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Engineered Derivatives of Maltose-Binding Protein

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1. Introduction

Maltose-binding protein (MBP), a member of the periplasmic binding protein family of Gram negative bacteria, is a versatile substrate for protein engineering. In common with other periplasmic proteins, it is extremely protease resistant, and it can fold properly in both the cytoplasmic and periplasmic compartments. It binds a variety of glucose-α1→4-glucose polysaccharides, from maltose and longer chain maltodextrins to β-cyclodextrin. Upon binding its ligand, it undergoes a large conformational change. These properties have made MBP attractive for a number of engineering studies that have elucidated its role in maltodextrin transport, tuned its properties as an affinity and solubility tag, and transformed it into an allosteric effector or a biosensor for both its natural ligand and for compounds as varied as zinc and TNT (Marvin & Hellinga, 2001, Naal et al., 2002, Wu et al., 1997).

1.1 MBP's function in *Escherichia coli*

MBP's native role in *E. coli* is to shepard maltodextrins from 2 to 7 glucose units in length through the periplasm to the transport apparatus in the cytoplasmic membrane. *E. coli* can grow on high concentrations of maltose in the absence of MBP, but at low concentrations it requires MBP for growth. In the present model of maltodextrin transport (Oldham & Chen, 2011), maltodextrins enter the periplasm by facilitated diffusion through the outer membrane porin LamB, where they are bound by MBP. In binding maltodextrin, MBP shifts from the open, unliganded conformation to the closed conformation. The liganded MBP diffuses to the inner membrane, where it binds to MalFG. Upon binding to MalFG, MBP shifts to the open conformation and releases the maltodextrin to its binding site in MalFG. This transmits a signal to the MalK ATPase subunit bound to MalFG on the cytoplasmic side, promoting ATP hydrolysis. Upon hydrolysis, MalFGK changes conformation and releases the maltodextrin on the cytoplasmic side of the membrane and MBP on the periplasmic side.

1.2 X-ray structures of MBP

The foundation of both understanding the function of MBP and protein engineering using MBP is a series of exquisite crystal structures from the Quiocho lab (Duan et al., 2001, Duan

& Quiocho, 2002, Quiocho et al., 1997, Sharff et al., 1992, Spurlino et al., 1991, Spurlino et al., 1992)(Fig.1). MBP consists of two globular domains, named domains I and II. The binding site is positioned in the cleft between the two domains. The binding site consists of regions that interact with the glucose residues, via nonpolar interactions with the sugar rings (primarily donated by domain II), and a large number of hydrogen bonds that interact with sugar hydroxyls (largely donated by residues in domain I). The structures of the liganded and unliganded forms show that it undergoes a large hinge-twist motion upon binding most of its ligands (Fig.2). Structures of MBP complexed with ligands that support the growth of *E. coli* show a fully closed conformation (Quiocho et al., 1997, Spurlino et al., 1991); the structure with β-cyclodextrin and one of the maltotetraitol structures, which will not support the growth of *E. coli*, show MBP in a open form (Sharff et al., 1993).

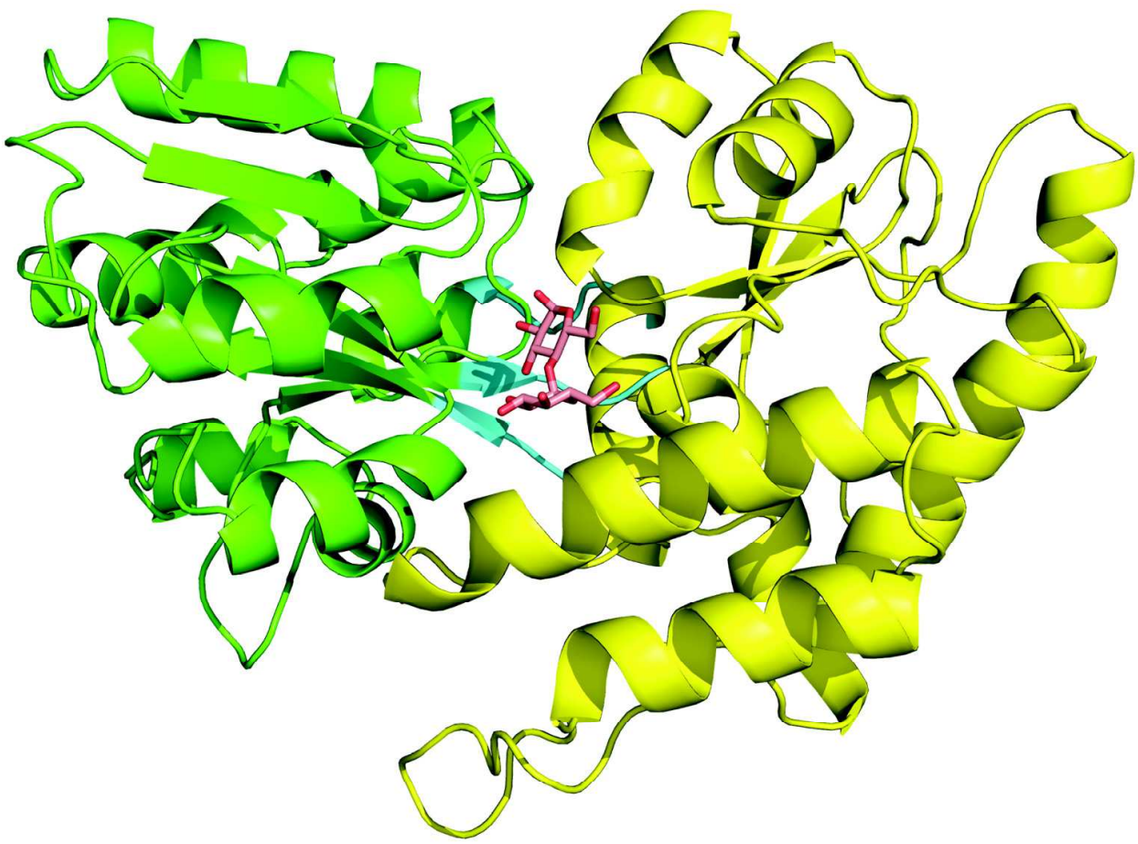

Fig. 1. Cartoon of the structure of MBP with bound maltose. Structure from PDB file 1anf rendered using PyMOL. Domain I is in green, domain II is in yellow, hinge regions are in cyan, and maltose carbons are in salmon.

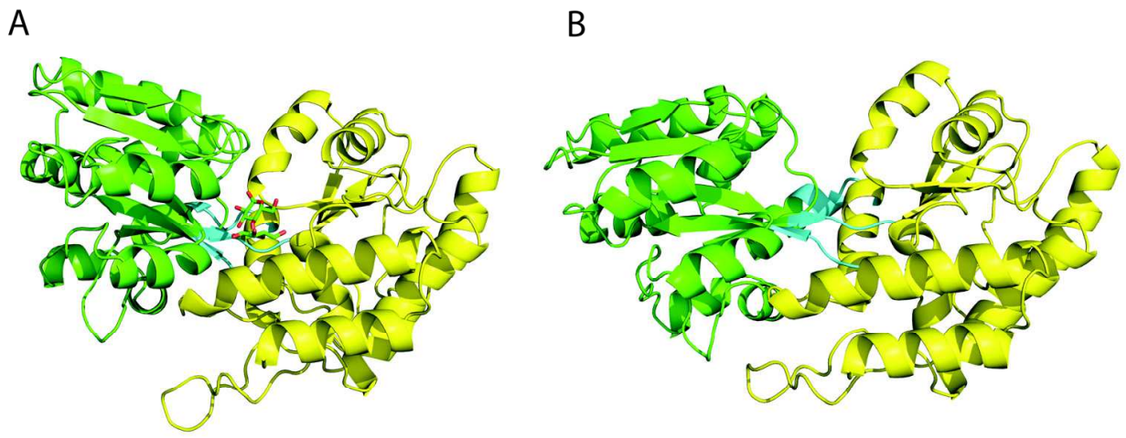

Fig. 2. Ribbon diagram of the two conformations of MBP. Colors are as in Fig 1
A. Closed, maltose-bound form. B. Open, unliganded form (PDB 1jw4)

2. Engineering to understand function

2.1 Examination of the open-closed equilibrium

The demonstration of the open and closed conformation of MBP by structural studies led to an examination of the role of this conformational change in the function of MBP. The Nikaido lab first showed that physical techniques such as fluorescence spectroscopy and electron paramagnetic resonance (EPR) spectroscopy could be used to probe the conformation of MBP (Hall et al., 1997a, Hall et al., 1997b). For EPR, they used an engineered MBP that contained an Asp41 to Cys (D41C) mutation in domain I and a Ser211 to Cys (S211C) mutation in domain II. This allowed them to attach spin labels to the cysteines via disulfide linkages. They showed that the double mutant, with and without spin labels, behaved normally in binding maltose. They were then able to measure the distance between the spin labels in the presence of maltose, maltotetraose, maltotrietol and β-cyclodextrin, and showed that upon binding ligands that allow the closed conformation in X-ray structures, the spin-labelled residues are closer together. The Clore lab extended this line of study using paramagnetic relaxation enhancement (PRE) to examine the conformational state of the ligand-bound and unliganded MBP (Tang et al., 2007). Using a different spin-label on the same two mutated cysteine residues, they measured the percentage of MBP in the closed and open form in both states. They found that while the PRE measurements of MBP-maltotriose were consistent with it being in the closed form, the unliganded MBP was a mixture of about 95% open and 5% in a modified closed form. They examined the structure of the modified closed form and found it to be slightly different from the ligand-bound closed form, with the two domains not completely closed and the sugar-binding site of domain II accessible. A third study examined the change in conformation mechanically. Choi et al. attached a single-stranded DNA linker to the two lobes of MBP via a N-terminal His-tag and an L202C mutation in domain II (Choi et al., 2005). Upon adding the complement of the DNA, the stiffness of the double strand exerts force on MBP to push it toward the open conformation. By varying the length of the DNA linker, they were able to measure a change in the Kd for maltose that could be explained by the physical properties of the DNA.

2.2 Use of mutant derivatives in structure-function studies

An understanding of the open-closed conformational shift has also led to an engineered derivative of MBP that has been used in structural studies to elucidate maltodextrin transport. Zhang et al. engineered a double mutant of MBP which locks it in the closed conformation. They substituted cysteines for G69 and S337, which are located on separate domains but adjacent to each other in the closed conformation. Upon purification, about 80% of this MBP mutant forms an intramolecular disulfide bond that locks it in the closed conformation. Two labs have taken advantage of this mutant MBP in their stuctural studies of the MalFGK transport apparatus (Oldham & Chen, 2011, Orelle et al., 2010). Orelle et al. used the locked MBP in EPR studies of the transport complex to show that the conformational changes that lead to ATP hydrolysis by MalK only take place after MBP in its closed conformation binds to MalFG. Oldham and Chen then followed this up with a crystal structure of MalFGK with the closed MBP bound, which along with their earlier structure of the open MBP-MalFGK complex (Oldham et al., 2007) gives a nearly complete picture of the steps in maltodextrin transport.

2.3 Probing folding and unfolding by mutation

In common with other periplasmic proteins, MBP is remarkably stable and resistant to proteolysis. In spite of this, it has been shown to fold relatively slowly, and becomes incompetent for export to the periplasm if it folds in the cytoplasm. Aspects of its folding/unfolding have been studied by mutating residues that disrupt the folding pathway or lower the energy barrier to unfolding (Betton, J. & Hofnung, 1996, Chang & Park, 2009). Betton and Hofnung found the MBP double mutant G32D I33P among random mutants that were unable to grow on maltose. The mutant MBP was expressed and secreted, but formed inclusion bodies in the periplasm; a derivative without a signal sequence formed inclusion bodies in the cytoplasm. If the inclusion bodies were purified, denatured and refolded they showed near normal affinity for maltose, indicating that the defect was in the folding pathway and not in the structure of the mutant protein. Chang and Park studied the unfolding pathway of MBP by examining its suceptibility to protease digestion during partial denaturation. Many proteins, when treated with a protease under partial denaturation conditions, give proteolytic fragments that indicate the domain or subdomain structure. However, some proteins, including MBP, show an all-or-nothing response to this treatment, where the first proteolytic cleavage leads to rapid unfolding and proteolysis of the entire protein. These researchers used a two step mutagenesis approach to identify the region of MBP that unfolds to allow the initial cleavage. They first surveyed the protein by making mutations in buried residues throughout its sequence to find mutations that destabilized the structure. Upon identifying the susceptible region, they made additional mutants that defined the final two C-terminal α-helices as a subdomain that unfolds to allow the first cleavage.

3. Engineered binding

3.1 High affinity derivatives

3.1.1 Site-directed based on open-closed conformations

The equilibrium between the open and closed form of MBP provides a route to altering its affinity for maltodextrins without changing the sugar binding site on the protein. Since MBP

is predominantly in the closed form when bound to maltodextrin, it is possible to alter its affinity for ligand by biasing the equilibrium of the unliganded MBP towards the closed form. In the open form an interface between the two domains forms in the area behind the hinge (Fig. 3). An examination of the interface shows close packing of the side chains. As the conformation shifts to the closed form this interface opens and becomes solvent accessible, and the binding energy of the contact surface is lost. Hellinga and coworkers were the first to take advantage of this by mutating a residue in the interface, I329, to residues with smaller or larger volume side chains, as well as to cysteine to allow attachment of bulkier substituents via the sulfhydryl. They found that larger groups at position 329 yielded proteins with higher affinity for maltose, while the one example with a smaller group (I329A) gave a lower affinity. The largest improvement with a natural substitution was found with I329Y, which gave a 23-fold tighter Kd that wild type. A cysteine at position 329 derivatized with thio-nitropyridine gave a protein with a Kd more than 100-fold tighter than wild type.

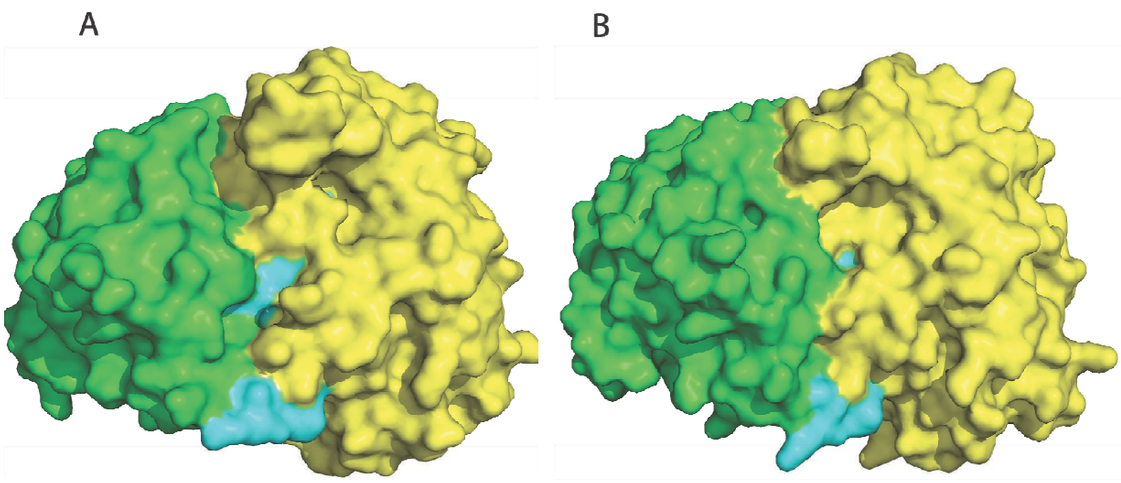

Fig. 3. Surface representation of MBP viewed from the opposite the binding cleft, showing the interface that forms behind the hinge region. A. Closed, maltose-bound form (PDB file 1anf). B. Open, unliganded form (PDB file 1jw4).

Telmer and Shilton took a slightly different approach, examining the interface for alterations that would disrupt the interface by removal of important contacts which stabilize the interface behind the hinge (Telmer & Shilton, 2003). Their analysis considered residues that had higher temperature factors in the closed conformation, indicating higher mobility, as well as the structural contacts of those residues that formed in the open conformation. They identified the side chain of M321 as fitting into a pocket formed by hydrophobic side chains on the opposite domain, and Q325 as an important sidechain that shields the M321 contact from solvent in the open conformation. An M321A Q325A double mutant increased the affinity of MBP for maltotriose about 6-fold. Another interaction their analysis identified was a loop consisting of residues 171-177, which makes contact with residues in domain II in the open conformation. Because G174 is part of a β-turn within the loop, they chose to preserve this residue and delete residues 172-173 and 175-176 on either side to shorten the loop. This deletion mutant also showed about a 6-fold increase in affinity for maltotriose. They combined the M321A/Q325A with the deletions on domain I to obtain a mutant they

called MBP-DM. This mutant could be produced *in vivo,* and had a 100-fold higher affinity for maltotriose than wild type.

3.1.2 Random mutagenesis for higher affinity

MBP, in addition to its attractiveness as a substrate for protein engineering for study of its function, is a useful affinity and solubility tag for recombinant protein expression. While MBP is one of the best tags for its ability to give high expression of soluble protein in *E. coli*, it affinity for maltodextrins is sometimes compromised when fused to another protein. The elegant studies that demonstrated higher affinity MBP's by manipulating the open/closed conformational equilibrium led experimenters to try two of these derivatives in the context of a fusion protein, to see if it could improve the yield during purification. Nallamsetty and Waugh fused MBP-DM and MBP (I329Y) to three proteins whose solubilty they had previously shown to be greatly enhanced by expression as an MBP fusion construct (Nallamsetty & Waugh, 2007). They found these derivatives of MBP, while soluble when expressed by themselves, completely loose the ability to enhance the solubility of the fusion partners.

The possibility that there might exist other mutations in MBP that would increase its affinity without damaging its solubility enhancement properties led Walker et al. to do a random mutagenesis and then screen for higher yield and solubility enhancement (Walker et al., 2010). A screen of 4000 random mutants yielded 19 that had increased yield in a small-scale purification, and five that retained solubility enhancement for two fusion proteins that had been demonstrated to tend toward insolubility. Mutations were found in residues that previous studies had identified as important in the open-closed equilibrium, namely M321 and Q325, as well as a number of others that could be rationalized to affect the conformational equilibrium similarly. Mutations that preserved the solubility enhancement of MBP showed modest improvements in affinity, in the range of 2- to 4-fold in both yield and Kd measurements. By combining two of these mutations, a mutant with a 10-fold tighter affinity was obtained that still functioned well as a solubility tag.

3.2 Altered affinity: Zn binding

Hellinga's lab has also done extensive work in altering the affinity of MBP from maltodextrins to zinc by computational design (Benson et al., 2001, Benson et al., 2002, Marvin & Hellinga, 2001). Using the design program *DEZYMER*, they searched the region in and around the maltose binding site in the cleft between the two domains for potential sites, modelled on tetrahedral zinc binding by three histidine residues and a water molecule. They started with the closed structure of MBP, and imposed the constraint that one of the three His residues be in the opposite domain from the other two. This biases the proposed sites towards those where zinc binding would cause the conformational shift from the open to closed form, similar to maltose binding for wild type. Twenty potential sites were identified, and they constructed four of these by site directed mutagenesis; two that replaced residues that make up part of the maltose-binding site and two that were located on the rim of the maltose binding site and might be compatible with retained affinity for maltose. By mutating a surface aspartate residue at position 95 to cysteine and attaching a fluorescent

label, they were able to monitor binding by fluorescence intensity. As predicted, the first class bound zinc but not maltose. The second class bound zinc only in the presence of maltose, suggesting that the zinc binding site depended on maltose- induced shift to the closed form for assembly of the zinc binding site. A closer examination of models of the first class indicated that some wild type residues were involved in binding, making fortuitous contacts to the zinc. This was confirmed by mutagenesis to improve the geometry of binding, with a site consisting of two His residues in domain I and two Glu residues in domain II giving the highest affinity for zinc. Telmer and Shilton examined this His_2 Glu_2 zinc-binding MBP by low angle X-ray scattering and crystallography, and to their surpise found that the MBP derivative bound zinc in the open conformation (Telmer & Shilton, 2005). All the zinc contacts were donated by sidechains from domain I. They confirmed the requirement of the Glu residues in domain II for zinc binding, but found them 8 A away from the zinc in the crystal structure, and concluded they must contribute to the electronegative environment of the binding site rather than providing direct zinc contacts.

4. MBP as a biosensensor

A number of labs have exploited MBP's specificity and the large conformational shift between the liganded and unliganded forms to develop biosensors. Both sensors that can be used in solution, *in vitro* or *in vivo*, and sensors immobilized on a surface have been explored. The most common readout for these sensors is a change in fluorescence, but alternatives that offer electronic and enzymatic read-outs have also been constructed. Besides solubility and the type of readout, important parameters include the strength of the signal, the dynamic range of ligand that can be detected, and whether the system is designed to use reagents or not.

4.1 Fluorescent biosensors with a single fluorescent reporter

The simplest form of MBP biosensor carries a single flourescent dye, such that a change of fluorescence intensity occurs when the protein undergoes the open/closed conformational shift. These biosensors use the fact that the local environment of the fluorescent group changes when the ligand is bound and the conformation of MBP changes. Gilardi et al. followed this approach with an S337C derivative of MBP by attaching a nitrobenzoxadiazole (NBD) group to the substituted cysteine (Gilardi et al., 1994, Gilardi et al., 1997). They found a increase in fluorescence intensity of 1.8-fold with their sensor. Hellinga and coworkers studied this kind of biosensor in detail, both with MBP and with their derivative that binds zinc (de Lorimier et al., 2002, Hellinga & Marvin, 1998, Marvin & Hellinga, 2001). They mutated a number of MBP residues to cysteine, then tested a number of fluorescent dyes to find the best combination of position and dye. The residue to attach the dye was either near the binding site of maltodextrin, e.g. S233, or one that contacts the opposite domain in one or the other conformation, e.g. D95, F92 and I329. A ratio ΔR was defined, which describes the difference in fluorescent intensity between ligated and unliganded sensor at two wavelength bands. With some experimentation as to fluorescent dye used and placement on MBP, ΔR's of 3 to 4 could be obtained. By mutating the MBP, they obtained a set of derivatives that could sense maltose at concentrations from 0.1 μM to 10 mM (Marvin et al., 1997). Sherman et al.

took a similar approach, attaching fluorescent dyes via different linker arms to an S237C mutant of MBP, and getting a maximum difference in fluorescence intensity of 3-fold between unliganded and liganded forms (Sherman et al., 2006). Jeong et al. produced a sensor that relies on a split green fluorescent protein, which they fused to the N- and C- termini of MBP. Upon maltose binding, the termini move closer together, allowing the split GFP to assemble and leading to a 5-fold increase in fluorescence (Jeong et al., 2006).

One way to make a biosensor convenient and reusable is to immobilize it on a surface. Topoglidis et al. used a nanocrystallin TiO_2 surface to immobilize a fluorophore-labeled MBP and showed a change in fluorescence in response to maltose (Topoglidis et al., 1998). Dattelbaum et al. incorporated a fluorophore-labeled MBP into a sol-gel silica matrix, and demonstrated fluorescence change in response to maltose at close to the same μmolar sensitivity as MBP in solution (Dattelbaum et al., 2009). They subsequently pegylated the MBP to help maintain it in an aqueous environment, and increased the intensity of the fluorescent signal. As we will see below, other forms of MBP-based biosensors can also be adapted to work as immobilized biosensors.

4.2 FRET sensors

Numerous MBP-based biosensors have been develped that use Förstner resonance energy transfer (FRET) to capture information about the binding state of MBP and its derivatives. FRET energy transfer is sensitive to both the distance between the two fluorophores and their relative orientations, making it attractive way to capture confomational information that changes upon ligand binding. Measures of FRET efficiency are quantified by measuring the change in the ratio of fluorescence intensity at the respective wavelengths of the two fluorophores.

4.2.1 MBP-GFP fusion FRET sensors

One way to arrange the FRET donor and acceptor is to fuse green fluorescent protein variants to the N- and C-terminus of MBP. Frommer and coworkers constructed enhanced cyan fluorescent protein-MBP-enhanced yellow fluorescent protein fusion (CFP-MBP-YFP) biosensors that they characterized *in vitro* and *in vivo* in yeast (Fehr et al., 2002, Fehr et al., 2005). The change in fluorescence ratio they observed for these sensors was around 0.1. They constructed CFP-MBP-YFP variants that had mutations in or near the maltose binding site of MBP to reduce its affinity and widen the dynamic range of the biosensor. In later work they improved their biosensor by shortening the linkers between the GFP variants and MBP, which improved its ratio to about 0.2 (Kaper et al., 2008). They used this improved sensor to detect sugar concentrations in *E. coli* upon the addition of maltose to the medium. Ha et al. took a similar approach with their CFP-MBP-YFP sensor, making systematic changes to the linkers to get a derivative with a fluorescence ratio of 0.5 (Ha et al., 2007). They then mutated Trp62 to decrease its affinity for maltose, once again to increase the sensor's dynamic range, and as an unexpected by-product got variants that showed fluorescence ratios of 0.7 and 1.0. They expressed their sensors in yeast and demonstrated the appropriate response to the addition of maltose to the medium. Park et al. also studied a CFP-MBP-YFP FRET sensor, using the characteristics of the energy transfer to measure the distance between the lobes of MBP (Park, K. et al., 2009b).

4.2.2 Dye-labeled FRET sensors

In principle, one should be able to construct a FRET biosensor by attaching fluorescent dyes to the lobes of MBP in place of the fluorescent proteins in the fusions described above. The difficulty lies in devising two site-specific labeling strategies so that each MBP gets labeled on domain I with one member of the donor/acceptor pair and on domain II with its partner. The most convenient and widely used method of site-specific labeleing, used in all studies described above, uses the sulfhydryl on cysteine substitutions as attachment sites on MBP. Hellinga and coworkers devised an elegant protocol to reversibly blocks a labeling site, by fusing a BZif or ZifQNK zinc finger domain to an MBP with a A141C substitution (Smith et al., 2005). Binding of zinc (BZif) or disulfide bond formation (ZifQNK) blocks the cysteines from reacting. The zinc finger domains were fused to the N- or C-terminus of MBP A141C, and in blocked form allowed specific labeling of the cysteine substitution at position 141. The block was then reversed and a second dye was conjugated to the cyteines in the zinc finger domain. A number of combinations of donor/acceptor at the three positions were constructed, including a triple-labelled MBP. With the dye tetramethylrhodamine-5-maleimide at position 141 and Cy5 at the C-terminus, a threefold change in the ratio of the donor:acceptor emission intensities was observed upon the addition of maltose.

4.2.3 Quantum dot FRET sensors

Quantum dots (QD) are colloidal nanocrystal fluorophores that have broad absorption spectra and tunable emmission spectra, making them particularly interesting for the development of FRET sensors. Medintz and coworkers have explored the properties of 555-nm emitting CdSe-ZnS quantum dots with MBP attached via a C-terminal His-tag (Medintz et al., 2003b, Medintz et al., 2004b, Medintz et al., 2005). In their initial experiments, they pre-bound the QD-MBP with β -cyclodextrin conjugated to Cy5 or the quencher QSY9, and measured the increase in fluorescence when the β-cyclodextrin was displaced by maltose (Medintz et al., 2003a). They followed this work with development of a reagentless biosensor, by attaching a Cy3 at a cystein substitution H41C, near the maltose binding site (Medintz et al., 2005), where conformational change upon maltose binding reduces the dyes efficiency as a FRET acceptor. Pons et al. extended this work by developing a single particle QD biosensor using the same MBP H41C labelled with Cy3 (Pons et al., 2006). Multiple MBP-Cy3 complexes were immobilized on the QD, and the response to maltose was compared in ensemble and in single particles.

4.2.4 Immobilized FRET sensors

As mentioned above, immobilizing a sensor on a surface has advantages in methodology and reusability, and could allow the fabrication of integrated microfluidic biosensing devices. One FRET biosensor has been adapted to work as a surface-tethered assembly by coating a glass slide with neutravidin and tethering a His-tagged MBP labelled with a quenching dye to the surface via a biotin-Ni-NTA linker (Fig. 4)(Medintz et al., 2004a). A signalling dye linked to β-cyclodextrin is tethered to the same surface via a biotinylated DNA linker. Upon addition of maltose, the β-cyclodextrin is displaced from the MBP binding site and thus removed from the vicinity of the quencher, and fluorescence intensity increases. Sapsford et al. used a similar tethering strategy to link a MBP-Cy3.5-quantum dot sensor to a glass slide, extending the advantages of quantum dot sensors to this format (Sapsford et al., 2004).

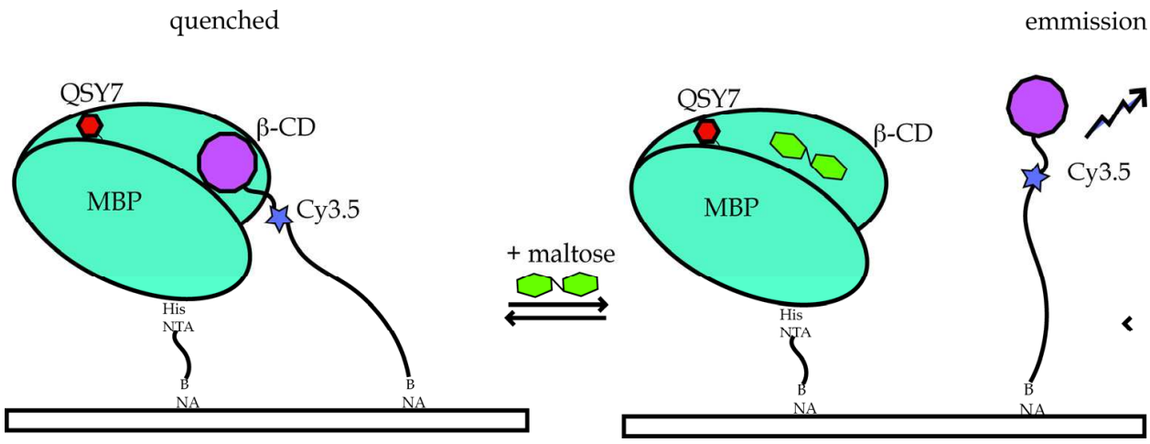

Fig 4. Reagentless surface tethered FRET sensor for maltose. When β-cyclodextrin (β–CD) is bound, the QSY7 on MBP 95C quenches the Cy3.5. Maltose displaces the β –CD and allows fluorescence.

4.3 Enzymatic sensors

Linking a biosensor to an enzyme allows a biochemical read-out that can greatly amplify the signal. Hofnung's lab has mapped certain sites, termed permissive sites, in MBP that can accept peptides without disturbing the folding and function of MBP (Clement et al., 1991). An enzyme encoding ampicillin resistance, TEM β-lactamase, can be inserted into two of these permissive sites, and the fusion protein retains activity for both maltose binding and ampicillin cleavage (Betton, J. M. et al., 1997). In order to make a bifunctional protein where enzymatic activity responds to ligand-binding, Guntas et al. isolated, circularized, then linearized the gene for β-lactamase, then inserted this circularly-permuted collection into a plasmid containing the gene for MBP (Guntas et al., 2004). They identified isolates from this library that exhibited both ampicillin resistance and conferred growth on maltose for a strain deficient in MBP, then screened in a microplate format for an isolate where β-lactamase activity depended on maltose. They obtained an isolate, called RG13, where enzymatic activity in the presence of maltose was 25-fold greater than in its absence. In later work it was demonstrated that mutations that effect MBP's conformational change also effect the β-lactamase activity of the bifunctional protein, strengthening the conclusion that β-lactamase activity was dependent on formation of the closed conformation of MBP (Kim & Ostermeier, 2006). This MBP-β-lactamase biosensor was subsequently immobilized via a C-terminal His-Tag on a gold surface derivatized with Ni-NTA, and maltose dependent β-lactamase activity was confirmed (Zayats et al., 2011).

4.4 Electrochemical sensors

Fluorescent and enzymatic sensors may suffer from the background of natural fluorescence and enzymatic activity that can be present in biological samples, making the development of biosensors with other forms of read-out attractive. Two MBP biosensors with that produce electrical signals have been prototyped, one that produces a electrochemical signal through a redox reaction and one that uses the conformational change in MBP to effect electrical

current directly. Hellinga and coworkers attached an MBP derivatized with a Ru(II) reporter to a Ni-NTA derivatized gold electrode via a C-terminal His tag (Benson et al., 2001). A change in redox potential dependent on maltose could be measured by monitoring current as a function of voltage. This general idea was extended to MBP-Ru(II) tethered to ZnS coated CdSe nanoparticles, where the photoluminescense of the nanoparticles responds to the MBP conformational shift (Sandros et al., 2005, Sandros et al., 2006). Park et al. took a different approach, fabricating an ion sensitive field effect transistor by coating a standard CMOS transistor with nickel and assembling MBP-His on the surface (Park, H. J. et al., 2009a). Upon addition of maltose, the charge on MBP effected the gate capacitance of the transistor differentially as the MBP changed from the open to close conformation, which led to a drop in current.

4.5 AFM sensor

While MBP biosensors with electrical read-out have advantages for fabrication of microfluidic devices, the relatively weak signal poses a challenge for detection of very low amounts of ligand. Staii et al. addressed this problem by placing and sensing an MBP derivative on a gold surface using an atomic force microscope (AFM) (Staii et al., 2008). A gold electrode was derivatized with a thiol compound, then activated by scanning with the AFM at a relatively high force. This allowed MBP fused to a Cys-Cys dipeptide at the C-terminus to immobilize at the activated spot by formation of a disulfide bond(s). The placement of the MBP-Cys-Cys could then be detected by the AFM probe scanning at low force. It was found that unliganded MBP, for unknown reasons, produced greater friction interacting with the probe than MBP liganded to maltose. These researchers used the difference in friction to measure the K_D of MBP for maltose at about 1 μM, in good agreement with measurements done on the wild type protein in solution. Their sensor could detect about 10^4 maltose molecules (10 nM concentration), a much higher sensitivity than can be obtained by electrochemical sensors, and uses an MBP derivative that can be purified and incorporated without *in vitro* modification with a dye or quantum dot.

4.6 Biosensors using MBP as a scaffold

MBP has been used by a number of labs as a scaffold in biosensors, not as a conformational switch but simply taking advantage of its robustness and ease of modification. Vardar Schara et al. modified a CFP-MBP-YFP fusion protein by cysteine substitution at several residues centrally located on domain I of MBP (Vardar-Schara et al., 2007). This allowed a binding domain to be attached between the GFP variants, and binding of its ligand could be detected by FRET. Another method of immobilizing a biosensor takes advantage of MBP itself as a linker. MBP has a natural affinity for a pyrolyl-propyl bipyridine surface, allowing an MBP-nitrate reuctase fusion protein to be immobilized on the surface (Naal et al., 2002, Takada et al., 2002). This immobilization strategy preserves the enzymatic activity of the nitrate reductase, and allows sensing of TNT by electrochemical detection in a potentiostat: the nitrate reductase reduces the NO_2 groups on TNT, with the electrons ultimately donated by the PBB layer. While these methods do not involve the conformational shift caused by binding maltose, the tools developed for MBP modification made them much easier to fabricate.

5. MBP fusion engineering

MBP has a twenty-year history as an expression, affinity and solublility tag for production of recombinant fusion proteins, and continues to be one of the best tags for producing soluble protein in *E. coli*. This has led to a number of variations on the basic fusion protein scheme that have facilitated research in diverse areas. An exploration of orthologs and mutants of MBP has extended its utility, and the foundation of so much research using MBP has made it an attractive tool for production of novel peptides and proteins.

5.1 Peptide production

At first glance, producing peptides as fusions to MBP appears to be unattractive, since for a 4 kDa peptide one needs to produce 10 mgs of fusion for every milligram of peptide produced. But the problems in producing synthetic or partially synthetic sequences in a soluble and stable form have led to a number of applications where MBP can be a useful scaffold. As related above, Hofnung and coworkers mapped regions in MBP that could accept insertions of foreign sequences (Clement et al., 1991). They made extensive use of this method to insert epitopes and study the immune response to poliovirus (LeClerc et al., 1990, Leclerc et al., 1991, Lo-Man et al., 1994, Martineau et al., 1996), and binding of HIV to its CD4 receptor (Clement et al., 1996, Lo-Man et al., 1994, Szmelcman et al., 1990), among other studies. Another way in which MBP has been used to study peptides is as a carrier for peptides identified from phage display libraries (Zwick et al., 1998). Restriction sites that allow subcloning from phage identified in commercially-available libraries simplify the transfer of DNA encoding the peptide to the N-terminus of the gene for MBP, and the peptide-MBP fusion can be affinity purified. In both these examples, fusion to MBP not only made purification of the peptide simpler, but most likely avoided problems of stability and solubility.

5.2 Exploration of solubility enhancement

The ability of MBP to enhance the solubility of recombinant fusion proteins in E. coli is one of its most attractive features, but the basis of this enhancement is not well understood. Waugh and coworkers examined the hypothesis that the somewhat hydrophobic binding cleft between the domains of MBP is responsible for solubility enhancement (Fox et al., 2001). They mutated hydrophobic residues that are exposed on the surface of MBP, and tested the mutants for their ability to enhance the solubility of three proteins that tend to insolubility when expressed in *E. coli*. They identified a region, not in but near one end of the binding cleft, that seemed to be important for this quality, but found the mutations also effected the global stability of MBP and thus were unable to distinguish between a chaperone effect of the folded protein and an effect on the folding pathway of the fusion proteins. Given the wider distribution of mutations that reduce or destroy solubility enhancement in the study of Walker et al. (Walker et al., 2010), it is difficult to imagine that the effect arises from a patch on the folded protein. It remains to be examined whether the effect stems from interactions during folding. In a later study, Waugh and coworkers cloned orthologs of MBP from five bacteria and archae and tested them as fusions with eight proteins that tend to be insoluble when expressed unfused in *E. coli* (Fox et al., 2003). All of the orthologs could confer solubility on the test proteins, although to different extents, so it seems that this is a common property of these periplasmic binding proteins. The availability

of thermostable MBP's may turn out to be attractive for the production of thermostable fusion proteins, as heat treatment is a useful purification step for production of these proteins in mesophiles such as *E. coli*.

5.3 Determining the structure of MBP fusions

The idea of crystallizing and determining the X-ray structure of a protein fused to MBP, similar to the production of peptides, may at first seem counterintuitive and unnecessarily complicated. The relatively large size of MBP (~40kDa) added to the protein whose structure is to be determined would result in a more complex diffraction pattern. However, the fact that MBP enhances solubility, the possibility that MBP might enhance the formation of crystal contacts, and the fact that the MBP structure is solved, all combine to make this approach one of the ways in which difficult protein structures are solved (Derewenda, 2010, Smyth et al., 2003). In most cases, conformational flexibility between the MBP and the target protein works against crystallization, so fusion proteins expressed in the most common commercial MBP vectors are often modified to shorten the spacer between MBP and the target. This approach has been used to solve the structures of a number of proteins, among them human T cell leukemia virus type 1 gp21 (Kobe et al., 1999), the SarR protein from Staphylococcus aureus (Liu et al., 2001), yeast MATa1 (Ke & Wolberger, 2003), and RACK1 from Arabidopsis thaliana (Ullah et al., 2008).

6. Conclusion

Maltose binding protein has seen wide and extensive use in protein engineering, and continues to be used in ways that could not be forseen when it was first discovered forty-odd years ago. Some of the properties that have made it so attractive for these studies are byproducts of its nature as a periplasmic binding protein in *E. coli*: it expresses well, it is naturally exported to the periplasm, it is very stable, and it undergoes a large conformational change upon binding maltodextrins. Other properties are somewhat fortuitous: it expresses and folds well when expressed in the cytoplasm, it has no cysteines (and thus no disulfide bonds), and it enhances the solubility of proteins to which it is fused. In addition, early studies that determined its structure and function in such detail made subsequent experiments much easier. All these characteristics have made MBP a prime component of the molecular biologist's toolkit, and will continue to keep it there in the foreseable future.

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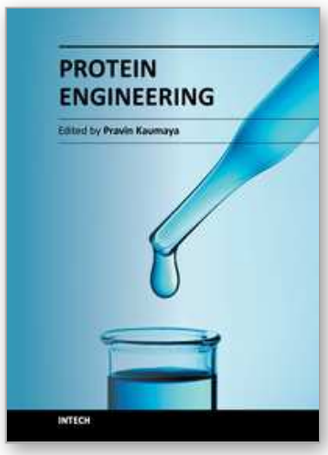

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