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Creation of an Allosteric Enzyme by Domain Insertion

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Two allosteric enzymes have been created by the covalent linkage of non-interacting, monomeric proteins with the prerequisite effector-binding and catalytic functionalities, respectively. This was achieved through a combinatorial process called random domain insertion. The fragment of the TEM-1 β-lactamase gene coding for the mature protein lacking its signal sequence was randomly inserted into the *Escherichia coli* maltose-binding protein (MBP) gene to create a domain insertion library. This library's diversity derived both from the site of insertion and from a distribution of tandem duplications or deletions of a portion of the MBP gene at the insertion site. From a library of $\sim 2 \times 10^4$ in-frame fusions, ~800 library members conferred a phenotype to *E. coli* cells that was consistent with the presence of bifunctional fusions that could hydrolyze ampicillin and transport maltose in *E. coli*. Partial screening of this bifunctional sub-library resulted in the identification of two enzymes in which the presence of maltose modulated the rate of nitrocefin hydrolysis. For one of these enzymes, the presence of maltose increased k_{cat} by 70% and k_{cat}/K_m by 80% and resulted in kinetic parameters that were almost identical to TEM-1 β-lactamase. Such an increase in activity was only observed with maltooligosaccharides whose binding to MBP is known to induce a conformational change. Modulation of the rate of nitrocefin hydrolysis could be detected at maltose concentrations less than 1 μM. Intrinsic protein fluorescence studies were consistent with a conformational change being responsible for the modulation of activity.

Keywords: allosteric enzyme; domain insertion; maltose binding protein; beta-lactamase; molecular switch

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Introduction

A hallmark of biological systems is the high degree of interactions amongst and within their constituent components. One advantage that such interactions bring is the establishment of coupling between different functions. A protein that couples two functions can be described as a molecular switch. For example, an allosteric enzyme is a switch that couples effector levels (input) to enzyme activity (output). In most general terms, a molecular switch couples signals (e.g. ligand-binding, protein–protein interactions, pH, covalent modification, temperature) to functionality (e.g. enzymatic activity, binding affinity, fluorescence). Molecular switches can be of an "on/off" nature or such that the signal modulates the function between two different levels of activity. The network of such molecular switches establishes the complex circuits that control cellular processes.

The design of molecular switches to modulate or report on biological functions has enormous potential for a variety of applications including the creation of biosensors[1–5] modulators of gene transcription and cell signaling pathways,[6–8] and novel biomaterials.[9] Despite their vast potential, molecular switches have not been explored extensively, in part due to the paucity of universal strategies for engineering them and the difficulty in engineering a switch that responds to a signal unrelated to the proteins' function and activates in the presence of the signal (as opposed to, for example, active-site inhibitors). In general, existing strategies for creating switches are inherently limited in the nature of the function that can be controlled, the signal that can be employed, the lack of reversibility, the lack of sensitivity, or the requirement for additional cellular components.

Domain insertion (the insertion of one protein

Abbreviations used: MBP, maltose binding protein; BLA, TEM1 β-lactamase.

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into another) is potentially a very general strategy for creating switches but has not been extensively explored. In this strategy, two existing proteins, one with the signal recognition function (protein A) and one with the function to be modulated (protein B), are fused so that the recognition of the signal by domain A is transmitted to domain B (e.g. by conformational change) such that the activity of domain B is affected. A wide variety of bifunctional proteins have been created by domain insertion.[10] More importantly, domain insertion has been successful at coupling conformational changes induced by ligand-binding[1,2] or by voltage-gated channels[3,11] to fluorescence of the green fluorescent protein to create molecular sensors. In addition, coupling between enzymatic sites in enzyme–enzyme fusions has been reported.[12] Recently, yeast sensors for ligand-binding were constructed by the insertion of FKBP12 and estrogen receptor-α ligand-binding domain (EPα-LBD) into a rationally chosen site in dihydrofolate reductase (DHFR)[13] in the first designed coupling of growth rate of an organism to a small molecule ligand by domain insertion. Yeast expressing the FKBP12-DHFR or ERα-DHFR fusion proteins had an approximate twofold increase in growth rate in the presence of their respective ligands (FK106 and estrogen) when DHFR activity limited growth. However, ligand-binding to the purified fusions did not significantly affect DHFR enzymatic activity or stability and the mechanism for increased growth rate of cells bearing these genes is not clear. Thus, to date, the creation of an allosteric enzyme (i.e. an enzymatic molecular switch) by domain insertion between a ligand-binding protein and an enzyme has not been achieved. However, a variety of different systems for conditionally controlling enzyme activity have been developed including split enzyme assays,[14,15] engineered reversible redox switches,[16] inhibitor-DNA–enzyme hybrids[17] and antibody-dependent modulation of epitope-displaying enzymes.[18]

Here we describe the creation of a random domain insertion library between TEM-1 β-lactamase (BLA) and the *Escherichia coli* maltose-binding protein (MBP) from which two β-lactamases were identified that can be allosterically modulated by maltooligosaccharides. In addition to serving as a model system for engineering molecular switches for biotechnological applications, these engineered allosteric enzymes should serve as an interesting platform for exploring theories on how allostery evolves in nature.

Results

We postulated that an allosteric enzyme could be created by fusing an enzyme and a ligand-binding protein by domain insertion. We sought to create an allosteric enzyme in which ligand-binding would increase the enzymatic activity of the fusion. For such a fusion, it was postulated that the enzyme domain would exist in a compromised, less active conformation in the absence of bound ligand. In the ligand-bound state, the enzyme domain would exist in a more normal, active conformation. The switching of the enzyme from one state to the other would be mediated by the ligand-dependent conformational change in the ligand-binding domain that would in turn affect the conformation of the enzyme domain.

Selection of model proteins

A model system consisting of *E. coli* MBP as the ligand-binding protein and the penicillin-hydrolyzing enzyme BLA as the enzyme was chosen to test this strategy. The desired property of the allosteric enzyme was the ability to modulate β-lactamase activity through maltose. This model system was chosen for a number of reasons: (i) MBP undergoes very large conformational changes upon binding maltose,[19] a conformational change that has previously been exploited for the creation of molecular sensors,[4,5] therefore, maltose-binding to MBP has a high potential for affecting the structure/activity of a protein domain inserted into MBP. (ii) MBP is a periplasmic-binding protein, a class of proteins whose binding sites have been redesigned to bind a wide variety of ligands by computation design,[20] (iii) β-lactamase has its N and C termini proximal, a proximity that may also be required for the domain insertion to be successful for geometric reasons, (iv) established selection methods exist for the activities of β-lactamase and MBP, and (v) β-lactamase has been successfully inserted into MBP in two surface loops chosen because of their tolerance to insertion of small peptides.[21] These insertional fusions, which contained long linkers, had activities essentially that of the individual wild-type enzymes. Although the stability of the β-lactamase activity to denaturants was found to increase in the presence of maltose, no coupling of the β-lactamase activity and maltose-binding was reported in the absence of denaturants. Furthermore, these fusions cannot be considered to be allosteric enzymes since the maltose is, in all likelihood, altering the equilibrium between folded and unfolded protein in the presence of denaturants.

Construction and characterization of random domain insertion libraries

In order to systematically explore the domain insertion strategy, we developed a methodology for constructing random domain insertion libraries (Figure 1). The *E. coli* MBP was cloned into plasmid pDIMC8[22] under control of the *tac* promoter to create plasmid pDIMC8-MBP. This vector is the target for insertion. The β-lactamase gene fragment *bla[24-286]* (codes for amino acid residues 24–286) was amplified by PCR from pBR322 such that it was flanked by *Ear* I restriction enzyme sites and cloned into the pTAdv to create the vector pTAdv-bla.

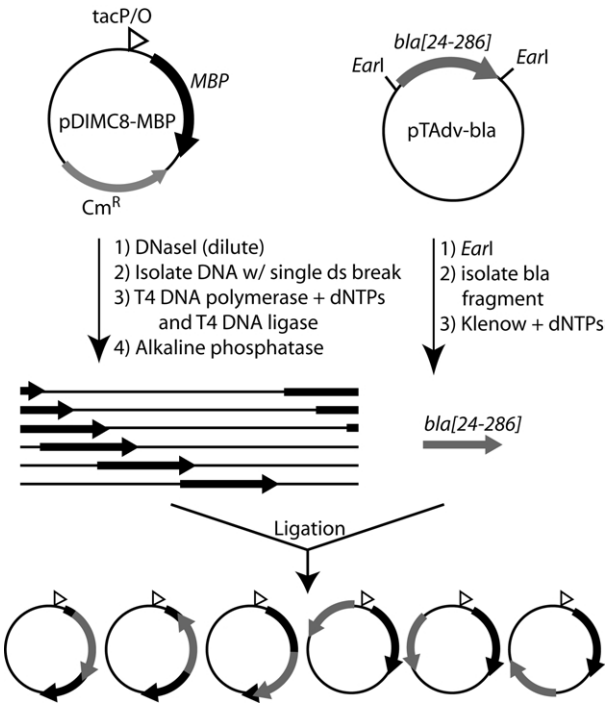

Figure 1. Schematic depiction of the construction of random domain insertion libraries. tacP/O, tac promoter/operator; Cm^R, chloramphenicol acetyltransferase; *MBP*, maltose binding protein gene (*malE*); *bla[24-286]*, fragment of TEM-1 β-lactamase gene coding for amino acid residues 24–286.

DNA coding for amino acid residues 1–23 was not desired because it codes for the signal sequence that targets β-lactamase to the periplasm. This sequence gets cleaved upon entering the periplasm and is not part of the mature, active β-lactamase. In the fusion constructs, the natural signal sequence of *malE* will direct the fusions to the periplasm. The gene fragment *bla[24-286]* was excised from pTAdv-bla with *Ear*I and the ends made blunt with Klenow polymerase. The desired fragment for insertion is achieved without extraneous bases by virtue of the fact that *Ear*I is a type IIS restriction enzyme that binds a non-palindromic sequence and cleaves outside this sequence. Note that the *bla[24-286]* fragment for insertion does not contain any sequence coding for a peptide linker.

Plasmid pDIMC8-MBP was randomly linearized using dilute concentrations of DNaseI in the presence of 1 mM Mn^{2+}. The digestions were controlled such that a significant fraction of DNA was undigested in order to maximize the amount of linear DNA that only had one double-strand break. DNA corresponding to one double-stranded break was purified by agarose gel electrophoresis. This DNA was repaired using T4 DNA ligase and T4 DNA polymerase and dephosphorylated. Ligation with the blunt *bla[24-286]* insert DNA and transformation into DH5α-E produced 1.06×10^6 transformants with 70% of the transformants containing the *bla[24-286]* insert.

Sequencing of 19 randomly selected library members indicated that the insertions were suitably random (all 19 were unique) and had a distribution of deletions and tandem duplications of the target DNA on either side of the inserted DNA (Figure 2). This important source of diversity in the library affects the distance and interactions between the two domains. A previously described method for random domain insertions was not observed to produce any tandem duplications.[23]

Selection of bifunctional library members

Approximately 0.8% (~8000 members) of the library could grow on plates containing ampicillin, indicating a functional β-lactamase protein. Sequencing of plasmid DNA from random Amp^R colonies showed that library members with an N-terminal fragment of the MBP gene fused in-frame to *bla[24-286]* with the remaining fragment of the MBP gene being out of frame dominated this sub-library. The plasmid DNA from all Amp^R colonies was isolated *en masse* and transformed into the MBP auxotroph PM9′, a strain unable to grow on minimal media with maltose as a sole carbon source unless the MBP is provided *in trans*.[24]

Approximately 10% (800 members) of the sub-library could grow on minimal plates containing maltose and ampicillin, indicating that the fusion could transport maltose in *E. coli*. Analysis of these bifunctional library members indicated that the insertions were predominantly localized to two locations in the MBP protein: near the C terminus and near residue 170, though sites outside of these were also found (Figure 3a). The sites for successful insertion correlated well with regions in MBP that have been previously shown to tolerate short insertions or deletions.[25]

Identification of bifunctional library members that are allosteric enzymes

As an initial examination of the behavior of these bifunctional proteins, overnight innocula of PM9F′ cells bearing nine of the sequenced members were lyzed by French press and the soluble fraction assayed for nitrocefin hydrolysis with and without 50 mM maltose. One member, T369-370 (i.e. MBP[1-370]-BLA-MBP[369-370]; amino acid residues 369 and 370 of MBP were tandemly duplicated on either side), exhibited an approximate 50% increase in velocity in the presence of maltose but not sucrose. Amino acid 370 is the last amino acid of MBP; thus, T369-370 was essentially an end-to-end fusion. Removal of 369 and 370 from the C terminus to produce an exact end-to-end fusion (MBP-BLA) resulted in a fusion that exhibited a similar stimulation of nitrocefin hydrolysis in the presence of maltose.

To identify other switches, a parallel assay was developed in which cultures of random bifunctional library members were grown in 96-well format in the presence of IPTG, resulting in the accumulation of the bifunctional protein in the

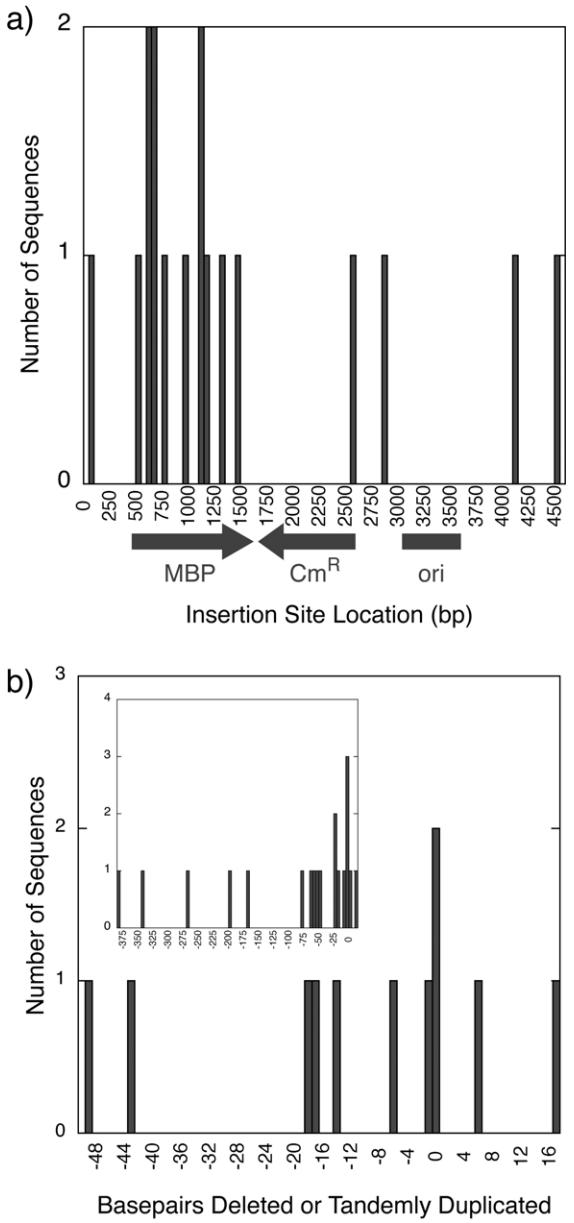

Figure 2. Characterization of the naïve random domain insertion library. Nineteen members of the naïve library that contained a β-lactamase gene insert were sequenced to identify the site of insertion in the plasmid and the number of bases deleted or tandemly duplicated. (a) Location of insertion sites on the plasmid. The locations of the gene coding for the maltose binding protein (MBP), the chloramphenicol acetyltransferase gene (Cm^R) and the origin of replication (ori) are indicated. Insertion in Cm^R and ori were not observed because most insertions in these regions will produce plasmids that do not confer chloramphenicol resistance or cannot be stably replicated, respectively. By chance, a disproportional number of the insertion sites were in the region coding for MBP. Restriction enzyme analysis of the library as a whole indicated that the frequency of insertions in the region coding for MBP was approximately 40–50%. (b) Number of base-pairs of the target plasmid deleted or tandemly duplicated at the insertion site. A negative number indicates a deletion and a positive number indicates a tandem duplication. The full-size graph indicates the range of deletions up to 50 bp and tandem duplications up to 18 bp. The small graph covers the full range of deletions found (up to 379 base-pairs).

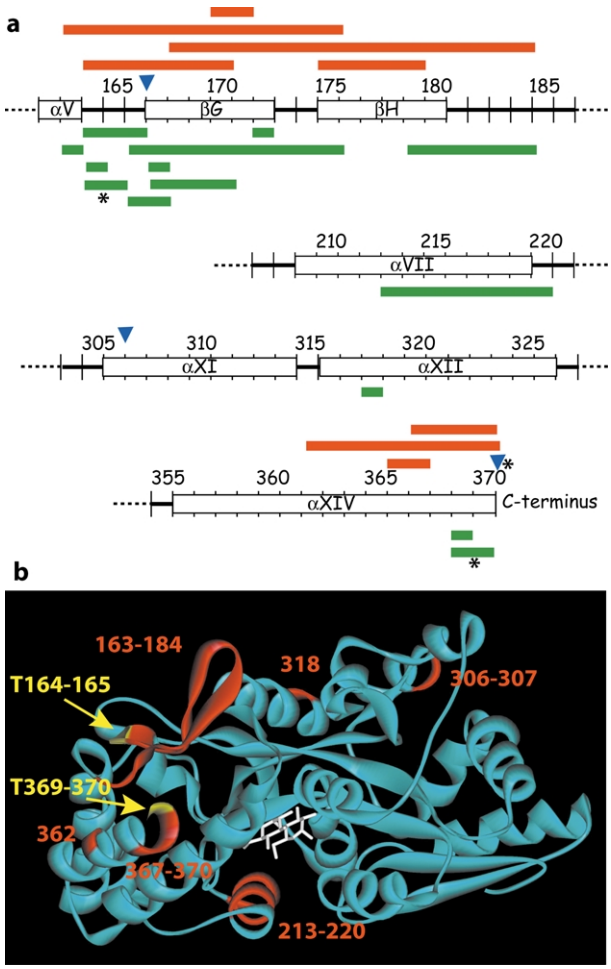

Figure 3. Insertion sites of bifunctional and allosteric enzymes. (a) Insertion sites of bifunctional proteins mapped on to the sequence of MBP. Only those regions in which bifunctional insertions were found are shown. Secondary structure elements are indicated on the sequence. An inverted blue triangle indicates a direct insertion of β-lactamase between the indicated amino acid residues. A red bar (positioned over the sequence) indicates that insertion of β-lactamase occurred with the deletion of the indicated MBP residues. A green bar (positioned under the sequence) indicates that insertion of β-lactamase occurred with the tandem duplication of the indicated MBP residues on either side of the β-lactamase domain. Sequences that are allosteric enzymes are indicated with an asterisk. (b) Insertion sites of bifunctional fusions and allosteric enzymes mapped onto the structure of MBP Sites where insertion of BLA into MBP creates bifunctional proteins (red) and allosteric enzymes (yellow) are shown on the structure of MBP with bound maltose (white).[28]

media. The media fraction was assayed spectrophotometrically in 96 well format for the velocity of β-lactamase hydrolysis of nitrocefin in the presence and absence of 5 mM maltose. The concentration of nitrocefin used was the same as the K_m for nitrocefin of wild-type β-lactamase so that switches in which maltose-binding affected either catalysis or substrate-binding might be identified. In a screening of ~400 bifunctional library members, a second library member that exhibited an ~40% increase in velocity of nitrocefin hydrolysis in the presence of maltose, but not in the presence of sucrose or glucose, was found three times: T164-165 (i.e. MBP[1-165]-BLA-MBP[164-370]; amino acid residues 164 and 165 of MBP were tandemly duplicated on either side). This site is within a hotspot for bifunctional proteins (Figure 3).

Initial kinetic characterization of allosteric enzymes

Initial kinetic characterization was performed on the media fraction of cells expressing the switches (Table 1).[26] As a negative control, an equal molar amount of purified MBP was added to the media fraction of cells expressing wild-type BLA and assayed for nitrocefin hydrolysis in the presence and absence of maltose. No difference in the kinetic parameters was detected. In contrast, the media fractions of cells expressing MBP-BLA or T164-165 showed an increase in k_{cat} in the presence of maltose but not with disaccharides known not to bind to the MBP (sucrose and lactose; data not shown). The presence of maltose resulted in an increase in K_m for MBP-BLA but no significant change for T164-165. Thus, only T164-165 showed an increase in k_{cat}/K_m in the presence of maltose. T164-165 was also the most sensitive switch, with a K_d for maltose close to that of the wild-type MBP. T164-165 was chosen for further characterization.

Purification and kinetic characterization of T164-165

Western blots (using anti-β-lactamase antibodies) of SDS-PAGE of the media fraction of cells expressing T164-165 showed a prominent band at the expected size of the fusion protein, but several bands with a size intermediate between the fusion and BLA. We ultimately chose to add a C-terminal His_6 tag to T164-165 (T164-165-H) and purify the protein by nickel affinity chromatography since purification *via* an amylose column resulted in poor yields and did not completely purify the full-length fusion from smaller fragments. Media fractions of cells expressing T164-165-H had very similar kinetic parameters to media fractions of cells expressing T164-165 (Table 1). Ni-column-purified T164-165-H was judged by Coomassie staining of SDS-PAGE gels to be >95% pure. N-terminal protein sequencing resulted in the sequence KIEEGK, the expected sequence of T164-165-H with its signal sequence removed.

The kinetic parameters of T164-165-H for nitrocefin hydrolysis at 30 °C in the presence of maltose ($k_{cat} = 1060\ s^1$; $K_m = 39\ \mu M$) were very similar to previously reported values for TEM-1 β-lactamase ($k_{cat} = 930\ s^1$; $K_m = 52\ \mu M$),[27] indicating that T164-165-H is a fully active TEM-1 β-lactamase (Table 2). The effect of maltose-binding on k_{cat} was more prominent at 22 °C than at 30 °C (70% *versus* 30% increase); however, at 30 °C a 20% decrease in K_m in the presence of maltose was observed. Switching behavior could be detected at concentrations of maltose less than 1 μM and the K_d for maltose-binding at 22 °C was 1.7(±0.5) μM, indistinguishable from the K_d previously reported for maltose-binding to MBP.[26]

Switching behavior correlates with a conformational change in MBP

MBP can bind many other maltooligosaccharides but the conformational change that is induced is ligand-dependent. The two structures that are commonly observed have been termed the open structure and the closed structure. MBP in the absence of ligand exists in the open structure. The closed structure, exemplified by that observed in the presence of maltose, is characterized by an approximately 35° closure and a −3.5° twist. Crystal structures show that maltose and maltotriose-binding results in an almost identical closed conformation.[28] Maltotriitol and maltotetraitol-binding results in a mixture of open and closed structures, the precise ratio of which is not known.[29] The crystal structure of MBP bound to

Table 1. Kinetic parameters of nitrocefin hydrolysis in *E. coli* culture supernatants

Protein	K_d maltose (μM)	K_m nitrocefin (μM)		k_{cat} ratio[a]	k_{cat}/K_m ratio[a]
		No. maltose	5 mM maltose		
BLA + MBP[b]	1–1.5[c]	35 ± 4	34 ± 2	1.0 ± 0.1	1.0 ± 0.2
MBP-BLA	14 ± 7	20 ± 1	32 ± 1	1.8 ± 0.1	1.1 ± 0.2
T164-165	3.2 ± 1.0	29 ± 3	28 ± 5	1.6 ± 0.1	1.6 ± 0.5
T164-165-H	nd	36 ± 3	34 ± 1	1.5 ± 0.1	1.6 ± 0.3

[a] (With maltose)/(without maltose).
[b] BLA and MBP present as separate proteins.
[c] Schwartz *et al.*[26]

Table 2. Kinetic parameters of T164-165-H molecular switch

Temp. (°C)	k_{cat} (s^{-1})			K_m (μM)			k_{cat}/K_m ratio[a]
	No. maltose	3.3 mM maltose	Ratio[a]	No. maltose	3.3 mM maltose	Ratio[a]	
30	790 ± 20	1060 ± 10	1.3 ± 0.1	51 ± 4	39 ± 1	0.8 ± 0.1	1.7 ± 0.3
22	450 ± 20	750 ± 20	1.7 ± 0.1	31 ± 3	30 ± 2	1.0 ± 0.1	1.8 ± 0.4

[a] (with maltose)/(without maltose).

β-cyclodextrin shows an open conformation very similar to that of the unbound protein;[30] however, NMR studies show a small closure of 13° and a twist of 5°.[31]

Only those ligands known to bind and induce conformational changes in MBP resulted in an increase in nitrocefin hydrolysis in T164-165-H and MBP-BLA (Figure 4a). Sugars known not to bind MBP (galactose, glucose, sucrose and lactose) had no effect at a concentration of 0.5 mM, which is a concentration $\sim 10^3$-fold higher than that at which maltose's effect could be observed. Although galactose, sucrose and lactose had no effect at 50 mM, glucose at this concentration resulted in a 45(±3)% increase in nitrocefin hydrolysis for T164-165H. However, intrinsic fluorescence data (below) suggest that trace maltose contamination in the glucose is the source of the increase. The increase in activity induced by maltose and maltotriose were statistically equivalent, consistent with the crystallographic data that shows that MBP exists in essentially the same closed conformation when bound to these sugars. β-Cyclodextrin, known to induce the smallest change in conformation, was observed to result in a small increase in the rate for T164-165H. Binding of β-cyclodextrin to T164-165-H was confirmed by competition experiments in which maltose's stimulation of β-lactamase activity could be completely competed away by β-cyclodextrin. β-Cyclodextrin had a larger effect on MBP-BLA, suggesting perhaps that MBP-BLA and T164-165H have different mechanisms for the allosteric effect observed. The switching effect induced by maltotriitol or maltotetraitol was intermediate, consistent with a mixture of both open and closed structures that are predicted to exist in MBP bound to these sugars based on crystallographic data.[29]

Since the circular dichroism spectrum of MBP changes very little upon binding of maltose or β-cyclodextrin,[32] changes in secondary structure of T164-165H upon sugar addition were monitored by intrinsic protein fluorescence, which has been shown to correlate with conformational change.[33] The emission maximum of MBP occurs at 348 nM. The binding of various maltooligosaccharides quenches the fluorescence of MBP on the order of 5–10% and alters the intrinsic fluorescence maximum of MBP. A 2.5 nM red shift is induced by maltose and a 6 nM blue shift is induced by β-cyclodextrin. Other maltooligosaccharides produce shifts between these values, with those known to bind in the closed form tending to be more red-shifted and those that are known to bind in both closed and open form tending to be more blue-shifted. The increase in velocity of nitrocefin hydrolysis correlated very well both with the shift in wavelength of maximum fluorescence previously reported for MBP[33] and the shift in wavelength of maximum fluorescence measured for T164-165-H (Figure 4b). All ligands induced a 5–10% quenching of the fluorescence of T164-165-H, much like that observed with MBP, with the exception of β-cyclodextrin, which induced only a 1% quenching. The presence of 50 mM glucose resulted in a red shift of 3 nM in T164-165-H, identical to the red shift produced by maltose. The fluorescence quenching with 50 mM glucose was 8% compared to 7% with maltose. This suggests that maltose is present as a contaminant in the glucose (at ~0.02% based on stimulation of nitrocefin activity) and is responsible for the stimulation of nitrocefin activity of T164-165-H in the presence of high levels of glucose.

Discussion

Domain insertion, unlike end-to-end fusion, allows the fusion of two proteins with a wide variety of geometric configurations. Such new orientations expand and enhance the applications of gene fusion such as protein purification, metabolic engineering and biochemical analysis.[34] Our criteria for bifunctionality were quite stringent: the fusions must have β-lactamase activity and must be able to transport maltose in *E. coli*. Transport requires maltose-binding, a conformational change in the MBP domain upon maltose-binding, and the requisite interactions with membrane proteins MalG and MalF. Thus, library members that bind maltose but cannot interact with MalG and MalF were not selected (and are not bifunctional by definition). The sites for successful insertion of BLA into MBP to make a bifunctional protein correlate quite well with permissive sites in MBP that tolerate short insertions/deletions[25] and protein bisection.[24] Thus, the striking observation of those studies (permissive sites were often within α-helical and β-strand structural elements) is repeated here. Bifunctional fusion Δ163-175 deletes an entire β-sheet and bifunctional fusion T213-220 tandemly duplicates two-thirds of an α-helix. Permissive sites for random insertions of GFP into the cAMP-dependent protein kinase regulatory subunit also included ones within α-helices.[23] It is worth noting

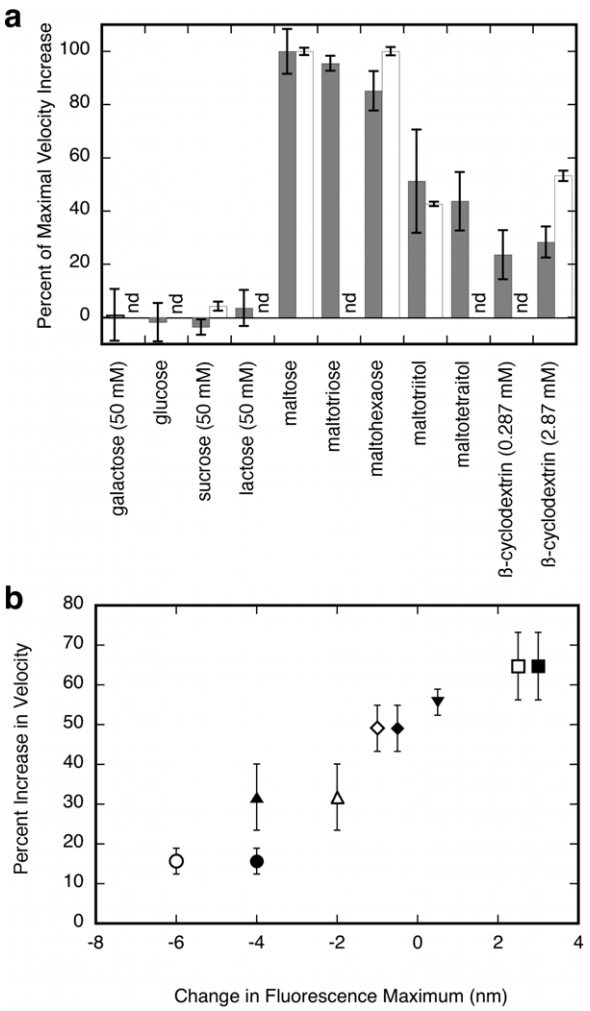

Figure 4. The rate of nitrocefin hydrolysis catalyzed by the domain fusions is only modulated by sugars that bind and induce a conformational change in MBP. (a) Percent of maximal increase in nitrocefin hydrolysis velocity (normalized to the increased caused by maltose) for T164-165H (filled bars) and MBP-BLA (open bars) in the presence of different sugars. The concentration of nitrocefin was 50 μM (T164-165H) or 200 μM (MBP-BLA). The concentration of sugar was 0.5 mM (for T164-165H) or 5 mM (for MBP-BLA) unless otherwise stated. A higher concentration of sugar was used for MBP-BLA since it has a lower affinity for maltodextrins. Experiments with T164-165H were performed with purified protein, whereas experiments with MBP-BLA were performed with lyzates of *E. coli* cells expressing MBP-BLA. nd, not determined; β-cyclo, β-cyclodextrin. (b) Percent increase in nitrocefin hydrolysis by T164-165-H as a function of shift in maximum intrinsic protein fluorescence upon sugar-binding to MBP (open symbols) and T164-165-H (filled symbols). The percent increase in velocity is in the presence of 5 mM sugar (except β-cyclodextrin, 2.87 mM sugar). The fluorescence maximum data were measured at 0.5 mM sugar (for MBP) or 1 mM sugar (for T164-165-H). β-Cyclodextrin (circle), maltotetraitol (triangle), maltotriose (inverted triangle), maltohexaose (diamond), maltose (square). The data points for maltotriose overlap.

that both the N terminus and C terminus of BLA are α-helices and it is possible that this could facilitate stable insertion within α-helices of MBP by offering a seamless continuation of the helix across the fusion point. Internal tandem duplications of α-helices and fragments thereof have been found to be tolerated in *Staphylococcal* nuclease.[35]

Two of the five permissive sites for linker scanning mutagenesis and protein fragment complementation (at about residues 133 and 285) were not observed to be permissive for domain insertion in this study. In a previous study, BLA with four to five amino acid linkers on each termini, was successfully inserted into MBP at residue 133.[21] The fact that linkers were not used in our study suggests that linkers may be required at this site or that insertion at this site interferes with interactions with MalG and MalF. However, only a small fraction of the bifunctional genes were sequenced. Thus, an alternate explanation is that more extensive sequencing of the bifunctional library members would identify bifunctional fusions in this region as well.

One of the allosteric enzymes identified by random domain insertion was essentially an end-to-end fusion of the two genes. It was unexpected that end-to-end fusion would result in a switch since end-to-end fusions of MBP and BLA (with linkers) have not been reported to behave as a switch.[21] In addition, the β-lactamase activity of another selected bifunctional protein in our library with a similar sequence (Δ367-368) was not modulated by maltose. It is not anticipated that, as a general rule, end-to-end fusions will behave as a switch. However, the occurrence here demonstrates that it is possible to functionally couple two proteins simply by end-to-end fusion.

The internal fusion T164-165 was the better of the two switches in the sense that it behaved as a switch both at high and low substrate concentrations and was more sensitive to low concentrations of maltose. The effects of maltose on the Michaelis–Menten parameters were found to be temperature-dependent. In addition, preliminary kinetic data using a variety of cephalosporins and penicillins as substrates indicates that the switching magnitude and direction of T164-165-H is substrate-dependent (P. L. Riva, G.G. and M.O., unpublished results). The interpretation of these results is complicated by the mechanism of β-lactamase. The acyl-enzyme mechanism (Scheme 1) for active-site serine β-lactamases like the TEM-1 β-lactamase is well-established.[27,36,37]

$$E + S \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} ES \xrightarrow{k_2} E\text{-acyl} \xrightarrow{k_3} E + P$$

Scheme 1.

For such a mechanism, the Michaelis–Menten equation still applies, the difference being that K_m and k_{cat} are now combinations of the rate

constants[36]:

$$K_m = \left(\frac{k_{-1} + k_2}{k_1}\right)\left(\frac{k_3}{k_2 + k_3}\right);$$

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3}$$

and

$$\frac{k_{cat}}{K_m} = \frac{k_1 k_2}{k_{-1} + k_2}.$$

Since k_2 is often within an order of magnitude of k_{-1}, the normal simplifications of $k_{-1} \gg k_2$ cannot be made.[36] Assuming the β-lactamase activity in the fusions follows this mechanism, one can only conclude that the presence of maltose increases the rate of one or both of the chemical steps and nothing definitive can be said about whether binding of substrate has been affected, even when K_m changes.

Several lines of evidence indicate that a conformational change in the MBP domain of T164-165-H upon maltose-binding is responsible for the differences in the enzymatic properties. First, T164-165 complements the *malE*$^-$ auxotroph, indicating it can transport maltose in *E. coli*. A conformational change in MBP upon maltose-binding is known to be required for maltose transport. Thus, T164-165 must undergo this conformational change upon binding maltose as well. Second, the level of increase in the rate of nitrocefin hydrolysis induced by different maltooligosaccharides correlates well with whether MBP undergoes a conformational change in the presence of those maltooligosaccharides. Finally, the magnitude and wavelength change in the maximum intrinsic fluorescence upon binding different maltooligosaccharides were very similar for MBP and T164-165-H.

The ability to create allosteric enzymes has applications in metabolic engineering. For example the coupling of an effector-binding domain to an enzyme active in a metabolic pathway could be used as a posttranslational method to control flux through that pathway. More generally, the ability to functionally couple two proteins has a wide range of potential therapeutic and biotechnological applications as well as applications as research tools. For example, protein molecular switches could be created that (i) couple a drug-binding protein to a protein that recognizes a molecular signature of a disease to create a switch for targeted drug delivery, (ii) couple an effector-binding domain to a transcription factor to create transcription factors that respond to effector concentration and (iii) couple a ligand-binding protein to a protein convenient for signal transduction to create molecular biosensors.

These engineered allosteric enzymes also serve as an interesting platform for exploring theories on how natural allosteric enzymes were first created and evolved. The models that dominate the view of allosteric regulation (the Monod–Wyman–Changeux (MWC) model[38] and the Kosland–Némethy–Filmer (KNF) model)[39] achieve allostery by deploying interactions between subunits of an oligomer. The recent report of a monomeric class II ribonucleotide reductase that is allosterically regulated[40] was viewed as somewhat of a conundrum[41] even through the monomer seemed to have re-established the dimer interface seen in homologs by a single 130-residue insertion. However, since it is widely appreciated that information can be transmitted through intramolecular interactions it seems obvious that an allosteric regulation in a monomeric enzyme can be established by coupling the binding site and the catalytic site through a chain of propagating interactions.[41–44] This leads to the question as to why nature, for the most part, evolved intermolecular instead of intramolecular allosteric interactions.

Although it remains to be conclusively proven, preliminary characterization of these engineered allosteric enzymes, which are derived from monomeric proteins, suggests that they are monomeric. Thus, the allostery achieved is a type of allostery that nature could achieve but has not. Furthermore, although the MWC and KNF models allow for effects on binding and catalysis, in practice almost all natural allosteric enzymes are regulated through effects on K_m (binding) alone. The engineered allosteric enzymes so far have only been shown to result from effects on k_{cat}. Although K_m has also been shown to be affected, this does not necessarily mean that binding has been affected, since K_m is a function of the catalytic rate constants as described above. Thus, the study of the nature of the allosteric effect created, the magnitude of the allostery that can be achieved and the mechanism by which allostery is achieved in these very "unconventional" allosteric enzymes may shed light on questions of the evolution of allosteric regulation by contrasting with "conventional" allosteric enzymes.

The domain insertion strategy presumably, but not necessarily, requires that one of the two proteins has its N and C termini near each other for geometric reasons; however, more than one-third of all proteins have their N and C termini proximal.[45] Many of the applications, including that of creating an allosteric enzyme, require a ligand-binding protein. However, ligand-binding is increasingly becoming a function that can be designed. In fact, periplasmic-binding proteins, of which MBP is an example, have proven to be excellent templates for structure-based computational design of novel receptors that bind their target ligands with high affinity and specificity.[20]

Approximately 70% of our library had BLA inserted somewhere in the plasmid. Since roughly half of the library has the BLA gene inserted into the MBP gene, half of the library will have BLA inserted in the correct orientation and one ninth of the library will have both crossovers in-frame, we estimate that our library contained

$(1.06 \times 10^6) \times 0.7 \times 1/2 \times 1/2 \times 1/9 = 20{,}600$ in-frame domain insertions of BLA into MBP. Of these approximately 4% (800/20,600) were bifunctional. After screening about 50% of the bifunctional members we found two switches, which corresponds to a frequency of switches among the in-frame domain insertions of approximately one in 5000. Such a frequency is remarkably high considering that we are creating a property (allostery) that did not exist in the starting proteins. The existing directed evolution toolbox is rich with combinatorial methods for improving protein function, if such a function exists. However, to our knowledge combinatorial methods such as random mutagenesis and DNA shuffling have not been demonstrated to be able to create a property, such as was achieved here with random domain insertion. Given the success of directed evolution to improve a wide variety of protein functions (particularly if the function is weak) it is reasonable to expect that the magnitude of the allosteric effects observed in the fusions engineered here can be improved further by established directed evolution methodologies.

Materials and Methods

Materials

DNaseI was purchased from Roche Biochemicals (Indianapolis, IN). All other enzymes and MBP were purchased from New England BioLabs (Beverly, MA). Nitrocefin was purchased from Oxoid (Hampshire, UK), anti-β-lactamase antibodies from Chemicon (Temecula, CA) and all saccharides from Sigma (St Louis, MO). The His-tag protein purification kit was purchased from Novagen (Madison, WI), Electromax DH5α-E from Invitrogen Life Technologies (Carlsbad, CA) and pT-Adv cloning kit from Clontech (Palo Alto, CA). All DNA purification kits were purchased from Qiagen (Valencia, CA).

Construction of random domain insertion libraries

The *malE* gene was PCR-amplified from *E. coli* chromosomal DNA and inserted into the low-copy plasmid pDIM-C8[22] under the control of the *tac* promoter to create pDIM-C8-MBP. An aliquot of 40 μg of pDIM-C8-MBP was digested with DNaseI (0.01 units) for eight minutes at 22 °C in the presence of 50 mM Tris–HCl (pH 7.4), 10 mM $MnCl_2$ and 50 μg/ml BSA in a total volume of 1 ml. The reaction was quenched by the addition of EDTA to a concentration of 5 mM and the solution was desalted using four Qiaquick PCR purification columns into 200 μl elution buffer which was subsequently concentrated by vacufuge. Nicks and gaps were repaired by incubating at 12 °C for one hour in a total volume of 120 μl in the presence of T4 DNA polymerase (15 units) and T4 DNA ligase (12 Weiss units) in the presence of 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 μg/ml BSA and 125 μM dNTPs. The reaction was stopped by incubating at 80 °C for ten minutes. Sodium chloride was added to 100 mM and the DNA was dephosphorylated by adding alkaline phosphatase (60 units) and incubating at 37 °C for one hour. The DNA was desalted as before and the linear DNA (corresponding to the randomly linearized pDIM-C8-MBP) was isolated from circular forms of the plasmid by agarose gel electrophoresis using the Qiaquick gel purification kit.

The TEM-1 β-lactamase gene was PCR-amplified from pBR322 such that it was flanked by *Ear* I sites and cloned into pTAdv T-vector to create pTAdv-bla. pTAdv-bla was digested with *Ear* I and treated with Klenow polymerase and dNTPs to create blunt ends. This DNA (85 ng) was ligated to the randomly linearized pDIM-C8-MBP (100 ng) at 22 °C overnight in the presence of T4 DNA ligase (30 Weiss units) and the ligase buffer provided by the manufacturer in a total volume of 13 μl. After ethanol-precipitation, 10% of the ligase-treated DNA was electroporated into 50 μl Electromax DH5α-E electrocompetent cells. Transformed cells were plated on a large (248 mm × 248 mm) LB agar plate supplemented with 50 μg/ml chloramphenicol (Cm). The naïve domain insertion library was recovered from the large plate[46] and stored in frozen aliquots.

Selection of bifunctional library members

Ten million colony forming units (CFU) were plated on a large LB agar plate containing 2% (w/v) maltose and 50 μg/ml ampicillin in order to obtain the ampicillin-resistant sublibrary. Cells were recovered as before and the plasmid DNA was isolated using the Qiagen midi-prep kit. Plasmid DNA was transformed into *E. coli* PM9F′ and cells were plated on a large LB agar plate supplemented with 50 μg/ml Cm. Recovered cells were frozen in aliquots, diluted and plated on M9 minimal plates containing 0.2% (w/v) maltose and 100 μg/ml ampicillin and incubated at 37 °C overnight.

Screening for allosteric enzymes

Colonies were picked and grown in LB overnight in 96 well plates (1 ml/well) in the presence of 0.1 mM IPTG and 50 μg/ml Cm. The cells were pelleted by centrifugation and supernatant was recovered. In 96-well format, 20 μl of supernatant was assayed for hydrolysis of nitrocefin (50 μM) by monitoring the increase in absorbance at 490 nm in 100 mM sodium phosphate buffer (pH 7.0), both with and without 5 mM maltose. Any supernatant in which there was a difference in rate of more than 25% (between with and without maltose) was selected for further investigation.

Protein purification and characterization

A GGSGHHHHHH His-tag was added to the C termini of T164-165. Two and a half liters of LB was inoculated with 2% overnight culture and 50 μg/ml Cm. Cells were grown at 37 °C until the A_{600} was 0.5. IPTG was added to 0.1 mM and the culture was shaken at 25 °C for another six hours. Cells were pelleted and resuspended in 33 mM Tris–HCl (pH 7.0) buffer. The periplasmic fraction was isolated by osmotic shock[47] and passed over the Ni^{2+} column. The protein was eluted with 120 mM imidazole solution and dialyzed at 4 °C against 300 volumes of 50 mM sodium phosphate buffer (pH 7.0) with 100 mM sodium chloride for two hours and then against 1000 volumes of the same buffer overnight. Glycerol was added to 10% (w/v) and the protein stored in aliquots at −80 °C. The molar extinction coefficient of T164-165-H at 280 nM was calculated to be 92,840 $M^{-1}\,cm^{-1}$.[48]

Kinetic characterization

The kinetic constants and binding constants were determined from Eadie–Hofstee plots and Eadie plot equivalents, respectively, using a spectrophotometric assay for nitrocefin hydrolysis.[49] Initial rates were determined from absorbance at 486 nm monitored as a function of time. The enzyme was incubated at the assay temperature in the absence or presence of saccharide for four minutes prior to performing the assay. All assays contained 100 mM sodium phosphate buffer (pH 7.0). The dissociation constant for maltose was determined using change in velocity of nitrocefin hydrolysis as a signal.

Fluorescence measurements

Fluorescence spectra were obtained at 22 °C on a Photon Technology QuantaMaster QM-4 spectrofluorometer in 5 mM sodium phosphate buffer (pH 7.0), containing 10 mM sodium chloride. The protein concentration was 130 nM. Excitation was at 280 nm and emission was scanned from 285 nm and 400 nm. Saccharides were used at a concentration of 1 mM except as noted.

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