Ligand binding and allostery can emerge simultaneously

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Abstract

A heterotropic allosteric effect involves an effector molecule that is distinct from the substrate or ligand of the protein. How heterotropic allostery originates is an unanswered question. We have previously created several heterotropic allosteric enzymes by recombining the genes for TEM1 β-lactamase (BLA) and maltose binding protein (MBP) to create BLAs that are positively or negatively regulated by maltose. We show here that one of these engineered enzymes has $\sim 10^6\ M^{-1}$ affinity for Zn^{2+}, a property that neither of the parental proteins possesses. Furthermore, Zn^{2+} is a negative effector that non-competitively switches off β-lactam hydrolysis activity. Mutagenesis experiments indicate that the Zn^{2+}-binding site does not involve a histidine or a cysteine, which is atypical of natural Zn^{2+}-binding sites. These studies also implicate helices 1 and 12 of the BLA domain in allosteric signal propagation. These results support a model for the evolution of heterotropic allostery in which effector affinity and allosteric signaling emerge simultaneously.

Keywords: allostery; maltose binding protein; β-lactamase; zinc; protein switch; protein evolution

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Allostery is the most direct, rapid, and efficient mechanism for the modulation and regulation of cellular function in response to changes in concentration of small molecules. However, allostery has not received as much attention as other mechanisms of regulation (e.g., transcriptional and translational regulation and posttranslational covalent modifications) because allosteric effectors are not easily identified and there is a misconception that only oligomeric proteins with multiple, identical subunits can be allosterically regulated (Lindsley and Rutter 2006). For heterotropic allostery, binding of a ligand or catalytic turnover of a substrate that occurs at one site on the protein is affected by binding of an effector molecule (different than the ligand or substrate) at a different site. As such, heterotropic allostery is a potential property of any protein (Gunasekaran et al. 2004). Interest in heterotropic allosteric effectors as drug molecules has increased in recent years (Hardy and Wells 2004; Lindsley and Rutter 2006).

It is reasonable to assume that allosteric enzymes (to use enzymes as an example) evolved from nonallosteric enzymes catalyzing the same reaction. Multifunctional, multidomain proteins can exhibit heterotropic allosteric effects that presumably arose through establishing domain interactions between the independently functioning, ancestral proteins—a process that can be facilitated by gene fusion (Ostermeier and Benkovic 2000). New allosteric proteins might also arise from pre-existing,

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multidomain, allosteric proteins by replacement of functional or effector-binding domains with new functional or effector-binding domains. Such reprogramming has been realized experimentally (Picard 2000; Dueber et al. 2003).

The above mechanisms of how heterotropic allostery can arise all employ ancestral proteins with pre-existing affinity for the future effector. How is allostery created in proteins that lack effector affinity? Conceptually, it is easier to understand the creation of homotropic allosteric effects because effector and substrate are one and the same, and thus, effector affinity does not need to evolve: Only allosteric signaling needs to evolve. Accordingly, it has been shown that single point mutations can convert nonallosteric homo-oligomeric enzymes into allosteric enzymes displaying cooperativity involving their substrates (Kuo et al. 1989; Scrutton et al. 1992; Stebbins and Kantrowitz 1992). However, the creation of heterotropic allostery from proteins that lack effector affinity seems a more difficult task since both effector affinity and an allosteric relationship with the active site must be created. Does this creation occur simultaneously, or does the ability to bind effector precede allostery? This question has not received extensive attention. One theory on the origins of the first allosteric proteins posits that primitive effector molecules would be those that mimic the functional groups of the amino acids found in proteins (Yousef et al. 2004). Allostery arises when binding of these effectors displaces intramolecular protein interactions, resulting in a conformational change. Using the aspartate receptor as a model, Yu and Koshland (2001) proposed that evolution first selects for an effector-binding competent state, which occurs with a conformational change that is probably not that of the mature allosteric protein. Subsequently, binding and allostery further evolve to be biologically relevant. This model seems to posit that effector binding precedes *effective* allostery; however, the selective pressure for evolving effector binding in the absence of effective allostery is not clear.

The ability to engineer heterotropic allosteric behavior into proteins has a number of potential biotechnological and biomedical applications (Ostermeier 2005). The study of such engineered allosteric proteins may inform the study of natural mechanisms of allostery evolution. We have previously engineered enzymes that exhibit heterotropic allostery by recruiting an enzyme into a protein with the desired effector-binding site (Guntas and Ostermeier 2004; Guntas et al. 2004, 2005). While attempting to engineer one of our allosteric enzymes to respond to a new effector, we discovered that it serendipitously already exhibited high affinity for the desired effector, a property the parental proteins lacked. Furthermore, binding of the effector negatively regulated the enzyme activity, completely inhibiting enzyme activity at saturating effector concentrations. This example of the simultaneous creation of ligand affinity and allosteric effects has implications for the evolution of heterotropic allostery.

Results

Engineered β-lactamases for which maltose is a positive effector

RG13 (Guntas et al. 2004) and MBP317–347 (Guntas et al. 2005) are engineered allosteric β-lactamases for which maltose is a positive effector. They are hybrid proteins between TEM1 BLA and maltose binding protein (MBP). Both were identified from combinatorial protein libraries. The diversity in these libraries derived from random circular permutations of the gene coding for BLA and random insertions of the *bla* gene into the gene for MBP. RG13 and MBP317–347 have different circular permutations of BLA inserted in the same location in MBP (in place of MBP residue 317) (Fig. 1). In RG13, the original N and C termini of BLA are joined by a GSGGG peptide linker and the BLA domain is circularly permuted between residues 226 and 227. In MBP317–347, the original N and C termini are joined by a DKS peptide linker and the BLA domain is circularly permuted near the BLA active site such that residue 170 is duplicated. Maltose binding to MBP is known to convert the open form of the protein to the closed form through conformational changes about the hinge region (Sharff et al. 1992).

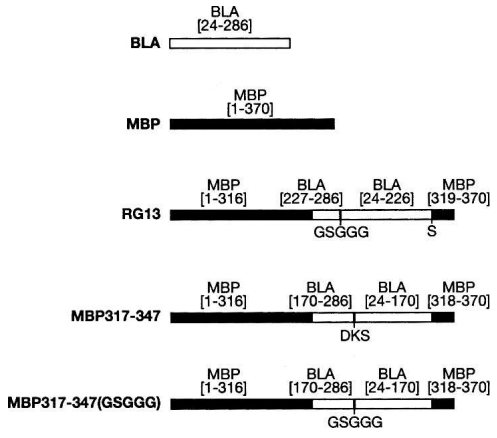

Figure 1. Schematic depiction of the amino acid sequences of the proteins used in this study. RG13, MBP317–347, and MBP317–347(GSGGG) are hybrid proteins derived from BLA (white) and MBP (black). The number in parenthesis indicates the amino acid number of the parental proteins. The numbering system for MBP does not include the signal sequence. The numbering system for BLA does include the signal sequence and does not follow the consensus numbering system for BLAs. The location of the GSGGG and DKS linkers and a serine derived from the fusion of partial codons is shown underneath.

A variety of evidence indicates that the MBP domains in RG13 and MBP317–347 undergo a similar change in conformation—a change that is required for switching activity (Guntas et al. 2004, 2005; Kim and Ostermeier 2006).

Strategy for altering effector specificity

We have previously used a genetic selection to identify variants of MBP317–347 that are activated by sucrose from a library in which five amino acid positions in the maltose-binding site of the MBP domain were varied (Guntas et al. 2005). Here, our intent was to convert our maltose-activated BLAs into Zn^{2+}-activated BLAs to test how flexible these switches are to adaptation to new effectors. We introduced into RG13's MBP domain the A* set of mutations (A63H, R66H, Y155E, and W340E) that previously have been shown to convert MBP into a Zn^{2+}-binding protein with a K_d of 5 μM (Marvin and Hellinga 2001). Marvin and Hellinga (2001) designed these mutations so that MBP bound Zn^{2+} in the closed conformation, with two mutations on each of the subdomains of MBP. We expected that such a zinc-driven conformational change in our switch should activate BLA activity. However, recent crystallographic studies indicate that MBP with the A* mutations unexpectedly binds Zn^{2+} in the open conformation with Zn^{2+} being directly contacted by the two histidines but not the two glutamates (Telmer and Shilton 2005). Based on these data, we would predict that introducing the A* mutations into RG13 would not create a zinc-activated BLA because the MBP domain would not undergo the large hinge bending conformational change necessary to activate the BLA domain in the presence of Zn^{2+}.

Zn^{2+} is an unexpected negative effector of RG13

Before the unexpected binding mode of MBP bearing the A* mutations was known, we tested the effect of Zn^{2+} on the β-lactam hydrolysis activity of RG13 bearing the A* set of mutations and, as a control, RG13 without the mutations. Surprisingly, both proteins' catalytic activities were inhibited by Zn^{2+} to an equal extent. The K_i of Zn^{2+} for RG13 (lacking the A* mutations) determined in the presence of maltose was 2.7 ± 0.1 μM (Fig. 2A). The K_i was not significantly different in the absence of maltose (2.1 ± 0.2 μM). Zn^{2+} inhibition of β-lactam hydrolysis was complete and reversible (i.e., the addition of EDTA to a solution of Zn^{2+}-inhibited RG13 turned enzyme activity back on). Subsaturating concentrations of zinc decreased k_{cat} but did not change K_m. This indicates that the inhibition is noncompetitive; thus, zinc does not bind at the same site as the substrate, and zinc is an allosteric inhibitor of RG13. RG13 was also inhibited by Ni^{2+} ($K_i = 92 \pm 4$ μM) and Co^{2+} ($K_i = 380 \pm 30$ μM) but not Ca^{2+} or Mg^{2+}.

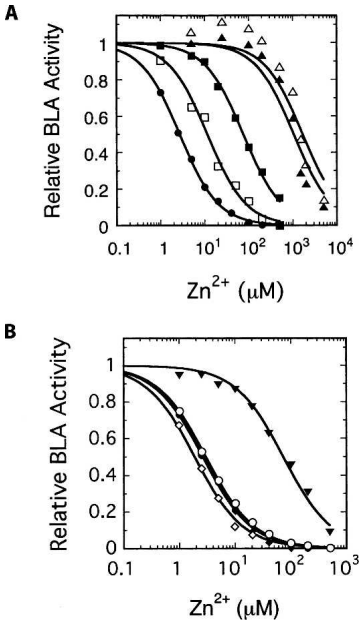

Figure 2. Zn^{2+} inhibition of β-lactam hydrolysis activity. The initial velocity of nitrocefin hydrolysis in the presence of Zn^{2+} was normalized to that in the absence of Zn^{2+} and fit to a model of a single Zn^{2+}-binding site for (*A*) switches and parental proteins and (*B*) mutants of RG13. RG13 (solid circles); BLA (solid triangles); BLA + MBP (open triangles); MBP317–347(GSGGG) with maltose (solid squares); MBP317–347(GSGGG) without maltose (open squares); RG13(C77A/C123A) (open diamonds); RG13(H96A/H112L) (solid diamonds); RG13(H153A/H158A) (open circles); RG13(H26A/H287A) (solid inverted triangles).

The inhibition of RG13 in the presence of low concentrations of zinc is surprising. MBP does not bind zinc (Marvin and Hellinga 2001; Telmer and Shilton 2005), and BLA is not known to bind Zn^{2+}, although it has been reported that BLA can be immobilized in a soluble, active form on a sepharose/Ni^{2+} column (Lawung et al. 2001). We purified BLA and found that although Zn^{2+} did inhibit BLA, inhibition of BLA required at least two orders of magnitude higher concentrations of Zn^{2+} than that required for inhibition of RG13 (Fig. 2A). The inhibition differed slightly between two independent preparations of BLA ($K_i = 340 \pm 50$ μM and 1.1 ± 0.2 mM). Zn^{2+} inhibition of a 1:1 mixture of MBP and BLA mirrored that of BLA, indicating that the Zn^{2+} sensitivity does not arise simply by having MBP and BLA in the same solution (Fig. 2A). Furthermore, the mechanism of inhibition is likely to be different for BLA and RG13. The inhibition curve of RG13 fit to a model of single binding site. The inhibition curve for BLA was steeper than that expected for a single binding site (Fig. 2A), suggesting a different inhibition mechanism involving cooperativity. Zinc inhibition of BLA was accompanied

by visible precipitation and by significant loss of soluble protein with secondary structure, as monitored by circular dichroism (CD) spectroscopy (Fig. 3A). Such precipitation is not surprising at high zinc concentrations since divalent metal cations are commonly used as protein precipitation agents. In contrast, zinc inhibition of RG13 was not accompanied by visible precipitation. Only very minor changes in RG13's CD spectrum were observed upon the addition of Zn^{2+} (Fig. 3B), even at zinc concentrations found to disrupt secondary structure and precipitate BLA.

Zn^{2+} binding to RG13

We next monitored Zn^{2+} binding in MBP, BLA, and RG13 using a fluorescent competition assay in which free Zn^{2+} is monitored using the Zn^{2+}-specific fluorophore FluoZin-1 (Fig. 4). The assay confirmed previous results (Marvin and Hellinga 2001; Telmer and Shilton 2005) that showed that MBP does not bind Zn^{2+} ($K_d \gg 50$ μM). Although the presence of BLA had a measurable effect on the amount of free Zn^{2+}, binding was significantly less than one zinc ion per protein molecule over the reliable concentration range of the assay (up to 30 μM Zn^{2+}), consistent with a low affinity (>100 μM K_d) interaction between Zn^{2+} and BLA. In contrast, the presence of RG13 significantly affected the amount of free Zn^{2+} (Fig. 4). The fluorescent titration curve fit well to a single, high-affinity binding site on the protein (Supplemental Fig. S1). Based on this assumption, the K_d of Zn^{2+} binding to RG13 determined by the fluorescent assay was 2.4 ± 0.5 μM, which matched the K_i of Zn^{2+} measured in the BLA activity assay. Protein-bound Zn^{2+} plateaus at about one Zn^{2+} per protein, and the experimentally determined binding curve approximates that expected if the protein had a single site with $K_d = 2.7$ μM (i.e., the value of K_i) (Fig. 4). The oligomeric state of RG13 was analyzed by gel filtration. RG13 behaved as a monomer in both the absence and presence of zinc, indicating that the zinc-binding site is intramolecular.

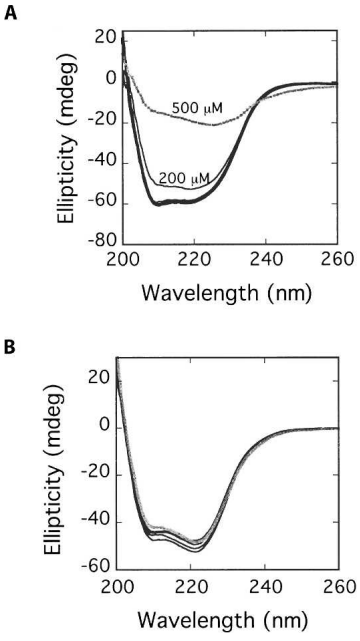

Figure 3. Circular dichroism spectra of (*A*) BLA and (*B*) RG13 in the presence of Zn^{2+}. The CD spectra (thick black line) in the absence and presence of increasing concentrations of Zn^{2+} up to 500 μM (thick gray line) were acquired. The concentrations of Zn^{2+} were 0 μM, 10 μM, 50 μM, 200 μM, and 500 μM. For BLA, the spectra for concentrations of Zn^{2+} from 0–50 μM overlay. For RG13, the spectra for 0 and 10 μM Zn^{2+} overlay, 50 μM Zn^{2+} produced the lowest spectra, 200 μM Zn^{2+} produced the next lowest spectra, and the spectra for 500 μM Zn^{2+} is slightly above the spectra in the absence of Zn^{2+}.

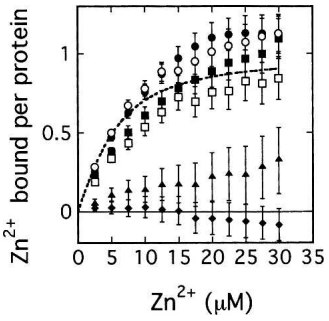

Figure 4. Zn^{2+} protein binding. The amount of protein-bound Zn^{2+} was determined using a fluorescence assay for free Zn^{2+} in the presence of known concentrations of protein and increasing concentrations of Zn^{2+}. The dashed line is the theoretical binding curve expected for a protein with a single Zn^{2+} binding site with a K_d of 2.7 μM (i.e., zinc's K_i for RG13). BLA (solid triangles); MBP (solid diamonds); RG13 (open and closed circles); RG13(H26A/H287A) (open and closed squares). The open and closed symbols for RG13 and RG13(H26A/H287A) represent results from two independent experiments.

Zn^{2+} inhibition requires the MBP domain and is less effective with MBP317–347

The BLA domain of RG13 is circularly permuted with a peptide linker joining the original N and C terminus. This led us to wonder if the circular permutation was responsible for the emergence of Zn^{2+} inhibition (i.e., was the MBP domain dispensable?). We constructed and purified (40% pure) the circular permuted variant of BLA found in RG13. This protein (cpBLA220) was compromised in catalytic activity relative to BLA and RG13 but was no more sensitive to Zn^{2+} than BLA. Thus, circular permutation of the BLA domain alone is not sufficient to create the Zn^{2+} inhibition found in RG13.

We next asked whether Zn^{2+} inhibition was unique to RG13 or occurred with other switches as well. Compared

with RG13, MBP317–347 has a different linker and circular permutation of BLA, but the BLA domain is inserted in the same site in the MBP domain (Fig. 1). MBP317–347 was inhibited by Zn^{2+} but required higher concentrations of zinc for equivalent inhibition. This experiment was done in the presence of maltose, since the weak catalytic activity and burst kinetics of MBP317–347 in the absence of maltose (Guntas et al. 2005) were problematic for quantifying zinc inhibition. To be sure that the weaker inhibition was not a result of differences in the circular permutation linker, a variant of MBP317–347, named MBP317–347(GSGGG), was created in which the DKS linker was replaced with the GSGGG linker of RG13. Interestingly, MBP317–347(GSGGG) was a noticeably poorer maltose switch than MBP317–347, having both higher activity in the absence of maltose and lower activity in the presence of maltose compared with MBP317–347 (Supplemental Fig. S2). This implicates the circular permutation linker in potentiating the maltose-dependent allosteric effect and suggests the region might have a direct role in the allosteric signaling for maltose. Zn^{2+} inhibition of MBP317–347(GSGGG) (in the presence of maltose) was essentially the same as that of MBP317–347 ($K_i = 75 \pm 3$ μM) and was intermediate between that of RG13 and BLA (Fig. 2A). The absence of maltose increased MBP317–347(GSGGG)'s sensitivity to zinc ($K_i = 12 \pm 1$ μM) (Fig. 2A), suggesting that maltose-induced conformational changes in MBP317–347(GSGGG) modulate affinity for zinc.

Zn^{2+}-binding site

We next sought to identify the Zn^{2+}-binding site in RG13 through mutagenesis. With very few exceptions, naturally occurring Zn^{2+}-binding sites contain at least one cysteine or histidine and typically have at least two of these residues (Auld 2001). RG13 contains two cysteines and nine histidines. The two cysteines (Cys77 and Cys123) are in the BLA domain and form a disulfide bond in BLA—a bond that is not necessary for catalytic activity (Vanhove et al. 1997). RG13 with the C77A/C123A mutations retained both maltose-switching activity and Zn^{2+} inhibition (Fig. 2B), indicating that the zinc-binding site does not involve these residues. Of the nine histidines, three are in the MBP domain and six occur in the BLA domain as three sets of vicinal pairs. All nine were replaced individually without losing maltose-switching activity. These mutations did not have a significant effect on the K_i for zinc with the exceptions of H26A and H287A in the BLA domain, which caused decreased zinc sensitivity. RG13 variants bearing double mutations in the vicinal pairs in the BLA domain (H96A/H112L, H153A/H158A, or H26A/H287A) retained maltose-switching activity, but only RG13(H26A/H287A) exhibited a loss of Zn^{2+} inhibition at low zinc concentrations (K_i increased 30-fold) (Fig. 2B). This suggested that H26 and H287 were involved in binding zinc. However, zinc binding to RG13(H26A/H287A), as measured by the fluorescent competition assay, was not significantly different than that observed with RG13 (Fig. 4).

Discussion

Allosteric signaling in RG13

It is surprising that RG13 has a high affinity Zn^{2+}-binding site considering that such a site does not exist in either of the parental proteins. With the exception of the GSGGG linker used to circularly permute the BLA domain and a serine at the 3′ gene fusion site, RG13 does not contain any amino acids not in MBP and BLA. Since RG13 is monomeric independent of the presence of zinc, either the specific fusion of MBP and BLA in RG13 causes a structural perturbation that creates a zinc-binding site or the two domains are properly oriented to form an interdomain binding site. The fact that both RG13 and MBP317–347(GSGGG) are inhibited by zinc argues against an interdomain binding site (provided zinc binds in the same site in both proteins) since the two proteins have very different geometric rearrangements of the two domains. Exhaustive mutagenic replacement of the usual suspects in natural zinc binding sites (cysteines and histidines) did not result in identification of mutants with decreased zinc affinity. This indicates that RG13's zinc-binding site is atypical compared with natural zinc-binding sites—perhaps one involving a subset of the 24 arginines in the protein.

Even more surprising than the creation of a zinc-binding site is the fact that zinc-dependent allostery was created simultaneously. The CD spectrum of RG13 changes only slightly in the presence of zinc, suggesting that zinc-binding induces more subtle changes in structure than those observed in designed zinc-dependent conformational switches (Cerasoli et al. 2005; Ambroggio and Kuhlman 2006). RG13's K_i and K_d for Zn^{2+} match, suggesting the Zn^{2+}-binding sites being observed by the two methods are the same. In contrast, although the H26A/H287A mutations shift the K_i 30-fold higher, zinc affinity changes comparatively little. A difference between K_d and K_i could be explained by substrate-binding decreasing the protein's affinity for zinc. However, zinc inhibition of RG13 was noncompetitive, and it would be surprising that the mutations would have altered this mechanism. A second possibility is that the H26A/H287A mutations may disrupt the allosteric signal but not Zn^{2+} binding. However, since zinc inhibition is still occurring with the H26A/H287A mutant, this would

require that its inhibition involve a different site than in RG13, which would also be surprising.

Although the reason for the effects of the H26 and H287 mutations is not clear, it is clear that they modulate the allosteric effect of zinc. Several lines of evidence implicate the region around these residues as being involved in allosteric effects. H26 and H287 are on helices 1 and 12 (the first and last helices) of BLA—helices that are connected through the peptide linker used to circularly permute the BLA sequence. The BLA activity of MBP317–347 can be modulated by the sequence of this linker, indicating a functional relationship between this region and the BLA active site (Supplemental Fig. S1). In addition, two previous studies suggest that these helices have the potential to be involved in allosteric effects in BLA: (1) Structure-based thermodynamic stability analysis of a variety of allosteric proteins indicates that allosteric regulatory sites are characterized by low stability in the absence of the regulator (Luque and Freire 2000). This stability analysis on BLA indicated that helices 1 and 12 have low stability (Luque and Freire 2000). (2) Horn and Shoichet (2004) identified two noncompetitive, reversible inhibitors of BLA with K_i's of ~500 μM. Crystal structures indicate that binding for both compounds involved helix 12 and resulted in the forcing apart of helices 11 and 12 (Horn and Shoichet 2004).

Origins of the Zn^{2+} allosteric effects in RG13

The fluorescent binding assay indicates that neither MBP nor BLA has a high affinity Zn^{2+}-binding site. Thus, three possibilities exist for the origin of the high affinity, allosteric Zn^{2+}-binding site of RG13: (1) A low affinity, allosteric site existed in BLA and was improved in affinity by protein fusion; (2) a low affinity, nonallosteric site existed in BLA or MBP, and both affinity improvement and allostery establishment occurred by protein fusion; and (3) the site did not exist in the parental proteins, and both Zn^{2+} binding and allostery were created by the protein fusion.

According to the first scenario, the site responsible for Zn^{2+} inactivation of BLA is the site of Zn^{2+} inactivation in RG13. The differences in inactivation mechanism between RG13 and BLA would argue against this possibility, but it cannot be ruled out. If allostery was created, did the high affinity Zn^{2+} binding site in RG13 arise from a low affinity site or was it created de novo? Ultimately, the argument comes down to the criteria for what constitutes a meaningful low affinity interaction. Imidazole has a K_d for Zn^{2+} of 2.6 mM (Sillen and Martell 1964). There are six histidines in BLA and three in MBP; thus, it would be expected that RG13 would have low affinity for Zn^{2+}. What is relevant is that a high-affinity site for Zn^{2+} binding was created by combining two proteins that lacked such a site. This affinity is substantial and comparable to that previously created in MBP by computational design (Marvin and Hellinga 2001). Furthermore, RG13 was not identified by applying a selective pressure for Zn^{2+} affinity, suggesting that zinc-binding sites might be easier to create than expected. Similarly, Keefe and Szostak (2001) found that one of their ATP-binding domains, evolved from a random-sequence library, bound zinc. These results support the theory that zinc-binding structures should have a high probability of occurring by chance, relative to many other folds (Sharpe et al. 2002).

Evolution of heterotropic allostery

Although the zinc affinity and enzymatic inhibition in an engineered protein such as RG13 have no direct physiological relevance, the *level* of Zn^{2+} affinity and allosteric regulation exhibited by RG13 is clearly sufficient to be relevant for biological systems. For example, Zn^{2+} allosterically attenuates the enzymatic activity of factor VIIa with a K_i of ~20–60 μM (Petersen et al. 2000) and inhibits $GABA_A$ receptor function with an IC_{50} concentration of 90 nM (Hosie et al. 2003). Thus, our results have relevance for the evolution of heterotropic allostery.

How heterotropic allostery originates is an unanswered question. The evolution of such allosteric sites must be more difficult than the evolution of nonallosteric ligand binding sites, since allosteric sites require both affinity for the intended effector and functional coupling between the effector-binding site and the active site. Since allostery involving the effector cannot exist without effector affinity, either affinity evolves prior to allosteric effects or these properties coevolve. However, since a protein having effector affinity but lacking allostery confers no apparent selective advantage, the seemingly more difficult path of coevolution of effector affinity and allostery may be the relevant one. An analogous argument has been made for the coevolution of protein fold and function, since fold alone provides no selective advantage (James and Tawfik 2003). In a protein that already has a site for a different effector, creation of allostery with the new effector may best proceed by creating effector affinity at that site (Hardy and Wells 2004).

One attractive theory for the evolution of new functions posits that nature does not evolve new functions ''out of nothing'' but gradually improves promiscuous activities already existing in proteins (Jensen 1976; James and Tawfik 2003). Evidence supporting this theory includes the identification of numerous proteins that exhibit promiscuity (O'Brien and Herschlag 1999; Copley 2003; Gerlt et al. 2005), the fact that some proteins can adopt different conformations to carry out different functions

(James and Tawfik 2003; James et al. 2003), the fact that multiple diverse ligands can bind at a single protein site (Ma et al. 2002), the occurrence of low level activity in unselected de novo protein libraries (Rojas et al. 1997; Wei and Hecht 2004) and "off-the-shelf" proteins (Hollfelder et al. 1996), and the observation from directed evolution experiments that the evolution of new functions results from mutations that have little effect on the native activity but large effects on the promiscuous functions (Aharoni et al. 2005; Yoshikuni et al. 2006). This theory skirts the need for de novo creation of an effector-binding site prior to (or concurrently with) allostery creation by postulating that evolvable, weak-affinity sites may commonly exist in proteins in the absence of selective advantages for having such sites.

The view that proteins exist in an ensemble of conformational states provides a mechanism for functional diversity at the protein level (Ma et al. 2002; James and Tawfik 2003). Mutations can alter this ensemble, allowing access to new conformers that are excluded from or rarely accessed in the native state ensemble. Furthermore, mutations are often destabilizing, which has important implications for allostery creation. Low stability regions in the effector-binding site (Luque and Freire 2000) and other regions (Streaker and Beckett 1999) have been implicated in playing a crucial role in allosteric signaling. In addition, destabilizing mutations may create an ensemble that has a larger number and a broader distribution of states. This increased conformational heterogeneity would allow the potential for more interactions with other molecules. Binding of the protein to these molecules may shift the protein to a new ensemble of states that has the potential to have significantly different properties than the ensemble in the absence of ligand (i.e., allostery is created) (Gunasekaran et al. 2004; Swain and Gierasch 2006). Thus, the same changes that result in the ability to bind a new ligand may predispose the mutant protein to exhibit allosteric effects involving that ligand. Recent work by Mathonet and coworkers (Mathonet et al. 2006a, b) supports this theory. Random peptides were inserted in three contiguous surface loops of BLA that were distal from the active site. From this phage-displayed library, transition metal ion-binding variants were isolated by panning on a metal ion affinity chromatography support. Of 30 selected library members, 10 exhibited metal-specific allosteric effects on catalytic activity (Mathonet et al. 2006a). Thus, the coevolution of effector affinity and allostery may not be as unlikely as it would first seem. This theory would predict that the evolution of new heterotropic effector sites would most likely proceed through evolutionary intermediates with increased conformational flexibility and heterogeneity. Given the relatively extreme nature of the changes involved in creating RG13 from MBP and BLA, it seems reasonable to expect that at least some regions of RG13 would have increased conformational flexibility compared to its parental proteins and this may have enabled the creation of the allosteric zinc site.

Materials and Methods

Protein mutagenesis

RG13 and MBP317–347 (and all variants thereof) in this work lacked the $GGSGH_9$ C-terminal extension described previously (Guntas et al. 2004) to avoid the potential binding of zinc to the poly his-tag. Removal of this extension did not appreciably affect the maltose-dependent switching activity. All mutations were constructed using Quikchange mutagenesis (Stratagene) and confirmed by sequencing the entire *RG13* gene. Mutations are indicated by the amino acid number of the parental protein.

Protein expression and purification

RG13, MBP317–347, and all variants were expressed in *malE*$^-$ auxotroph PM9F′ *Escherichia coli* (Betton and Hofnung 1994). Three liters of LB was inoculated with 2% overnight culture and 50 μg/mL of chloramphenicol and was grown at 37°C until OD_{600} was 0.6–0.8. After induction with 1 mM of IPTG, the culture was grown overnight at 16°C–20°C. Cells were lysed with a French press in amylose column buffer (20 mM Tris-Cl/200 mM NaCl at pH 7.0) and passed over amylose affinity resin (New England Biolabs). After washing three times with 10-column volumes of column buffer, the protein was eluted using 20 mM Tris-Cl/200 mM NaCl/20 mM maltose and dialyzed at 4°C against 300 volumes of 20 mM Tris-Cl (pH 7.0) five times. BLA and cpBLA220 were produced in DH5α under the same conditions as RG13. Three liters of bacterial culture were concentrated and then lysed with a French press in PBA column buffer (20 mM Tris-Cl/0.5M NaCl at pH 7.0) and passed over 3 mL of phenylboronic acid-agarose (Sigma-Aldrich). After four washes of 30 mL of PBA column buffer each, the protein was eluted with 12 mL of 0.5 M sodium borate/0.5 M NaCl (pH 7.0). The elution was then dialyzed three times against 400 volumes of 20 mM Tris-Cl (pH 7.0), concentrated to 1.5 mL with Amicon Ultra-15 ultrafilters (Millipore), and further purified using FPLC on a 16/60 Sephacryl S-200 gel-filtration column (GE HealthCare).

Nitrocefin assay for β-lactam hydrolysis and divalent metal inhibition

Nitrocefin hydrolysis was monitored at 486 nm as previously described (Guntas et al. 2004) in 10 mM MOPS (pH 7.0) with 2–20 nM protein, 100 μM nitrocefin, and increasing concentrations of $ZnCl_2$, $NiSO_4$, $CoCl_2$, $MgCl_2$, or $CaCl_2$. Initial velocities (v) were recorded and normalized to the velocity in the absence of added compounds (v_o). The normalized data were fitted to the equation: $v/v_o = 1 - (\{[M^{2+}]/K_i\}/\{1 + ([M^{2+}]/K_i)\})$. EDTA rescue was performed by adding 10 mM of EDTA to a solution of RG13 and 1 mM of Zn^{2+}.

CD spectroscopy

CD spectra were recorded using a Jasco J-710 CD Spectropolarimeter. The measurements were carried out using quartz

cuvettes (pathlength of 0.1 cm) in 10 mM MOPS (pH 7.0) with increasing concentrations of $ZnCl_2$ (0, 10, 50, 200, 500 μM). Protein concentrations were 15.9 μM (BLA) or 6.9 μM (RG13). Samples were incubated with zinc 5 min before acquiring the spectra.

Gel filtration

Using a Biologic HR FPLC (Bio-Rad), 100 μL of 20 μM RG13 was applied to a Superdex 200 10/300 GL column (1 × 30 cm; GE Healthcare) equilibrated with 20 mM Tris-Cl (pH 7.5), with or without 200 μM $ZnCl_2$. The protein was eluted with the same buffer. Vitamin B12, equine myoglobin, chicken ovalbumin, bovine γ-globulin, and thyroglobulin were used as molecular weight standards (Bio-Rad). RG13 eluted as a single peak at a time corresponding to a molecular weight of 70 kDa in the absence of zinc and 59 kDa in the presence of zinc. The expected molecular weight of RG13 is 72 kDa.

Fluorescent competition assay for zinc

Free Zn^{2+} was monitored in a fashion similar to previous studies (Telmer and Shilton 2005) using the fluorophore FluZin-1 (Invitrogen), which is reported by the manufacturer to have a 8 μM K_d for Zn^{2+}. Fluorescence titrations were carried out using a Photon Technology QuantaMaster QM-4 spectrofluorometer at 22°C in ~3 mL of 50 mM MOPS (pH 7.0) with 50 nM FluoZin-1 using excitation and emission wavelengths of 485 nm and 515 nm. Buffers and solutions were prepared with NANOpure (Barnstead) deionized water and filtered with 0.2 μm syringe filters. Because the concentration of FluoZin-1 was so low, the amount of zinc bound to FluoZin-1 is negligible compared to total zinc concentration and can be approximated as zero. Titrations were first performed in the absence of protein to determine the K_d of FluoZin ($K_{d,FluoZin}$) under the assay conditions. The $K_{d,FluoZin}$ was determined to be 9.59 ± 0.49 μM from five independent titrations in which the fluorescent data were fitted to Equation 1.

$$F - F_0 = (F_{max} - F_0)\frac{[Zn^{2+}]}{[Zn^{2+}] + K_{d,FluoZin}} \quad (1)$$

F is the fluorescence, F_{max} is the maximum fluorescence, and F_0 is the background fluorescence in the absence of added Zn^{2+}. Slit widths were adjusted such that ~10^6 counts were observed under saturating Zn^{2+} concentrations. Next, titrations were performed in the presence of 5 μM protein. The fluorescence, F, is a function of $[Zn]_{free}$ (the free zinc concentration) according to Equation 2:

$$F = \frac{F_{max}[Zn]_{free} + F_0 K_{d,FluoZin}}{K_{d,FluoZin} + [Zn]_{free}} \quad (2)$$

For some proteins, precipitation occurred at high zinc concentrations, resulting in the inability to experimentally determine F_{max}. F_{max} and K_d (the dissociation constant for zinc and the protein) were fit using Equation 2 (after substitution for $[Zn]_{free}$ from Equation 3) using the curve fitting function in Kaleidagraph (Synergy Software). This

$$[Zn]_{free} = \frac{1}{2}\Big(-K_d + [Zn]_{total} - [P]_{total} + \sqrt{(K_d - [Zn]_{total} + [P]_{total})^2 - 4K_d[Zn]_{total}}\Big) \quad (3)$$

fitting was done assuming a single zinc-binding site on the protein. $[Zn]_{total}$ is the total zinc concentration, and $[P]_{total}$ is the total protein concentration. The amount of protein-bound zinc was found by subtracting $[Zn]_{free}$ from $[Zn]_{total}$. The error in determining protein bound zinc primarily results from imprecision in the value of $K_{d,FluoZin}$.

Electronic supplemental material

Two figures are available electronically as supplemental material.

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