	0 ± 0
BU	37	490 ± 95	394 ± 76	168 ± 23	159 ± 32	122 ± 19	0 ± 0
Bn	15	260 ± 68	203 ± 51	25 ± 46	1.2 ± 2	0 ± 0	0 ± 0
Bn	37	444 ± 99	385 ± 80	225 ± 15	180 ± 44	0 ± 0	0 ± 0

E. coli BL21(DE3) were transformed with plasmids bearing BU or Bn, grown to $A_{600} = 0.2$ in Luria–Bertani medium (50 μg/ml of ampicillin, 37 °C). Cells were then diluted 10^4-fold and 0.2 ml was spread onto Petri dishes containing 50 μg/ml of ampicillin and the indicated concentration of IPTG. The values are the averages and standard deviations obtained from five plates.

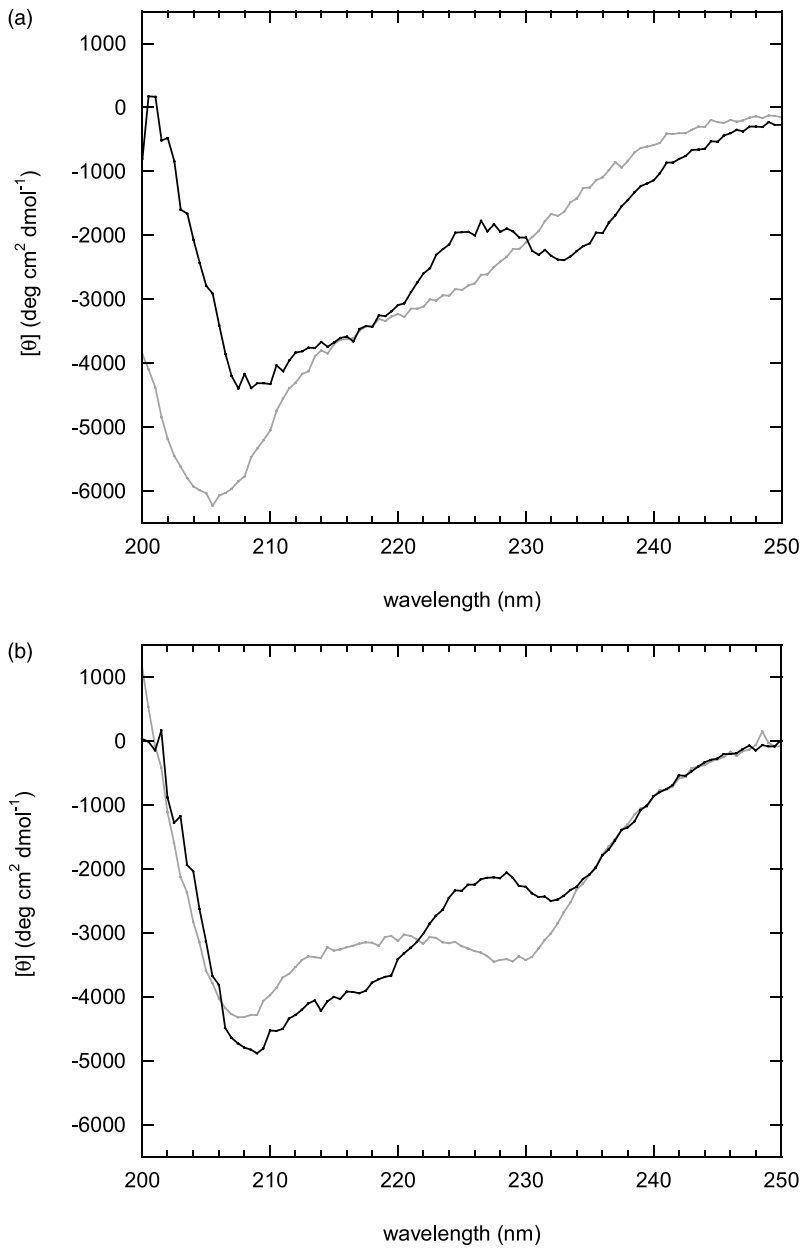


Figure 5. Bs binding induces folding of the Bn domain and unfolding of the Ub domain. CD spectra were recorded at (a) 40 °C, (b) 15 °C. Gray and black lines represent 10 μM BU in the absence and in the presence of 12.5 μM Bs, respectively. Black lines were generated by subtracting the CD spectra of free Bs.

in an efficient and decisive manner. This strategy is general and can be applied to proteins other than Ub, including those that are too large to be expressed on the phage surface. The primary consideration is that the N to C-terminal distance must be significantly greater than ~20 Å. If necessary, known stabilizing or destabilizing mutations can be introduced into the Bn domain[10,23] in order to make the system optimally responsive to the inserted protein.

Finally, the system can be modified to generate a class of cytotoxic molecules with novel sensor capabilities. Ribonucleases have emerged as an important class of anti-cancer and anti-viral molecules.[24] Their efficacy, however, is hampered by their lack of specificity. They cleave RNA with the same remarkable efficiency whether inside healthy or diseased cells. The mutually exclusive folding design allows ribonuclease activity to be turned on and off by ligand binding to an engineered regulatory domain. Ub does not possess a high-affinity, small-molecule binding site; however, receptor domains from any one of a large number of proteins can perform this function as long as they meet the simple topological criterion described above. This switching mechanism forms the basis for developing cytotoxic proteins that are activated by a wide variety of cell-specific effector molecules, and can thus potentially target cancerous or virally infected cells for destruction.

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