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A Molecular Switch Created by In Vitro Recombination of Nonhomologous Genes

Brief Communication

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Summary

We have created a molecular switch by the in vitro recombination of nonhomologous genes and subjecting the recombined genes to evolutionary pressure. The gene encoding TEM1 β-lactamase was circularly permuted in a random fashion and subsequently randomly inserted into the gene encoding *Escherichia coli* maltose binding protein. From this library, a switch (RG13) was identified in which its β-lactam hydrolysis activity was compromised in the absence of maltose but increased 25-fold in the presence of maltose. Upon removal of maltose, RG13's catalytic activity returned to its premaltose level, illustrating that the switching is reversible. The modularity of RG13 was demonstrated by increasing maltose affinity while preserving switching activity. RG13 gave rise to a novel cellular phenotype, illustrating the potential of molecular switches to rewire the cellular circuitry.

Introduction

The incredible complexity of biological systems derives to a large extent from the high degree of interactions among their constituent components. As such, the cell is often described as a complex circuit consisting of an interacting network of molecules. A key component of these networks are protein "switches" that serve to couple cellular functions. A switch recognizes an input signal (e.g., ligand concentration, pH, covalent modification), and, as a result, its output signal (e.g., enzyme activity, ligand affinity, oligomeric state) is modified. Examples of natural switches include allosteric enzymes that couple effector levels to enzymatic activity and ligand-dependent transcription factors that couple ligand concentration to gene expression. The ability to create novel switches or to modify existing switches by coupling hitherto uncoupled functions would enable the rewiring of the cellular circuitry to our own design. In addition, the ability to create protein switches has tremendous practical potential for developing novel molecular sensors, "smart" materials, and as a tool for elucidating molecular and cellular function.

We sought a general approach for creating switches in which one could select from natural or engineered proteins with the desired input and output functions and, by combining the proteins in a systematic fashion, create switches in which their functions were tightly coupled. In addition, we sought switches in which the presence of the signal resulted in an increase in the output function. Existing approaches [1–16] either require existing switches as starting points, are limited to specific input functions and mechanisms, lack reversibility, or lack practicality in vitro or in vivo. In addition, most existing approaches have not been shown to produce switches with large changes in output (>10-fold) in response to a signal.

Our approach involves recombination between two genes encoding the prerequisite input and output functions. Such an approach is inspired by the evolutionary mechanism of domain recombination [17], a major facilitator in the natural evolution of protein function [18]. Domain recombination strategies have been applied to the construction of switches [2, 7, 10–16], but the very limited manner in which the domains were recombined has restricted the success of this approach. We reasoned that a more diverse exploration of fusion geometries between two proteins would enable the creation of switches with superior properties. The structural space that we sought to explore can be conceptualized as "rolling" the two proteins across each other's surface and fusing them through peptide bonds at the points where their surfaces meet. We developed a homology-independent, combinatorial method for recombining genes that samples such a structural space. The method involves two basic steps. First, the gene to be inserted is circularly permuted in a random fashion to vary the fusion site on the insert gene. Second, the library of the circularly permuted gene is randomly inserted into the target gene.

Results and Discussion

We applied this approach to the recombination of the genes encoding TEM-1 β-lactamase (BLA) and the *E. coli* maltose binding protein (MBP). BLA and MBP lack any sequence, structural, or functional relationship except for the fact that they are periplasmic proteins of bacterial origin. BLA is a monomeric enzyme that hydrolyzes the amide bond of the β-lactam ring of β-lactam antibiotics. The presence of maltose has no effect on wild-type BLA enzymatic activity, with or without the presence of an equimolar amount of MBP [15]. MBP is a member of the periplasmic binding protein superfamily and is involved in chemotactic response and the transport of maltodextrins. MBP consists of a single polypeptide chain that folds into two domains connected by a hinge region. The single binding site for maltose is at the interface of these two domains. In the absence of maltose, MBP exists in an open form. Maltose binding is concomitant with a 35° bending motion about the hinge, resulting in the closed form of the protein [19]. We sought to create a molecular switch by combining BLA and MBP in such a manner that the rate of β-lactam hydrolysis was coupled to maltose binding

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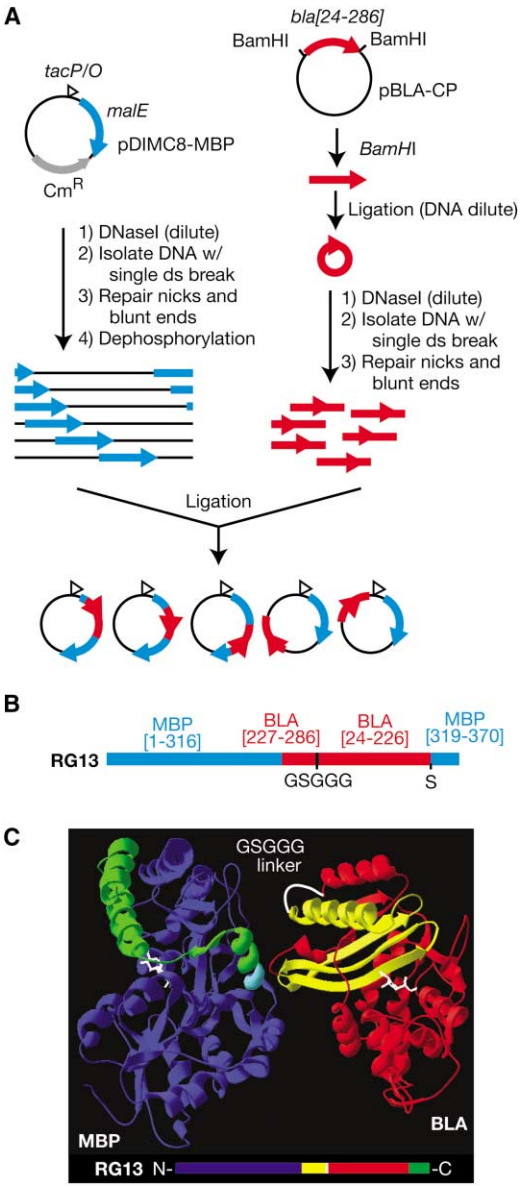

Figure 1. Creation of MBP-BLA Molecular Switches by Nonhomologous Recombination

(A) The fragment of the BLA gene coding for the mature protein (codons 24–286) is flanked by sequences coding for a GSGGG linker (each of which contains a BamHI site). The fragment is excised by digestion with BamHI and cyclized by ligation under dilute DNA concentrations. A single, randomly located double-strand break is introduced by DNaseI digestion to create the circularly permuted library. This library is randomly inserted into plasmid pDIMC8-MBP containing the MBP gene (*malE*) under control of the *tac* promoter (*tacP/O*). The site for insertion in pDIMC8-MBP is created by introduction of a randomly located double-stranded break by digestion with dilute concentrations of DNaseI.

(B) Sequence of switch. Regions derived from MBP are shown in blue, and those from BLA are shown in red. The GSGGG linker and a serine derived from the fusion of partial codons are shown in black. The number in parentheses indicates the amino acid number of the starting 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 β-lactamases.

(C) Structures of maltose-bound MBP [22] and BLA bound to an active-site inhibitor [23] oriented such that the fusion sites in RG13 are proximal. Dark blue, MBP[1-316]; yellow, BLA[227-286]; white, GSGGG linker; red, BLA[24-226]; green, MBP[319-370]; light blue, MBP deleted residues 317 and 318.

and maltose concentration. We reasoned that in such a switch the conformational change in the MBP domain upon maltose binding would propagate to the active site of the BLA domain and alter its catalytic properties, a mechanism analogous to natural allosteric effects.

The fragment of the *BLA* gene coding for the mature protein was circularly permuted in a random fashion [20, 21] and subsequently randomly inserted [15] into a plasmid containing the *E. coli malE* gene that codes for MBP (Figure 1A). For the random circular permutation of *bla[24-286]*, we fused the 5′ and 3′ ends by an oligonucleotide sequence that would result in a GSGGG flexible peptide linker between the original N and C termini of the protein. This linker was designed to be of sufficient length to connect the termini without perturbing BLA structure. Statistical analysis on the resulting library indicated that a minimum of 27,000 members contained a circularly permuted *bla[24-286]* inserted into *malE* in the correct orientation with both fusion points in-frame with *malE*. Approximately 0.33% of these members were able to form colonies on rich media plates containing 200 μg/ml ampicillin and 50 mM maltose. These library members were screened in 96-well format for a maltose dependence on β-lactamase activity using a colorimetric assay for nitrocefin hydrolysis. We identified one protein (RG13; Figure 1B) in which the initial velocity of nitrocefin hydrolysis (at 50 μM nitrocefin) increased by 17-fold in the presence of maltose. In RG13, the BLA was circularly permuted in a loop that precedes a β sheet that lines the active site of the enzyme. The circular-permuted BLA was inserted at the beginning of an α helix of MBP such that two MBP residues were deleted (Figure 1C).

Using purified RG13, we confirmed that the increase in catalytic activity occurred only in the presence of sugars that are known to bind and induce a conformational change in MBP (Figure 2A). Sugars known to induce a large conformational change [22] (maltose and maltotriose; 35° closure angle) produced a 15- to 20-fold increase in the rate of nitrocefin hydrolysis. β-cyclodextrin, which only induces a 10° hinge bending motion in MBP [24], increased the rate 2-fold. Nonligands such as sucrose, lactose, and galactose had no effect. We next determined that the switching was reversible (i.e., upon removing maltose, the activity returns to its premaltose level). This was demonstrated both by competing maltose off RG13 using β-cyclodextrin (Figure 2B) and by subjecting RG13 to repeated rounds of dialysis and addition of maltose to cycle between low and high levels of enzymatic activity (Figure 2C). This reversibility is one of the features that differentiates our approach from methods such as conditional protein splicing [8, 16], which produce nonreversible switches that control the *production* of active protein rather than a protein's activity per se.

From steady-state kinetics experiments, we determined RG13's Michaelis-Menten parameters for nitrocefin hydrolysis at 25°C in the absence and presence

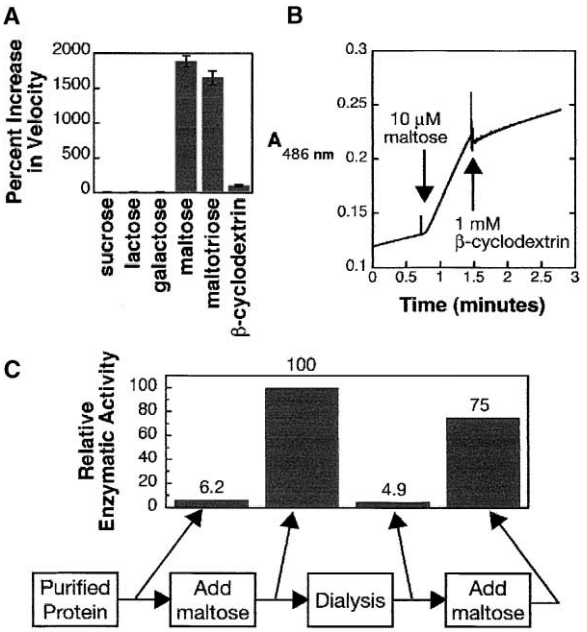

Figure 2. Switch Activity in RG13 Is Specific to Ligands of MBP and Is Reversible

(A) The percent increase in the initial velocity of nitrocefin hydrolysis at 20 μM nitrocefin upon addition 5 mM of the indicated ligands (maltose, maltotriose, and β-cyclodextrin) and nonligands (sucrose, lactose, and galactose).

(B) Reversible switching using competing ligand. During the enzymatic hydrolysis of nitrocefin, formation of product is monitored by absorbance at 486 nm. At time zero, the reaction is started in 2 ml phosphate buffer (0.1 M) with 20 μM nitrocefin and 2.5 nM RG13. At the time indicated by the first arrow, 20 μl of 1 M maltose was added, resulting in a 10-fold increase in the reaction rate. This maltose concentration is above the K_d for maltose but is subsaturating. At the time indicated by the second arrow, 230 μl of 10 mM β-cyclodextrin was added (final concentrations are 1.0 mM β-cyclodextrin and 8.9 μM maltose). Because RG13 has similar affinities for maltose and β-cyclodextrin but β-cyclodextrin is present at a >100-fold higher concentration, the β-cyclodextrin preferentially replaces the maltose bound to RG13, and the rate of reaction decreases to a level consistent with β-cyclodextrin's modest effect on nitrocefin hydrolysis.

(C) Reversible switching after dialysis. The initial rate of nitrocefin hydrolysis at 25 μM nitrocefin was measured at the indicated steps. Maltose was added to a final concentration of 5 mM.

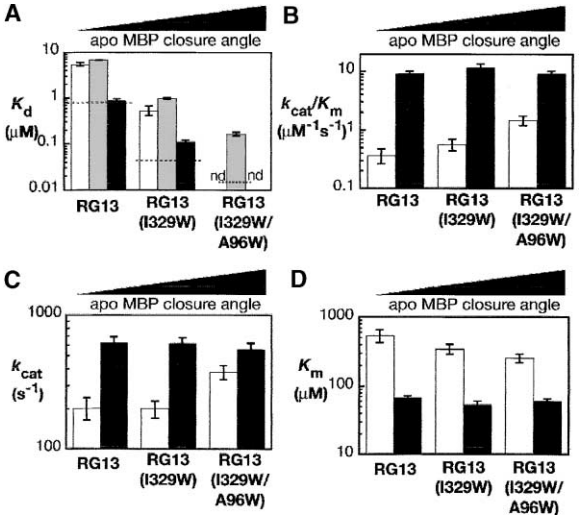

Figure 3. Characterization of Switches

(A) Dissociation constants for maltose were determined in the absence (white bars) and presence (black bars) of saturating concentrations of carbenicillin. The apparent dissociation constants in the presence of subsaturating concentrations (25 μM) of nitrocefin (gray bars) were also determined. The dissociation constants for maltose of MBP, MBP(I329W), and MBP(I329W/A96W) (dashed line) reported by Marvin and Hellinga [28] are shown for comparison.

(B–D) Steady-state kinetic parameters of nitrocefin hydrolysis for RG13, RG13(I329W), and RG13(I329W/A96W) in the presence (black bars) or absence (white bars) of saturating concentrations of maltose. Experimental conditions were as follows: 100 mM sodium phosphate buffer (pH 7.0), 25°C.

of maltose. In the absence of maltose, the catalytic constants were k_{cat} = 200 ± 40 s^{-1} and K_m = 550 ± 120 μM. With the addition of saturating amounts of maltose, k_{cat} increased 3-fold and K_m decreased 8-fold, resulting in a 25-fold increase in k_{cat}/K_m. The kinetic constants of RG13 in the presence of saturating concentrations of maltose (k_{cat} = 620 ± 60 s^{-1} and K_m = 68 ± 4 μM) were comparable to that previously reported for BLA at 24°C (k_{cat} = 900 s^{-1} and K_m = 110 μM; [25]), indicating that RG13 is a very active TEM1 β-lactamase in the presence of maltose. RG13 has exhibited switching behavior with all seven BLA substrates tested to date, including ampicillin (16-fold rate increase at 50 μM ampicillin) and carbenicillin (12-fold rate increase at 50 μM carbenicillin).

The increase in k_{cat} indicates that maltose binding affects the catalytic steps. However, since K_m is a combination of the rate constants for substrate binding as well as catalysis [26], K_m could not be directly used to ascertain the effect of maltose on substrate binding. Instead, the effect of maltose on substrate binding was determined indirectly by measuring the effect of substrate on maltose binding using intrinsic protein fluorescence. These studies suggested that RG13 undergoes a conformational change much like MBP does upon maltodextrin binding, since maltose-induced quenching of total fluorescence (~10%) and shifting of the maximum fluorescence wavelength (a 1.5 nm red-shift for maltose and a 4 nm blue-shift for β-cyclodextrin) were similar to that previously reported for MBP [27]. The presence of saturating amounts of the substrate carbenicillin decreased the dissociation constant of maltose and RG13 from 5.5 ± 0.5 μM to 1.3 ± 0.5 μM; thus, maltose binding must decrease the dissociation constant of carbenicillin and RG13 by the same factor (Supplemental Figure S1). This corresponds to a coupling energy of approximately 1 kcal/mol and offers an additional explanation for the increase in β-lactam hydrolysis in the presence of maltose: a positive heterotropic allosteric effect on substrate binding.

Presumably, the BLA domain of the apo, open form of RG13 exists in a compromised, less active conformation. In the ligand-bound state, the BLA domain exists in a more normal, active conformation. But what is the state of the BLA domain in the process of closing? At what closure angle do the catalytic properties of RG13 improve? To address these questions, we took advantage of mutations in the hinge region of MBP that manip-

Table 1. Ampicillin Resistance of *E. coli* Cells in the Presence and Absence of Maltose

Expressed Protein	Minimum Inhibitory Concentration of Ampicillin (μg/ml)[a]	
	No Maltose	50 μM Maltose
None	4	4
RG13	128	512
BLA(W208G)[b]	32	32
BLA	≥2000	≥2000

[a] Conditions: DH5α-E cells on LB plates (with or without maltose) incubated at 37°C for 20 hr.
[b] A mutant of BLA with reduced activity.

ulate the conformational equilibria between the open and closed state [28]. Residual dipolar couplings have been used to establish that the apo forms of these mutants are partially closed relative to the apo wild-type MBP with the ensemble average closure angles being 9.5° and 28.4° for I329W and I329W/A96W, respectively [29]. The ligand-bound closed forms of MBP, MBP (I329W) and MBP(I329W/A96W), have closure angles of 35°. Partial closing shifts the equilibrium toward the ligand-bound state, and thus the mutations increase the affinity for maltose [28].

Introduction of these mutations into RG13 resulted in the creation of more sensitive switches, switches that respond to lower concentrations of maltose (Figures 3A and 3B). The fact that we observed qualitatively similar changes in maltose affinity when the mutations are introduced into RG13 strongly suggests that the relative order and magnitude of the angles of closure of RG13, RG13(I329W), and RG13(I329W/A96W) are similar to that of MBP, MBP(I329W), and MBP(I329W/A96W). Thus, the apo forms of the two RG13 mutants offer conformations intermediate between the open to the closed form of RG13, conformations that may reflect that of RG13 in the process of closing. Assuming that the process of closing in RG13 passes through the conformations of the apo forms of the two RG13 mutants, kinetic characterization of RG13(I329W) and RG13(I329W/A96W) suggested that the initial stages of closing do not result in changes in the BLA domain that substantially affect catalysis (Figures 3B–3D). Both k_{cat} and K_m improved during the intermediate stages of closing, but the majority of the effect on K_m occurred during the final stages of closing.

As the magnitude of the allosteric effect was on the same order as that of many natural allosteric enzymes, we next examined the biological effects of RG13. The switching activity was sufficient to result in an observable phenotype: maltose-dependent resistance to ampicillin (Table 1). *E. coli* cells expressing RG13 had a minimum inhibitory concentration (MIC) for ampicillin that was 4-fold higher in the presence of 50 μM maltose. In contrast, the addition of the same concentration of sucrose or glucose to a plate did not affect the MIC. Thus, RG13 serves to couple the previously unrelated functions of ampicillin resistance and maltose concentration. *E. coli* cells expressing RG13 function as a growth/no growth sensor for maltose.

Significance

We have shown that two unrelated proteins can be systematically recombined in order to link their respective functions and create molecular switches. A combination of random circular permutation and random domain insertion enabled the creation of a MBP-BLA fusion geometry in which conformational changes induced upon maltose binding could propagate to the active site of BLA and increase BLA enzymatic activity up to 25-fold. The functional coupling of two proteins with no evolutionary or functional relationship is a powerful strategy for engineering novel molecular function. For example, coupling a ligand binding protein and a protein with good signal transduction properties would result in the creation of a molecular sensor for the ligand. Furthermore, switches that establish connections between cellular components with no previous relationship can result in novel cellular circuitry and phenotypes. We envision, for example, that such switches might establish connections between molecular signatures of disease (e.g., abnormal concentrations of proteins, metabolites, signaling, or other molecules) and functions that serve to treat the disease (e.g., delivery of drugs, modulation of signaling pathways, or modulation of gene expression) and therefore possess selective therapeutic properties.

Supplemental Data

Supplemental Data including Experimental Procedures and a figure are available at http://www.chembiol.com/cgi/content/full/11/11/1483/DC1/.

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References

1. Spencer, D.M., Wandless, T.J., Schreiber, S.L., and Crabtree, G.R. (1993). Controlling signal transduction with synthetic ligands. Science *262*, 1019–1024.
2. Picard, D. (2000). Posttranslational regulation of proteins by fusions to steroid-binding domains. Methods Enzymol. *327*, 385–401.
3. Posey, K.L., and Gimble, F.S. (2002). Insertion of a reversible redox switch into a rare-cutting DNA endonuclease. Biochemistry *41*, 2184–2190.
4. Saghatelian, A., Guckian, K.M., Thayer, D.A., and Ghadiri, M.R. (2003). DNA detection and signal amplification via an engineered allosteric enzyme. J. Am. Chem. Soc. *125*, 344–345.
5. Brennan, C., Cristianson, K., Surowy, T., and Mandecki, W. (1994). Modulation of enzyme activity by antibody binding to an alkaline phosphatase-epitope hybrid protein. Protein Eng. *7*, 509–514.
6. Pelletier, J.N., Campbell-Valois, F.X., and Michnick, S.W. (1998). Oligomerization domain-directed reassembly of active dihydrofolate reductase from rationally designed fragments. Proc. Natl. Acad. Sci. USA *95*, 12141–12146.
7. Dueber, J.E., Yeh, B.J., Chak, K., and Lim, W.A. (2003). Repro-

gramming control of an allosteric signaling switch through modular recombination. Science *301*, 1904–1908.
8. Mootz, H.D., and Muir, T.W. (2002). Protein splicing triggered by a small molecule. J. Am. Chem. Soc. *124*, 9044–9045.
9. Guo, Z., Zhou, D., and Schultz, P.G. (2000). Designing small-molecule switches for protein-protein interactions. Science *288*, 2042–2045.
10. Gryczynski, U., and Schleif, R. (2004). A portable allosteric mechanism. Proteins *57*, 9–11.
11. Baird, G.S., Zacharias, D.A., and Tsien, R.Y. (1999). Circular permutation and receptor insertion within green fluorescent proteins. Proc. Natl. Acad. Sci. USA *96*, 11241–11246.
12. Tucker, C.L., and Fields, S. (2001). A yeast sensor of ligand binding. Nat. Biotechnol. *19*, 1042–1046.
13. Doi, N., and Yanagawa, H. (1999). Insertional gene fusion technology. FEBS Lett. *457*, 1–4.
14. Radley, T.L., Markowska, A.I., Bettinger, B.T., Ha, J.H., and Loh, S.N. (2003). Allosteric switching by mutually exclusive folding of protein domains. J. Mol. Biol. *332*, 529–536.
15. Guntas, G., and Ostermeier, M. (2004). Creation of an allosteric enzyme by domain insertion. J. Mol. Biol. *336*, 263–273.
16. Buskirk, A.R., Ong, Y.C., Gartner, Z.J., and Liu, D.R. (2004). Directed evolution of ligand dependence: Small-molecule-activated protein splicing. Proc. Natl. Acad. Sci. USA *101*, 10505–10510.
17. Ostermeier, M., and Benkovic, S.J. (2000). Evolution of protein function by domain swapping. Adv. Protein Chem. *55*, 29–77.
18. Apic, G., Gough, J., and Teichmann, S.A. (2001). Domain combinations in archaeal, eubacterial and eukaryotic proteomes. J. Mol. Biol. *310*, 311–325.
19. Sharff, A.J., Rodseth, L.E., Spurlino, J.C., and Quiocho, F.A. (1992). Crystallographic evidence of a large ligand-induced hinge-twist motion between the two domains of the maltodextrin binding protein involved in active transport and chemotzxis. Biochemistry *31*, 10657–10663.
20. Graf, R., and Schachman, H.K. (1996). Random circular permutation of genes and expressed polypeptide chains: Application of the method to the catalytic chains of aspartate transcarbamoylase. Proc. Natl. Acad. Sci. USA *93*, 11591–11596.
21. Ostermeier, M., and Benkovic, S.J. (2001). Construction of hybrid gene libraries involving the circular permutation of DNA. Biotechnol. Lett. *23*, 303–310.
22. Quiocho, F.A., Spurlino, J.C., and Rodseth, L.E. (1997). Extensive features of tight oligosaccharide binding revealed in high-resolution structures of the maltodextrin transport/chemosensory receptor. Structure *5*, 997–1015.
23. Maveyraud, L., Massova, I., Birck, C., Miyashita, K., Samama, J.-P., and Mobashery, S. (1996). Crystal structure of 6alpha-(hydroxymethyl)penicillanate complexed to the TEM-1 β-lactamase from Escherichia coli: evidence on the mechanism of action of a novel inhibitor designed by a computer-aided process. J. Am. Chem. Soc. *118*, 7435–7440.
24. Evenas, J., Tugarinov, V., Skrynnikov, N.R., Goto, N.K., Muhandiram, R., and Kay, L.E. (2001). Ligand-induced structural changes to maltodextrin-binding protein as studied by solution NMR spectroscopy. J. Mol. Biol. *309*, 961–974.
25. Sigal, I.S., DeGrado, W.F., Thomas, B.J., and Petteway, S.R., Jr. (1984). Purification and properties of thiol beta-lactamase. A mutant of pBR322 beta-lactamase in which the active site serine has been replaced with cysteine. J. Biol. Chem. *259*, 5327–5332.
26. Christensen, H., Martin, M.T., and Waley, S.G. (1990). Beta-lactamases as fully efficient enzymes. Determination of all the rate constants in the acyl-enzyme mechanism. Biochem. J. *266*, 853–861.
27. Hall, J.A., Gehring, K., and Nikaido, H. (1997). Two modes of ligand binding in maltose-binding protein of *Eschericia coli*: correlation with the structure of ligands and the structure of binding protein. J. Biol. Chem. *272*, 17605–17609.
28. Marvin, J.S., and Hellinga, H.W. (2001). Manipulation of ligand binding affinity by exploitation of conformational coupling. Nat. Struct. Biol. *8*, 795–798.
29. Millet, O., Hudson, R.P., and Kay, L.E. (2003). The energetic cost of domain reorientation in maltose-binding protein as studied by NMR and fluorescence spectroscopy. Proc. Natl. Acad. Sci. USA *100*, 12700–12705.