Designing redox potential-controlled protein switches based on mutually exclusive proteins

Qing Peng, Na Kong, Hui-Chuan Eileen Wang, and Hongbin Li*

Department of Chemistry, University of British Columbia, Vancouver, British Columbia V6T 1Z1, Canada

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Abstract: Synthetic/artificial protein switches provide an efficient means of controlling protein functions using chemical signals and stimuli. Mutually exclusive proteins, in which only the host or guest domain can remain folded at a given time owing to conformational strain, have been used to engineer novel protein switches that can switch enzymatic functions on and off in response to ligand binding. To further explore the potential of mutually exclusive proteins as protein switches and sensors, we report here a new redox-based approach to engineer a mutually exclusive folding-based protein switch. By introducing a disulfide bond into the host domain of a mutually exclusive protein, we demonstrate that it is feasible to use redox potential to switch the host domain between its folded and unfolded conformations via the mutually exclusive folding mechanism, and thus switching the functionality of the host domain on and off. Our study opens a new and potentially general avenue that uses mutually exclusive proteins to design novel switches able to control the function of a variety of proteins.

Keywords: mutually exclusive protein; protein switch; stopped flow spectrofluorometry; protein folding and unfolding

Introduction

Many enzymes in biological systems can be switched on and off in response to signaling events to precisely regulate their biological functions.^{1–5} Engineering synthetic/artificial protein switches, whose function can be controlled by chemical signals and stimuli, is of tremendous importance toward the understanding of basic biological problems, as well as for the development of novel therapeutics and biosensors that could find application over a wide range of applications.^{6–14} Over the last decade, tremendous progress has been made in this field.^{6–12,15–20} Creating fusion proteins has become a commonly used

approach toward engineering protein switches, where a signal-induced conformational change in one domain affects the structure and thus the function of the second domain.^{6–12,15–20}

Among the fusion protein-based approaches, the mutually exclusive folding-based approach is a newly developed method that encompasses several unique features and advantages.^{21–25} Mutually exclusive proteins are engineered artificial domain-insertion proteins. In a typical mutually exclusive protein, the guest domain, which has a longer distance between its N–C-termini, is inserted into one of the loops of a host domain, whose loop length is significantly shorter than the N–C distance of the guest domain.^{21,25} Such structural incompatibility leads to a folding tug-of-war between the host and the guest domains, with the result that only one domain can remain folded at a given time.^{21,25} Whether the host or guest domain is folded at the equilibrium conformation of the mutually exclusive protein depends on the relative thermodynamic

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*Correspondence to: Hongbin Li, Department of Chemistry, University of British Columbia, Vancouver, British Columbia V6T 1Z1, Canada. E-mail: Hongbin@chem.ubc.ca

Figure 1. Design principle of the redox potential-dependent protein switch based on a mutually exclusive protein design. (A) Three-dimensional structure of GB1 (PDB code: 1PGB). The hFc binding epitope of GB1 is highlighted in yellow. Residues that are in the hFc binding epitope of GB1 and in direct contact with hFc are highlighted in yellow. (B) The three-dimensional structure of the GC2/hFc complex (PDB code: 1FCC). GC2 is highly homologous to GB1, only differing by two residues. The structure of GC2/hFc has been used as a model for the GB1/hFc complex. GC2 is colored in green and hFc is colored in yellow. In (A and B), the loop where I27w34f is inserted to construct the mutually exclusive protein is highlighted in blue. (C) The schematic showing the equilibrium of the conformational change between the oxidized form and the reduced form of the mutually exclusive protein GL5CC–I27w34f. The equilibrium can be controlled using either oxidizing and reducing reagents. GL5CC is colored in green, and I27w34f is colored in pink. The two cysteine residues are colored in yellow. In the oxidized conformation GL5CC(F)–I27w34f(U), the mutually exclusive protein should have a high binding affinity to hFc; in the reduced state GL5CC(U)–I27w34f(F), the mutually exclusive protein should not bind to hFc.

stability of the two domains. It is possible to use ligand binding to reverse the relative stability of the two domains, and thus change the conformational state of the mutually exclusive protein. Taking advantage of this feature, Loh and coworkers designed the first mutually exclusive protein-based switch/sensor, in which a GCN4-DNA-binding domain was inserted into one of the surface loops of ribonuclease barnase.²³ DNA binding forced the unfolding of the host domain barnase, thus switching the ribonuclease activity of barnase off.²³ This study demonstrated the great potential of using mutually exclusive proteins to design novel protein switches/sensors for various applications. To further explore the potential of mutually exclusive proteins as applied in the design of novel protein switches/sensors, we report a new approach toward engineering a mutually exclusive folding-based protein switch. This new approach uses an engineered disulfide bond as a modulating motif to regulate protein function by controlling the conformation of the

mutually exclusive protein via an applied redox potential. As a proof-of-principle, here we engineer a redox potential-dependent protein switch to regulate the IgG-binding affinity of the small protein GB1.

Results and Discussion

Design of the mutually exclusive folding-based protein switch

The principle of our approach is to use chemical stimuli to shift the equilibrium between the two conformations of the mutually exclusive protein to regulate protein function. In our previous study, we designed a mutually exclusive protein GL5–I27w34f,²⁵ in which a W34F mutant of the 27th Ig domain from the giant muscle protein titin was inserted into the host domain GL5, a loop insertion mutant of the B1 IgG binding domain of protein G (GB1).^{26,27} Using stopped-flow spectrofluorometry and single molecule atomic force microscopy techniques, we observed a tug-of-war between the two

proteins that occurs during the folding of this mutually exclusive protein.²⁵ Owing to its fast folding kinetics, GL5 folds first, resulting in the GL5(F)–I27w34f(U) conformation, where GL5(F) indicates that GL5 is folded, whereas I27w34f(U) is unfolded. As I27w34f is thermodynamically more stable than GL5, the subsequent slow folding of I27w34f results in the forced unfolding of the GL5 host domain, resulting in the GL5(U)–I27w34f(F) conformation, where GL5 is unfolded and I27w34f is folded. Our results showed that GL5(U)–I27w34f(F) is the dominant conformation in equilibrium.²⁵

GL5 retains the high binding affinity of GB1, binding to the hFc fragment of the IgG antibody with high affinity. Figure 1(A, B) shows the crystal structure of GB1 and its homologue GC2 in complex with the Fc fragment of human IgG antibody (hFc).²⁸ GC2, the C2 IgG binding domain of protein G, is highly homologous to GB1, where the structure of GC2/hFc has been used as a model for the structure of the GB1/hFc complex.^{28,29} As the Fc binding epitope ([Fig. 1(A)], colored in yellow) is encoded in the folded structure of GB1, we argue that the binding affinity can be switched off (or inactivated) if GL5 is unfolded. Based on this hypothesis, we used the mutually exclusive protein approach to design a protein switch to regulate the Fc binding affinity of GL5. The key for this design was the ability to regulate the conformational state of GL5 in the mutually exclusive protein using a chemical stimulus to control the Fc binding properties of GL5.

Figure 1(C) shows the protein switch design, engineered to be controlled by its redox potential. In this design, we make use of a disulfide mutant of GL5–C41 and 43 (GL5CC). We found that residues 41 and 43, when mutated into cysteines, can readily form a disulfide bond under oxidizing conditions.³⁰ In the oxidized state, the formation of the disulfide bond between C41 and C43 will drive the equilibrium toward the GL5(F)–I27w34f(U) conformation, allowing GL5 to bind Fc; under reducing conditions, the disulfide bond will break and the folding of I27w34f will forcibly unfold GL5, shifting the equilibrium toward the GL5(U)–I27w34f(F) conformation. In this form, the Fc binding ability of GL5 should be switched off, allowing the Fc binding ability of GL5–I27w34f to be modulated by redox potential; thus, the new protein can serve as a protein switch controlled by redox potential. To realize this design, two conditions have to be met: (1) the thermodynamic stability of I27w34f must be higher than that of the GL5/Fc complex; (2) the folding and unfolding of I27w34f must be controlled by the redox potential.

I27w34f is thermodynamically more stable than GL5+hFc complex

It is well known that ligand binding will increase the thermodynamic stability of protein participating

Figure 2. Typical tryptophan fluorescence traces showing the folding of GL5–I27w34f in 0.3M GdmCl. Black trace is in the absence of hFc and gray trace is in the presence of 1.75 mg/mL hFc. The presence of hFc increased the relative population of GL5(F)–I27w34f(U), but GL5(U)–I27w34f(F) is still the dominant conformation of the mutually exclusive protein, suggesting that I27w34f is thermodynamically more stable than the GL5–hFc complex.

in binding. To switch off the Fc binding ability of GL5–I27w34f even when the hFc fragment is bound via the mutually exclusive folding mechanism, the thermodynamic stability of I27w34f must be higher than that of GL5–hFc complex. To qualitatively determine the relative thermodynamic stability of the GL5–hFc complex versus I27w34f, we used stopped-flow spectrofluorometry. In these experiments, the tryptophan fluorescence was used as a probe. As I27w34f does not contain tryptophan residues, the tryptophan fluorescence measured from GL5CC–I27w34f solely reflects the folding and unfolding dynamics of the host protein GL5.²⁵ Figure 2 shows the time evolution of tryptophan fluorescence from GL5–I27w34f as it folds in 0.3M GdmCl and in the absence (black trace) and presence of saturating concentration of hFc (gray trace).

As we demonstrated before, the folding of the mutually exclusive protein GL5–I27w34f shows characteristic tug-of-war folding behavior: the tryptophan fluorescence shows a rapid rising phase, which corresponds to the folding of GL5 and the formation of the conformation GL5(F)–I27w34f(U), followed by a slower decay phase, which corresponds to the subsequent unfolding of GL5 and the formation of GL5(U)–I27w34f(F) conformation (black trace, Fig. 2). At equilibrium, ~80% of the tryptophan fluorescence has decayed from its peak amplitude during the folding of GL5 domain, suggesting that 80% of the mutually exclusive protein exists as GL5(U)–I27w34f(F) under equilibrium conditions. Our previous single-molecule atomic force microscopy experiments also confirmed this conclusion.²⁵

It is worth noting that several groups reported that there exists an on-pathway folding intermediate

state in the folding of GB1 although the nature of this intermediate state is still under debate.^{31–33} The existence of an intermediate state could potentially complicate the assignment of the conformational state of GL5 in the mutually exclusive protein in stopped-flow experiments. However, the folding intermediate state observed for GB1 occurs at a sub-millisecond time scale. This time scale is orders of magnitude faster than the folding kinetics of GL5 used in our current study. Moreover, owing to the loop insertion, GL5 folds slower than GB1. Thus, from a practical point of view, the change of tryptophan fluorescence in our stopped-flow experiments can be largely attributed to the folding/unfolding of GL5. Accordingly, we can assign the high tryptophan fluorescence state in our stopped-flow experiments as the folded state of GL5, and low-fluorescence state as the unfolded state of GL5. Accordingly, we can use stopped-flow spectrometry to determine the conformational state of the mutually exclusive protein GL5–I27w34f. Moreover, our previous study on GL5–I27w34F showed that accompanying the tryptophan fluorescence decay phase (the unfolding phase of GL5), the secondary structure of GL5 in the mutually exclusive protein also shows a similar exponential decay phase (as monitored by the CD signal of GL5 at 221 nm), whereas I27w34f showed a similar exponential increasing phase (as monitored by the characteristic ellipticity of folded I27 domain at 230 nm), strongly suggesting that the fluorescence decay phase observed in our stopped-flow experiments indeed correspond to the unfolding of GL5 (and folding of I27w34f).

In the presence of saturating concentration of hFc, the folding of the mutually exclusive protein GL5–I27w34f exhibited similar tug-of-war folding behaviors (gray trace, Fig. 2). However, at equilibrium, tryptophan fluorescence of the mutually exclusive protein in the presence of hFc is higher than in the absence of hFc, suggesting that the binding of hFc indeed stabilizes GL5(F)–I27w34f(U) conformation. Saturating concentration of hFc can lead to a ~10% increase of the GL5(F)–I27w34f(U) population. However, the conformation GL5(U)–I27w34f(F) remains the dominant form (~70%) in equilibrium, suggesting that the thermodynamic stability of the GL5–hFc complex is lower than I27w34f, providing the possibility that the mutually exclusive folding mechanism may be utilized to unfold GL5 and switch off its Fc binding ability.

The conformation of the mutually exclusive protein can be regulated by redox potential

To establish that the conformation of the mutually exclusive protein can be modulated by redox potential, we engineered a disulfide bond mutant GL5CC and used it as the host domain for the mutually exclusive protein. We then carried out stopped-flow spectrofluorometry experiments to characterize the

Figure 3. Typical tryptophan fluorescence traces for the folding of GL5CC–I27w34f in 0.3M GdmCl. The gray curve represents the oxidized form, where the black curve is from the reduced form. The tryptophan fluorescence of GL5CC–I27w34f exhibits a fast rising phase followed by a much slower decay phase. The amplitude of the decay phase in the oxidized mutually exclusive protein is significantly smaller than that in the reduced conformation. In the oxidized mutually exclusive protein, a double exponential fit to the folding phase of GL5 domain results in rate constants of 3.9 and 22.6 s^{−1} in 0.3M GdmCl (gray). The unfolding phase of GL5 is best described by a double exponential fit with rate constants of 0.087 and 0.022 s^{−1} in 0.3M GdmCl (gray). In the reduced mutually exclusive protein, the folding phase of the GL5 domain can be well-described by a single exponential with a rate constants of 7.8 s^{−1} (black). At the same time, the decay phase is best described by a double exponential fit with rate constants of 0.30 and 0.039 s^{−1}, respectively (black).

folding kinetics of the mutually exclusive protein GL5CC–I27w34f and obtain information about its conformational state. If the disulfide bond can indeed form under oxidizing conditions, the conformation GL5CC(F)–I27w34f(U) should be the dominant form in equilibrium. Thus, the folding kinetics of the mutually exclusive protein should be characterized by the folding kinetics of the host GL5CC domain, as the disulfide bond will prevent the folding of the guest I27w34f domain. Under reducing conditions, the disulfide bond will be reduced and the conformation GL5CC(U)–I27w34f(F) should be dominant in equilibrium. Thus, the folding kinetics of the mutually exclusive protein under reducing conditions should behave in a similar way to GL5–I27w34f.

Figure 3 shows the time evolution of the tryptophan fluorescence of GL5CC–I27w34f during the folding reaction in 0.3M GdmCl under oxidizing (colored in gray) and reducing conditions (colored in black). When the folding reaction of the oxidized GL5CC–I27w34f was initiated (gray line), we observed a rapid rising phase of the tryptophan fluorescence, followed by a small decay phase. The amplitude of the decay phase is only ~20% of that of the rising phase. This suggests that GL5CC quickly

Figure 4. Far UV CD spectra of the mutually exclusive protein show that the reduction of the disulfide can convert GL5CC(F)–I27w34f(U) to GL5CC(U)–I27w34f(F). (A) Far UV CD spectra of GL5CC–I27w34f in the oxidized and reduced condition. (B) Time evolution of the ellipticity at 221 and 230 nm of the mutually exclusive protein after addition of 6 mM DTT.

refolds, with only ~20% of GL5CC subsequently unfolding after refolding. In comparison, under reducing conditions (black line), the tryptophan fluorescence of the mutually exclusive protein exhibited very different behaviors: after a rapid rising phase, ~70% of the tryptophan fluorescence decayed. This behavior is similar to that of GL5–I27w34f, which results from the initial folding of the host GL5 domain followed by its unfolding (which is driven by the folding of the I27w34f domain). This drastic difference in the amplitude of the decay phase under oxidizing and reducing conditions clearly shows the significant difference in folding ability of I27w34f between the two conditions. Under oxidizing conditions, only ~20% of GL5 can unfold, whereas ~70% can unfold under reducing conditions. This difference can be readily attributed to the formation of a disulfide bond in GL5CC under oxidizing conditions. Our results suggest that under oxidizing conditions, ~80% of the mutually exclusive protein exists in the GL5CC(F)–I27w34f(U) conformation; meanwhile 20% remains in the GL5CC(U)–I27w34f(F) conformation, giving rise to the 20% decay phase exhibited. Under reducing conditions, driven by the folding of the I27w34f domain, ~70% GL5CC unfolded for a second time after refolding, giving rise to the observed ~70% decay of tryptophan fluorescence.

To further confirm the formation of a disulfide bond by residues Cys41 and Cys43 in GL5CC–I27w34f under oxidizing conditions, we also carried out Ellman's test. Ellman's test showed that only ~20% of cysteines exist as free thiols, suggesting that 80% of the cysteines in the mutually exclusive protein exist as disulfide bonds and GL5CC(F)–I27w34f(U) is the dominant conformation under oxidizing conditions. This result is in close agreement with stopped-flow results.

Our results clearly indicate that the formation of the disulfide bond shifts the equilibrium

GL5CC(F)–I27w34f(U) $\rightleftharpoons$ GL5CC(U)–I27w34f(F) toward the GL5CC(F)–I27w34f(U) conformation, and thus preventing the folding of I27w34f and unfolding of GL5CC. In contrast, under reducing conditions, the disulfide bond is absent and there is no constraint on the folding of I27w34f. Thus, GL5CC–I27w34f shows a typical tug-of-war behavior between I27w34f and GL5CC. After GL5CC folds, folding of the thermodynamically more stable I27w34f drives the unfolding of GL5 and shift the equilibrium toward the GL5CC(U)–I27w34f(F) conformation, providing a good explanation as to why 73% of the GL5 tryptophan signal decayed.

Having established that the formation of the disulfide bond can lead to the conformation GL5CC(F)–I27w34f(U) under oxidative condition, we then used circular dichroism spectroscopy to directly demonstrate that the addition of reducing agent dithiothreitol (DTT) can shift the conformation from GL5CC(F)–I27w34f(U) to GL5CC(U)–I27w34f(F). Figure 4(A) shows the far-UV CD spectra of GL5CC–I27w34f in the absence and presence of DTT. The CD spectra of both oxidized and reduced conformations are different from that of standalone GL5 and I27w34f, and are convolution of the CD signal of GL5CC and I27w34f domains in the mutually exclusive protein. It is evident that the reduction of disulfide bond in the mutually exclusive protein results in large secondary structure change. To monitor the change of secondary structures of the mutually exclusive protein upon reduction of the disulfide bond, we used the ellipticity at 221 nm to monitor the folding/unfolding transition of the GL5CC domain, and ellipticity at 230 nm to monitor the conformation state of I27w34f (which is characteristic of the folded I27 domain). As shown in Figure 4(B,C), ellipticity at 221 and 230 nm increases as a function of time, indicative of the loss of the folded structure of GL5 (α/β) and formation of folded I27w34f (all β).

These results suggest that the reduction of the disulfide bond leads to the unfolding of GL5CC and refolding of I27w34f, and thus converting the mutually exclusive protein from GL5CC(F)–I27w34f(U) to GL5CC(U)–I27w34f(F).

Switching off the IgG binding ability of GL5CC–I27w34f by reduction

Having established that we can modulate the folded state of GL5 in the mutually exclusive protein, and that I27w34f is thermodynamically more stable than the GL5/hFc complex, we tested the feasibility of using the mutually exclusive protein as a redox-dependent switch to regulate the Fc binding ability of GL5. For this purpose, we used surface plasmon resonance (SPR) spectroscopy to characterize the binding of GL5CC–I27w34f to the Fc fragment of hFc. GL5, like wild-type GB1, binds the hFc fragment of IgG antibody with high affinity. If GL5 is folded in the mutually exclusive protein, we expect that the mutually exclusive protein will be able to bind hFc fragment with similar affinity; if the GL5 is unfolded, the IgG binding ability of the mutually exclusive protein should be switched off. Figure 5(A) shows a sensorgram of the mutually exclusive protein under oxidizing conditions. It is evident that under oxidizing conditions, the mutually exclusive protein binds the hFc fragment with high affinity. Fitting the SPR sensorgram to a 1:1 Langmuir association model yielded a dissociation constant K_d of 233 nM for the binding of GL5CC–I27w34f to hFc under oxidizing conditions. However, in the presence of 20 mM DTT, the mutually exclusive protein does not show hFc binding, suggesting that the folding of I27w34f “forced” unfolding of GL5CC, and in the process switched the hFc binding affinity of GL5CC off.

To confirm that the loss of hFc binding affinity of the mutually exclusive protein under reducing conditions is not owing to the interference of the reducing agent DTT, we characterized the binding affinity of a control protein GL15CC in the absence and presence of DTT using SPR. Instead of GL5CC, GL15CC, which is a loop insertion mutant of GB1 and carries a flexible loop of 15 residues, was used in this control experiment to mimic the potential effect of the unfolded I27w34f on the binding affinity of GL5 to hFc. Figure 6 shows the SPR sensorgrams of the binding of GL15CC to hFc under oxidizing and reducing conditions. Fitting the resultant SPR sensorgrams resulted in a K_d of 184 nM for the oxidized GL15CC and 371 nM for the reduced form, respectively. It is clear that the presence of DTT does not affect the binding affinity of GL5 to hFc fragment, and that the loss of hFc binding affinity in the mutually exclusive protein under reducing conditions is owing to the structural disruption of GL5CC. It is worth noting that the dissociation constant of the oxidized mutually exclusive protein

Figure 5. SPR sensorgrams for the binding and dissociation of oxidized (A) and reduced (B) forms of mutually exclusive protein GL5CC–I27w34f to hFc. In SPR experiments, hFc was immobilized onto the SPR chip. Concentrations of analytes are indicated on each individual curve. Using the Langmuir 1:1 association model, the dissociation constant K_d for the oxidized form of GL5CC–I27w34f to hFc was determined to be 233 nM. In contrast, the reduced form of GL5CC–I27w34f does not show significant binding to hFc.

GL5CC–I27w34f is close to the K_d of isolated GL15CC under similar conditions (233 versus 184 nM), suggesting that the binding epitope of the oxidized GL5CC–I27w34f remains largely intact as compared to isolated GL15CC.

These results clearly indicate that by introducing the disulfide bond in the host domain, we can now control the conformational state of the host domain through mutually exclusive folding mechanism, allowing us to switch the functionality of the host domain off. This approach is different from traditional methods in that it does not use a combination of sensing and effector domains.^{6–12} Instead, the engineered disulfide bond serves as a locking mechanism to trap the mutually exclusive protein in a conformation that is otherwise unfavorable, where the guest domain serves as the trigger domain to convert the mutually exclusive domain to its favorable conformation once the disulfide bond is ruptured. In addition, this method does not depend on ligand binding. As the disulfide bond can be introduced in

Figure 6. SPR sensorgrams tracking the binding and dissociation of oxidized (A) and reduced (B) GL15CC to hFc. Analyte concentration is indicated on individual curves. Using the Langmuir 1:1 association model, the dissociation constants of hFc were determined to be 184 nM for the oxidized GL15CC, and 371 nM for the reduced GL15CC.

an unstructured loop of the host domain relatively easily, the method reported here has the potential to serve as a general switch mechanism to design novel protein switches that can control the functions of other proteins of interest. This approach has enormous potential toward the construction of smart functional protein-based devices and/or surfaces.

The protein switch reported here clearly demonstrates a proof-of-principle that redox potential and mutually exclusive proteins may be successfully used to modulate protein function. However, it is important to note that this protein switch is yet to be optimized. The response of this protein switch is relatively slow and not fully reversible. Much work needs to be done to improve the response of such protein switches.

The response of this protein switch depends on two main factors: (1) redox kinetics of the disulfide bond; and (2) folding rate of the host and guest domains. The reduction of the disulfide bond occurs on a time scale of minutes, and can be tuned readily by adjusting the concentration of the reducing agent. However, the oxidation process is much slower than the reduction process. It is necessary to use more efficient oxidation processes such that the “on” state of the mutually exclusive protein switch may be more efficiently regenerated. For such purposes, efficient enzymes, such as protein disulfide isomerase, can be

employed to accelerate the oxidation of disulfide bond and thus the rate of switch. Modulating the folding rate of the guest and host domains is another potential approach to improve the response of the protein switch. It has been shown that the folding rate of GB1 domain depends on pH.³³ Thus, it is possible to use pH as an additional means to tune the response of this protein switch.

Recently, Loh and co-workers explored the use of circular permutants that are conformationally constrained by a disulfide bond at the original N–C termini to regulate enzymatic activity.³⁴ Our method shares a similar activation mechanism as Loh’s method. However, in our method the disulfide bond does not introduce conformational strain on the functional protein itself; instead, it imposes conformational strain on the guest control domain. In addition, upon cleavage of the disulfide bond, the mutually exclusive protein remains a continuous polypeptide chain. Thus, the switching does not depend on the complexation stability of the cleaved protein as encountered in Loh’s method. This advantage makes it possible to use the mutually exclusive protein switch reversibly over a number of cycles. We believe that our new approach, together with Loh’s constrained circular permutant approach, represents a new general mechanism toward regulating enzymatic functions through chemical stimuli.

Materials and Methods

Protein engineering

The gene of the mutually exclusive protein GL5CC-I27w34f was constructed following the previously described methods.²⁵ Residues 41 and 43 of GL5 were mutated into cysteines using a standard site directed mutagenesis method. The DNA sequence of GL5CC-I27w34f was confirmed by direct DNA sequencing. The final construct (GL5CC–I27w34f), which carries an N-terminal His tag for purification, had the following amino acid sequence:

MRGSHHHHHHGSMDTYKLILNGKTLKGETT
TEAVDAATAEKVFKQYANDNGVGCGLGLIEVEKP
LYGVEVVFVGETAHFEIELSEPDVHGQFKLKGQ
PLAASPDCEIHEDGKKHILILHNCQLGMTGEVS
FQAANTKSAANLKVKELCGDGEWTYDDATKTFT
VTERS, where the sequence in *italics* is from the host domain GL5CC, and the sequence in **bold** is from the guest domain I27w34f. The Leu-Gly junction between GL5CC and I27w34f resulted from *Ava*I site, where the N-terminal Gly-Ser resulted from the *Bam*HI cleavage site and the C-terminal Arg-Ser resulted from the *Bgl*II site. The mutually exclusive protein GL5CC–I27w34f was overexpressed in the *Escherichia coli* strain DH5 α and purified by Ni²⁺ affinity chromatography. We found that, although slow, ambient oxygen was sufficient

to oxidize GL5CC–I27w34f to obtain the disulfide-bonded GL5CC(F)–I27w34f(U) after 2 days.

The gene of GL15CC, which is a loop insertion mutant of GB1 and carries a flexible loop of 15 residues, was constructed as a control protein using a similar method.²⁵ The parent protein GL15 was constructed as described previously.²⁷ Residues 2 and 14 of the inserted loop (equivalent to the residues 41 and 43 of GL5) were mutated into cysteines using standard site directed mutagenesis method. DNA sequence of GL15CC was confirmed by direct DNA sequencing. The final construct GL15CC, which carries an N-terminal His tag for purification, has the following amino acid sequence:

MRGSHHHHHHGSM~~DTYKLILNGKTLKGETTT~~
EAVDAATAEKVFKQYANDNGV**GCGSGASGASGAS**
CGDGEWTYDDATKTFTVTERS, where the sequence in italic is from the host domain GB1, and the sequence in bold is the inserted loop. The N-terminal Gly-Ser is resulted from *Bam*HI site and the C-terminal Arg-Ser is resulted from *Bgl*II site. The protein GL15 was overexpressed in *E. coli* strain DH5 α and purified by Ni²⁺ affinity chromatography.

Stopped-flow fluorescence and circular dichroism spectroscopy measurements

Folding kinetics of the reduced and oxidized forms of GL5CC–I27w34f was monitored by following its tryptophan fluorescence at 350 nm. The folding reaction was initiated by diluting the denatured protein from 4M GdmCl to 0.3M GdmCl. For experiments with oxidized GL5CC–I27w34f, the protein was oxidized by ambient oxygen for 2 days before adding 4M GdmCl to denature the protein. The folding kinetics was then monitored in 0.3M GdmCl/PBS. For experiments using reduced GL5CC–I27w34f, 4M GdmCl and 20 mM DTT was added to the protein solution to completely denature the protein. Folding kinetics was then monitored in a 0.3M GdmCl/20 mM DTT/PBS buffer. Stopped-flow experiments were carried out on a BioLogic SFM-4 stopped-flow instrument.

Far UV circular dichroism experiments were carried out on a Jasco-J810 spectropolarimeter flushed with nitrogen gas. To monitor the conversion of GL5CC(F)–I27w34f(U) to GL5CC(U)–I27w34f(F), 12 mM DTT was added to the oxidized mutually exclusive protein sample and the ellipticity at 221 and 230 nm were monitored as a function of time.

SPR spectroscopy measurements

SPR experiments were carried out using a Biacore3000 SPR instrument. Fc fragment of human IgG antibody (hFc) was immobilized onto a CM5 chip (Biacore) until the SPR signal reached ~1,000 RU (resonance units). Different concentrations of GL5CC–I27w34f or GL15CC proteins were then passed through the CM5 chip to measure their bind-

ing to hFc. The dissociation of GL5CC–I27w34f or GL15CC from hFc was monitored by passing buffer through the CM5 chip. Oxidized GL5CC–I27w34f or GL15CC was obtained by air oxidation of the sample in PBS buffer, and reduced GL5CC–I27w34f or GL15CC was obtained by incubating the protein solution with 20 mM DTT for 30 min.

References

1. Donaldson JG, Jackson CL (2011) ARF family G proteins and their regulators: roles in membrane transport, development and disease. *Nat Rev Mol Cell Biol* 12:362–375.
2. Garcia-Mata R, Boulter E, Burridge K (2011) The 'invisible hand': regulation of RHO GTPases by RHOGDIs. *Nat Rev Mol Cell Biol* 12:493–504.
3. Hergovich A (2011) MOB control: reviewing a conserved family of kinase regulators. *Cell Signal* 23:1433–1440.
4. Inman GJ (2011) Switching TGF β from a tumor suppressor to a tumor promoter. *Curr Opin Genet Dev* 21:93–99.
5. Stavnezer J (2011) Complex regulation and function of activation-induced cytidine deaminase. *Trends Immunol* 32:194–201.
6. Baird GS, Zacharias DA, Tsien RY (1999) Circular permutation and receptor insertion within green fluorescent proteins. *Proc Natl Acad Sci USA* 96:11241–11246.
7. Ambroggio XI, Kuhlman B (2006) Design of protein conformational switches. *Curr Opin Struct Biol* 16:525–530.
8. Wright CM, Heins RA, Ostermeier M (2007) As easy as flipping a switch? *Curr Opin Chem Biol* 11:342–346.
9. Ostermeier M (2009) Designing switchable enzymes. *Curr Opin Struct Biol* 19:442–448.
10. Koide S (2009) Generation of new protein functions by nonhomologous combinations and rearrangements of domains and modules. *Curr Opin Biotech* 20:398–404.
11. Vallee-Belisle A, Plaxco KW (2010) Structure-switching biosensors: inspired by Nature. *Curr Opin Struct Biol* 20:518–526.
12. Stratton MM, Loh SN (2011) Converting a protein into a switch for biosensing and functional regulation. *Protein Sci* 20:19–29.
13. Palmer AE, Qin Y, Park JG, McCombs JE (2011) Design and application of genetically encoded biosensors. *Trends Biotech* 29:144–152.
14. Oh KJ, Cash KJ, Plaxco KW (2009) Beyond molecular beacons: optical sensors based on the binding-induced folding of proteins and polypeptides. *Chemistry* 15:2244–2251.
15. Cerasoli E, Sharpe BK, Woolfson DN (2005) ZiCo: a peptide designed to switch folded state upon binding zinc. *J Am Chem Soc* 127:15008–15009.
16. Dueber JE, Yeh BJ, Chak K, Lim WA (2003) Reprogramming control of an allosteric signaling switch through modular recombination. *Science* 301:1904–1908.
17. Guntas G, Mansell TJ, Kim JR, Ostermeier M (2005) Directed evolution of protein switches and their application to the creation of ligand-binding proteins. *Proc Natl Acad Sci USA* 102:11224–11229.
18. Sallee NA, Yeh BJ, Lim WA (2007) Engineering modular protein interaction switches by sequence overlap. *J Am Chem Soc* 129:4606–4611.
19. Strickland D, Moffat K, Sosnick TR (2008) Light-activated DNA binding in a designed allosteric protein. *Proc Natl Acad Sci USA* 105:10709–10714.

20. Yousef MS, Baase WA, Matthews BW (2004) Use of sequence duplication to engineer a ligand-triggered, long-distance molecular switch in T4 lysozyme. *Proc Natl Acad Sci USA* 101:11583–11586.
21. Radley TL, Markowska AI, Bettinger BT, Ha JH, Loh SN (2003) Allosteric switching by mutually exclusive folding of protein domains. *J Mol Biol* 332:529–536.
22. Cutler TA, Loh SN (2007) Thermodynamic analysis of an antagonistic folding-unfolding equilibrium between two protein domains. *J Mol Biol* 371:308–316.
23. Ha JH, Butler JS, Mitrea DM, Loh SN (2006) Modular enzyme design: regulation by mutually exclusive protein folding. *J Mol Biol* 357:1058–1062.
24. Cutler TA, Mills BM, Lubin DJ, Chong LT, Loh SN (2009) Effect of interdomain linker length on an antagonistic folding-unfolding equilibrium between two protein domains. *J Mol Biol* 386:854–868.
25. Peng Q, Li H (2009) Direct observation of tug-of-war during the folding of a mutually exclusive protein. *J Am Chem Soc* 131:13347–13354.
26. Akerstrom B, Brodin T, Reis K, Bjorck L (1985) Protein G: a powerful tool for binding and detection of monoclonal and polyclonal antibodies. *J Immunol* 135: 2589–2592.
27. Li H, Wang HC, Cao Y, Sharma D, Wang M (2008) Configurational entropy modulates the mechanical stability of protein GB1. *J Mol Biol* 379:871–880.
28. Sauer-Eriksson AE, Kleywegt GJ, Uhlen M, Jones TA (1995) Crystal structure of the C2 fragment of streptococcal protein G in complex with the Fc domain of human IgG. *Structure* 3:265–278.
29. Cao Y, Yoo T, Zhuang S, Li H (2008) Protein-protein interaction regulates proteins' mechanical stability. *J Mol Biol* 378:1132–1141.
30. Wang H-C (2009) Studies on the mechanical stability of a protein by single molecule atomic force microscopy. Chemistry. University of British Columbia.
31. Morrone A, McCully ME, Bryan PN, Brunori M, Daggett V, Gianni S, Travaglini-Allocatelli C (2011) The denatured state dictates the topology of two proteins with almost identical sequence but different native structure and function. *J Biol Chem* 286:3863–3872.
32. Park SH, Shastry MC, Roder H (1999) Folding dynamics of the B1 domain of protein G explored by ultra-rapid mixing. *Nat Struct Biol* 6:943–947.
33. Tcherkasskaya O, Knutson JR, Bowley SA, Frank MK, Gronenborn AM (2000) Nanosecond dynamics of the single tryptophan reveals multi-state equilibrium unfolding of protein GB1. *Biochemistry* 39: 11216–11226.
34. Butler JS, Mitrea DM, Mitrousis G, Cingolani G, Loh SN (2009) Structural and thermodynamic analysis of a conformationally strained circular permutant of barnase. *Biochemistry* 48:3497–3507.