The affinity of maltose-binding protein (MBP) for maltose and related carbohydrates was greatly increased by removal of groups in the interface opposite the ligand binding cleft. The wild-type protein has a $K_D$ of 1200 nM for maltose; mutation of residues Met-321 and Gln-325, both to alanine, resulted in a $K_D$ for maltose of 70 nM; deletion of 4 residues, Glu-172, Asn-173, Lys-175, and Tyr-176, which are part of a poorly ordered loop, results in a $K_D$ for maltose of 110 nM. Combining these mutations yields an increased affinity for maltodextrins and a $K_D$ of 6 nM for maltotriose. Comparison of ligand binding by the mutants, using surface plasmon resonance spectroscopy, indicates that decreases in the off-rate are responsible for the increased affinity. Small-angle x-ray scattering was used to demonstrate that the mutations do not significantly affect the solution conformation of MBP in either the presence or absence of maltose. The crystal structures of selected mutants showed that the mutations do not cause significant structural changes in either the closed or open conformation of MBP. These studies show that interactions in the interface opposite the ligand binding cleft, which we term the “balancing interface,” are responsible for modulating the affinity of MBP for its ligand. Our results are consistent with a model in which the ligand-bound protein alternates between the closed and open conformations, and removal of interactions in the balancing interface decreases the stability of the open conformation, without affecting the closed conformation.

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The affinity of maltose-binding protein (MBP) for maltose and related carbohydrates was greatly increased by removal of groups in the interface opposite the ligand binding cleft. The wild-type protein has a $K_D$ of 1200 nM for maltose; mutation of residues Met-321 and Gln-325, both to alanine, resulted in a $K_D$ for maltose of 70 nM; deletion of 4 residues, Glu-172, Asn-173, Lys-175, and Tyr-176, which are part of a poorly ordered loop, results in a $K_D$ for maltose of 110 nM. Combining these mutations yields an increased affinity for maltodextrins and a $K_D$ of 6 nM for maltotriose. Comparison of ligand binding by the mutants, using surface plasmon resonance spectroscopy, indicates that decreases in the off-rate are responsible for the increased affinity. Small-angle x-ray scattering was used to demonstrate that the mutations do not significantly affect the solution conformation of MBP in either the presence or absence of maltose. The crystal structures of selected mutants showed that the mutations do not cause significant structural changes in either the closed or open conformation of MBP. These studies show that interactions in the interface opposite the ligand binding cleft, which we term the “balancing interface,” are responsible for modulating the affinity of MBP for its ligand. Our results are consistent with a model in which the ligand-bound protein alternates between the closed and open conformations, and removal of interactions in the balancing interface decreases the stability of the open conformation, without affecting the closed conformation.

The atomic coordinates and structure factors (code MBP-Del unliganded, MBP-Del with bound maltose, MBP-DM unliganded, and MBP-DM with bound maltose with codes 1NX3, 1N3W, 1PEB, and 1NLS, respectively) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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ATP binding cassette transporters couple ATP hydrolysis to the transmembrane transport of a diverse range of compounds. Members of the ATP binding cassette transporter superfamily are characterized by two membrane-integral domains that each contain 6 or more membrane spanning helices, but are otherwise poorly conserved, and two peripheral ATP binding cassette domains that display sequence conservation across the entire superfamily (1). In addition to the membrane complex, ATP binding cassette systems that catalyze nutrient uptake have primary receptors (binding proteins) that serve two functions: they provide a high affinity binding site for the transported molecule and they regulate the ATPase activity of the integral membrane complex.

We are interested in the function of the primary receptors in the transport process. As a group, these proteins have been intensively studied by x-ray crystallography and other biophysical techniques (for a review, see Ref. 2). They typically contain two domains separated by a hinge region; the substrate binds in the cleft between the two domains, and the protein undergoes a large conformational change, leading to closure of the cleft. With respect to the maltose transport system, domain closure in the binding protein is thought to be the first step toward molecular shape recognition by the membrane complex (3, 4), although it has been shown that both substrate-loaded and substrate-free binding proteins have a role in the transport cycle (5–8). The association and dissociation of the substrate, and attendant conformational changes in the binding protein (MBP), 5 may have direct effects on transport kinetics and regulation of ATP hydrolysis by MalFGK2. To investigate the role of binding protein affinity on the transport process, our goal was to engineer MBP molecules with greater affinity for maltose, without changing residues in either the maltose binding site or in regions thought to interact with MalFGK2.

Crystal structures of MBP in both the closed and open conformations have been solved (9, 10), and they show that binding of maltose results in a large conformational change of the protein, bringing the two domains together such that the substrate is buried inside the cleft. In solution, unliganded MBP is in the open conformation (11); however, there is no obvious energetic barrier to closure of the ligand binding cleft, either in the hinge or in the interface surrounding the ligand binding site. Rather, an interface on the opposite side of the hinge from the ligand binding site appears to maintain the protein in an open conformation.

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open conformation (Fig. 1): in the closed conformation, this interface is broken and becomes solvent exposed.

Changes to this interface should affect the open-closed equilibrium, and consequently the maltose affinity of the protein. This idea is supported by a study by Marvin and Hellenga (12), in which they demonstrated that introduction of large, branched groups into the interface (either as natural amino acid substitutions or specific chemical modification) increased the affinity of the protein for maltose. We have found that by simply removing certain interactions in this interface, we could increase the affinity for maltose by approximately 2 orders of magnitude. On this basis, the interface is postulated to play an active role in maintaining the open conformation of MBP, and we have therefore called this region the “balancing interface” (Fig. 1). In an effort to understand how removal of interactions in the balancing interface affects such a pronounced change in ligand affinity, we have characterized the binding properties, solution conformations, and crystal structures of MBP molecules harboring mutations in the balancing interface. The results from these studies are consistent with the idea that the mutations have increased maltose affinity by destabilizing the open conformation.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**—Mutant MBP molecules MBP-Ala (M321A/Q325A) and MBP-Del (E47III/H9232) were produced using oligonucleotide-directed mutagenesis. This was carried out by two rounds of PCR, following standard methods (13). The first round of PCR consisted of two reactions, each including either a 5’ or 3’ mutagenic primer and a corresponding non-mutagenic flanking primer. The PCR products obtained from this first round were pooled together and used as template for the second round to obtain the full-length mutantified product. PCR products were then subcloned back into the parent plasmid pLH1 (4) to yield the mutant plasmids as follows: pLH1-Ala was made by insertion of a BglII/Ndel fragment from the PCR product into pLH1, whereas pLH1-Del was made by insertion of a BglII/Ncol fragment from the PCR product into pLH1. To make plasmid pLH1-DM, which harbors the combined mutations, a BglII/Eco47III fragment from pLH1-Del was subcloned into pLH1-Ala. All mutations were confirmed by automated DNA sequencing.

**Expression and Purification of MBP**—All chromatographic media were purchased from Amersham Biosciences. Plasmids containing wild-type or mutant *E. coli* coding regions were transformed into *Escherichia coli* strain HSS309, which does not produce MBP (14). Cultures were grown with vigorous shaking at 37 °C in LB broth containing 100 μg/ml ampicillin for 16–18 h. Periplasmic proteins were extracted by osmotic shock (15) and dialyzed against 50 mM Tris-HCl, pH 8.5, prior to ion exchange chromatography. The extract was applied onto a 2.6 × 15-cm column packed with Q-Sepharose Fast Flow and eluted with a linear gradient from 0 to 1 M NaCl. Fractions containing MBP were pooled, dialyzed against 50 mM Tris-HCl, pH 8.5, and further purified on DEAE-Sepharose resin, 2.6 × 30 cm, using a linear gradient from 0 to 1 M NaCl. The DEAE-Sepharose step was not effective for purification of MBP-DM. In this case, fractions from the Q-Sepharose column were applied to a 1.6 × 15-cm column of amyllose affinity resin (New England Biolabs) and eluted with 10 mM maltose.

**Preparation of Maltose-free Protein**—To prepare maltose-free binding protein, the MBP was concentrated by ion-exchange chromatography using a 1-ml HiTrap Q column (Amersham Biosciences) and then denatured by adding guanidine HCl to a concentration of 6 M. The concentrated and denatured protein was loaded onto a 2.6 × 60-cm column of Superdex 200 Prep Grade gel filtration resin, which had been prepacked with 50 mM guanidine HCl. This column was developed with 6 M guanidine HCl at a flow rate of 1 ml/min; fractions containing denatured protein were pooled and dialyzed exhaustively against 50 mM Tris-HCl, pH 8.5. To remove aggregates, the refolded MBP was again concentrated and applied to a 2.6 × 60-cm column of Superdex 200 Prep Grade gel filtration resin, previously equilibrated with 100 mM Tris-HCl, pH 8.5, 1 mM EDTA. Fluorescence Titrations—Fluorescence titrations were carried out at protein concentrations of 10–100 nM, in 3 ml of 50 mM Heps, 100 mM NaCl, 5 mM NaN3, pH 7.5, using a Fluorolog 3 spectrofluorimeter (ISA Instruments). The excitation and emission wavelengths were 280 and 349 nm, respectively, with 0.25 nm excitation and 15 nm emission slit widths. The value for relative minimum fluorescence (F_{0}^\text{min}) was obtained using Equation 1 (16).

\[
\frac{F_0 - F}{F_0} = \frac{K_0 + [\text{ligand}]}{[\text{ligand}] + c}
\]

Where F_0 is the starting fluorescence, F is the fluorescence after addition of ligand (maltose or maltotriose, both from Sigma), and c is a constant. The K_0 was then calculated using the following equation (17), with the K_0 as the single variable.

\[
K_0 + [\text{ligand}] + [\text{MBP}] = \sqrt{K_0 + [\text{ligand}] + [\text{MBP}]} - 4[\text{ligand}][\text{MBP}]
\]

The “solver” function in Microsoft Excel was used to fit the data to the equations and obtain values for the relevant parameters.

**Surface Plasmon Resonance Analysis of Maltodextrin Binding**—Surface plasmon resonance analyses were performed using a BIAcore X instrument. A CM5 sensor chip (BIAcore AB) was derivatized with either amylose or pullulan as follows. Amylose (Sigma) was dissolved in 1 M NaOH to yield a concentration of 50 mg/ml, and then diluted with 0.1 M NaOH, and the pH adjusted to 5.5 with acetic acid to yield a final amylose concentration of 1 mg/ml. A 1 ml of 1 mg/ml amylose solution was treated with NaIO4 at a concentration of 0.2 mM. The solution was incubated for 2 h at 20 °C to effect periodate cleavage of the amylose, and then 400 μl of the amylose solution was desalted on a 2-ml column of Sephadex G-25, equilibrated, and developed with 0.1 mM sodium acetate, pH 4.5. Prior to derivatization, the CM5 chip was equilibrated with 10 mM Heps, 0.15 M NaCl, 3 mM EDTA, 0.005% polysorbate 20, pH 7.4. The periodate-oxidized amylose was coupled to the surface in flow cell 2 by sequential injection of the following solutions at a flow rate of 5 ml/min: 28 μl of a solution of 10 μg/ml N-hydroxysuccinimide and 50 μl of 5 mM N-ethyl-N-(dimethylaminopropyl)carbodiimide, 35 μl of 5 μl of hydrazide, 2 × 100 μl injections of the oxidized amylose solution, 35 μl of 1 M ethanolamine, pH 8.5. Finally, the chip was stabilized with 40 μl of 0.1 M NaNBH4, in 0.1 mM sodium acetate, pH 4.5, at a flow rate of 2 μl/min. A total of 1300 response units (RU) of amylose immobilized to flow cell 2, whereas flow cell 1 was left unmodified for use as a reference cell. Derivatization of a CM5 chip with pullulan followed essentially the same procedure, except that the pullulan, which is highly soluble, could be dissolved directly in 0.1 mM sodium acetate, pH 5.5.

Samples of wild-type and engineered MBP molecules were dialyzed against 50 mM Heps, 150 mM NaCl, 5 mM NaN3, pH 7.4, and serially diluted to yield concentrations ranging from 4 nM to 4 μM. To minimize changes in bulk refractive index, the running buffer for the SPR experiments was exactly the same as the dialysis buffer, with the exception that for some experiments the SPR running buffer contained 100 μM maltose to abrogate rebinding effects. For experiments with the amylose-coupled chip, the flow rate was 50 μl/min, and the injection volumes were 100 μl; for the pullulan-coupled chip, the flow rate was 25 μl/min and the injection volume was 40 μl. For both the amylose and pullulan chips, the response from flow cell 1 (the flow cell with a non-derivatized surface) was subtracted from the response from flow cell 2.

**Small Angle x-ray Scattering**—To remove aggregates prior to SAXS analysis, concentrated protein samples were pooled using a 2.6 × 65-cm Superdex 200 Prep Grade gel filtration column (Amersham Biosciences), equilibrated and developed with 100 mM KCl, 20 mM Tris-HCl, 1 mM EDTA, pH 8.5. Samples for SAXS were dialyzed against 100 mM Tris-HCl, 100 mM NaCl, 5 mM NaN3, pH 8.0. The dialysis buffers were reserved for background measurements. Measurements of MBP-WT, MBP-Ala, and MBP-Del were made at the European Molecular Biology Laboratory Outstation at the Deutsches Elektronen Synchrotron (Hamburg, Germany), beamline X33 (18). Measurements of MBP-WT and MBP-DM proteins were made at BioCAT (beamline 18ID) of the Advanced Photon Source. Details of SAXS measurements and data processing have been described previously (19, 20).

**Crystalization and Crystal Structure Determination**—MBP was crystallized by vapor diffusion using the hanging drop method; equal volumes of protein and reservoir solutions were mixed to yield drop sizes of 2 to 4 μl. For maltose-bound MBP-DM, the reservoir solution was comprised of 18–24% PEG 5K monomethyl ether (Hampton Re-
In the open conformation of MBP, contacts between the two domains are formed through an interface on the opposite side of the hinge from the ligand binding cleft, which we term the balancing interface. In the presence of maltose, the protein adopts a closed conformation in which maltose is buried within the ligand binding cleft, and domain contacts are broken at the balancing interface. The strategy to increase the affinity of MBP for maltose is based on the idea that removal of important contacts in the balancing interface will destabilize the open conformation of the binding protein.

We located two potentially important interactions in the balancing interface; in both cases, these interactions only take place when the protein is in the open conformation. The first interaction occurs between the side chains of Met-321 on the C-terminal domain, and Tyr-90, Phe-92, Glu-308, and Ala-301 on the N-terminal domain: in the ligand-free conformation, the Met-321 SD and CE atoms are projected into a pocket formed by the hydrophobic parts of the other four side chains, resembling a ball-and-socket joint. In addition, the side chain of Gln-325 sits over the side chain of Met-321, protecting the assembly from solvent. In the ligand-bound conformation, this interaction is disrupted: the Met-321 side chain, the “ball,” moves almost 4 Å of the “socket” formed by Tyr-90, Phe-92, Glu-308, and Ala-301, and the hydrophobic surfaces that were buried become solvent exposed. Further evidence for important structural changes in this region comes from an analysis of the temperature factors, which provide an indication of atomic mobility. Temperature factors for the side chain atoms of Met-321 and Gln-325 are greater in the ligand-bound conformation (Table I), indicating an increase in mobility for these atoms when the protein binds maltose. These observations suggested that Met-321 and Gln-325 mediate important stabilizing interactions in the ligand-free, but not the ligand-bound, conformation. We mutated both residues to alanine to abrogate these interactions and selectively destabilize the open conformation. The M321A/Q325A mutant will be referred to as MBP-Ala.

The loop containing residues 171 to 177 was also identified as a potential stabilizing factor for the open conformation. In various crystal structures of MBP, this loop appears in different conformations with relatively high temperature factors, indicating that it is probably not important for the overall structure or fold of MBP. Nevertheless, this loop is retained with moderate sequence conservation in MBP molecules from various organisms, suggesting that it may have an important function. This loop is part of the C-terminal domain, but in the ligand-free conformation it makes a number of contacts with the N-terminal domain; these contacts are broken when the protein binds ligand. As with the side chains of Met-321 and Gln-325, the temperature factors of residues 171–177 are greater in the ligand-bound conformation (Table I), indicating that the loop becomes more mobile when the protein binds maltose. Note that the loop does not participate in crystal contacts in the structures analyzed. Based on reasoning similar to that given for the MBP-Ala mutant, we decided to shorten this loop to selectively destabilize the ligand-free, open conformation of MBP. Residue 174, a glycine, is located in the middle of the β-turn of the loop, and given its unique properties, we decided to shorten the loop by removing two residues on either side of Glu-174, namely Glu-172, Asn-173, Lys-175, and Tyr-176. This deletion mutant will be referred to as MBP-Del.

The mutations in MBP-Ala and MBP-Del caused an increase in the mobility of the protein, and therefore our strategy to increase the affinity of MBP for maltose was to remove important contacts in the balancing interface (Fig. 1B). The assumption here was that the interface has an active role in stabilizing the open conformation.

| Group                     | Average B-factor | Ligand-free | Maltose-bound |
|---------------------------|------------------|-------------|---------------|
| Met-321 side chain atoms  | 16.7             | 26.3        |
| Gln-325 side chain atoms  | 33.7             | 60.6        |
| Residues 171–177          | 43.4             | 59.2        |
| Whole protein             | 25.9             | 22.1        |

**Table I.** Temperature factor analysis of ligand-free and ligand-bound MBP.

**Fig. 1.** Maintenance of the open-closed equilibrium of MBP. A, in the open conformation of MBP, contacts between the two domains are formed through an interface on the opposite side of the hinge from the ligand binding cleft, which we term the balancing interface. In the presence of maltose, the protein adopts a closed conformation in which maltose is buried within the ligand binding cleft, and domain contacts are broken at the balancing interface. B, the strategy to increase the affinity of MBP for maltose is based on the idea that removal of important contacts in the balancing interface will destabilize the open conformation of the binding protein.
in affinity for maltose (see below). Because the mutations in MBP-Ala and MBP-Del are completely independent, we decided to combine them in an attempt to further increase the affinity for maltose. The MBP molecule produced by combining the mutations in MBP-Ala with the deletions in MBP-Del will be referred to as MBP-DM (for “double mutant”).

**Functional Characteristics of MBP and Engineered Mutants**—MBP molecules that cannot bind maltose, or interact productively with MalFGK₂, will not function in transport, and we first tested the mutants for their ability to participate in maltose transport. The MBP mutants were put into a background, E. coli HS3309 (14), which has a disruption of the maltose operon such that there is no chromosomally encoded MBP present. In this background, all of the mutants were able to support growth on minimal maltose media, indicating that they are fully functional.

We monitored binding of maltose to both MBP-WT and the engineered mutants MBP-Ala and MBP-Del by changes in intrinsic fluorescence. To ensure that the proteins were absolutely free from maltose, they were unfolded in 6 M guanidine HCl, purified by gel filtration chromatography, and refolded (see “Experimental Procedures”). The mutations attenuated the fluorescence quenching normally caused by maltose binding: upon addition of a saturating concentration of maltose, wild-type MBP exhibited a decrease in fluorescence of 14%, whereas the decrease for MBP-Ala and MBP-Del, under exactly the same conditions, was 8% (Table II). The change in fluorescence for MBP-Ala and MBP-Del, although modest, was still sufficient to carry out fluorescence titrations to determine the $K_D$ for maltose binding (Fig. 2). The $K_D$ values obtained from these experiments are listed in Table II.

The MBP-DM showed no significant change in fluorescence upon addition of maltose, but there was an 11–14% decrease in fluorescence upon addition of a saturating concentration of maltotriose (Table II). This change in fluorescence for MBP-Ala and MBP-Del, although modest, was still sufficient to carry out fluorescence titrations to determine the $K_D$ for maltose binding (Fig. 2). The $K_D$ values obtained from these experiments are listed in Table II.

The mutations produced an additive attenuation in the ligand-induced quenching of tryptophan fluorescence (Table II). None of the mutations produced an observable change in structure in the region of any tryptophan residue (see crystal structure analysis, below), and therefore it would appear that the strategy of destabilizing the open conformation of the protein has also had a general effect on ligand-induced fluorescence quenching. Of the eight tryptophan residues in MBP, three region, and therefore contacts with neighboring residues are different in the open and closed conformations. For example, in the open conformation, NE1 of the indole ring of Trp-158 can make a hydrogen bond with OE1 of Glu-326; this hydrogen bond is lost in the closed conformation because the side chain of

### Table II

| Protein | Maltose | Maltotriose |
|---------|---------|-------------|
|         | Quenching\(^a\) | $K_D$ | Quenching\(^a\) | $K_D$ |
| MBP-WT  | 14 ± 1 | 1200 ± 200 | 22 ± 2 | 660 ± 60 |
| MBP-Ala | 8 ± 2  | 70 ± 10  | 16 ± 2  | 120 ± 10 |
| MBP-Del | 8 ± 3  | 110 ± 20 | 18 ± 2  | 100 ± 10 |
| MBP-DM  | <2     | ND        | 12 ± 3  | 6 ± 1   |

\(^a\) Average ± S.D. for three determinations.
Trp-158 moves ~4 Å away from that of Glu-238. If our mutations have destabilized the open conformation, then interactions between Trp-158 and neighboring residues, such as Glu-328, will also be affected, and this could explain the observed attenuation of ligand-induced quenching.

**Binding Kinetics Measured Using Surface Plasmon Resonance Spectroscopy**—The kinetics of binding between MBP and longer maltodextrins were investigated using surface plasmon resonance (SPR) spectroscopy. Amylose, a component of starch consisting of linear polymers of glucose connected by 1,4-glycosidic linkages, was coupled to a sensor chip. Note that maltose consists of two glucose molecules attached through an 1,4-glycosidic linkage, and therefore amylose can be viewed as a linear polymer of maltose. Our goal in these experiments was to measure the relative affinities of MBP-WT and the three mutant proteins for long maltodextrins, and to discover whether the proteins were affected in substrate association, dissociation, or both. The same amylose-coupled sensor chip was used for all experiments. In these experiments, the refractive index of the media in the vicinity of the chip surface was monitored as solution was passed over the surface of the chip. The refractive index is sensitive to the composition of the chip surface, and therefore binding or dissociation of MBP produced a change in refractive index, which was quantified as arbitrary “response units” by the BIAcore instrument. Each experiment involved running a buffer across the sensor chip at a flow rate of 50 µl/min (for example), and then injecting a volume (100 µl) of solution containing MBP. A typical “sensorgram” (Fig. 3) consists of three phases: the first is an associative phase, where MBP binds to the immobilized amylose; the second is a “steady-state” phase, where a plateau in the response factor may occur, indicating that the rate of MBP association is exactly offset by dissociation; the third phase, dissociation, occurs at the end of the injection when MBP-free buffer again passes over the chip, and bound MBP

![Figure 3](image-url)
dissociates from the chip surface. Each of the four proteins (MBP-WT, MBP-Del, MBP-Ala, and MBP-DM) ranging in concentration from 4 nM to 4 μM, was analyzed.

For a fixed MBP concentration, the response maximum will be proportional to the affinity of the MBP for the immobilized ligand; examples for MBP molecules at 63 and 500 nM are given in Fig. 3, panels A and B. We analyzed the sensorgrams using the BIAevaluation software supplied with the instrument and found that, when a global analysis incorporating both the associative and dissociative phases was attempted, there were systematic deviations from the models. The most likely explanation for these systematic deviations is that amylose, a rather rigid and insoluble polymer, may present a collection of heterogeneous binding sites when coupled to the chip surface. Despite our inability to carry out a quantitative global kinetic analysis, in qualitative terms the results were unambiguous: the response maximum at any given protein concentration was always highest with MBP-DM and lowest with MBP-WT, with MBP-Del and MBP-Ala roughly equal and reaching response maxima intermediate between MBP-WT and MBP-DM (Fig. 4). The relative affinities of the protein for immobilized amylose, as measured using the BIAcore instrument, resemble the relative affinities of the proteins for maltose and maltotriose observed in solution by fluorescence titrations.

Sensorgrams can also be used to investigate the kinetics of association and dissociation. For a fixed solute concentration, the initial rate of association will be determined solely by $k_{ON}$, and therefore comparison of sensorgrams recorded with MBP molecules at a fixed concentration will show whether there are any changes in $k_{ON}$ produced by the mutations. In Fig. 3, panels A and C, sensorgrams recorded using 63 nM MBP-WT, MBP-Del, or MBP-DM were carefully aligned to illustrate the difference in $k_{ON}$ between the three proteins; the same analysis was carried out with 500 nM protein (Fig. 3, panels B and D). Note that MBP-Ala gave similar results to MBP-Del, but has been omitted from these plots for clarity. The initial rate of association for each experiment was estimated using the linear region of the sensorgram, which is typically comprised of the first 3–5 data points after the start of injection. The rate constants ($k_{ON}$) were independent of protein concentration, allowing us to calculate an average $k_{ON}$ for each protein, determined from 6 sensorgrams with protein concentrations ranging from 63 nM to 4 μM. These $k_{ON}$ values are listed in Table III. There is no significant difference in $k_{ON}$ values between the three mutants, but there is a 1.5–1.9-fold increase in the $k_{ON}$ of the mutants compared with MBP-WT. The $k_{ON}$ value determined for association of MBP-WT and maltose in solution, by stopped-flow fluorescence spectroscopy, is $2.3 \times 10^{-7}$ M$^{-1}$ s$^{-1}$ (17). The $k_{ON}$ values determined with the BIAcore instrument are 5–10-fold higher, which may be because of a relatively high concentration of binding sites on the chip surface, and the fact that the MBP solution is actively flowing across the chip.

The rate of dissociation is determined by the amount of MBP bound to the sensor chip and by $k_{OFF}$, the dissociation rate constant; therefore, at a fixed response (i.e. a fixed amount of bound MBP), the rate of dissociation should be determined solely by $k_{OFF}$. In Fig. 5, sensorgrams are matched according to the response units at the beginning of the dissociative phase. In Fig. 5, A and C, MBP-WT, MBP-Del, and MBP-DM were present at concentrations of 250, 63, and 32 nM, respectively, producing a response maximum of 40 units in each case, and it is clear that the rate of dissociation is greatest for MBP-WT and smallest for MBP-DM. Similar results were obtained for a response maximum of 220 units (obtained from MBP-WT, MBP-Del, and MBP-DM at concentrations of 2000, 500, and 125 nM, respectively; Fig. 5, B and D). We attempted to measure the rate constant for dissociation, but we were unable to fit the dissociation curve to a single exponential decay (which corresponds to the expected first order process), most likely because the immobilized amylose presents a mixed population of binding sites. In lieu of rate constants, we measured the time it took for each protein to dissociate from the immobilized amylose (Table III). Here, data from the experiment illustrated in Fig. 5 were used to determine the time it took for 50, 75, and 90% of each protein to dissociate from the immobilized amylose. With this type of analysis, one expects that the time it takes for a given percentage of the protein to dissociate will be independent of the starting response; that is, 50% dissociation should occur in 5 s (for example), regardless of whether 50 or 500 RU are present at the start of dissociation. However, we found that the rate of dissociation increased as the chip became saturated (compare the values for 40 RU with those for 220 RU). We included 100 μM maltose in the running buffer to abrogate re-binding effects during the dissociative phase, and therefore the correlation between dissociation rate and RU is likely because of occupation of the lower affinity binding sites as the chip surface becomes saturated. Nevertheless, across the whole range of RU values measured, both MBP-Del and MBP-Ala show a decreased rate of dissociation compared with MBP-WT, and MBP-DM shows a strikingly slow rate of dissociation, taking up to 30–50 times longer (compared with MBP-WT) for the protein to dissociate from the chip surface (Table III).

To conclude, the relative affinities of the four proteins for immobilized amylose correlate with their relative affinities for maltose and maltotriose in solution. The $k_{ON}$ values for the three mutants are not significantly different from each other, and are only 1.5–1.9-fold greater than the $k_{ON}$ value for MBP-WT. Therefore changes in $k_{ON}$ are insufficient to account for the increased affinity caused by the mutations. Although dissociation rate constants ($k_{OFF}$) could not be determined using the amylose affinity chip, because of hetero-
High Affinity MBP Mutants

**Table III**

Binding and dissociation of MBP molecules to immobilized amylose

| Protein      | $k_{\text{ON}}^{a,b}$ | Dissociation times$^c$ (s) |          |          |          |
|--------------|------------------------|---------------------------|----------|----------|----------|
|              | $\times 10^{-7} \text{M}^{-1} \text{s}^{-1}$ | 40 RU$^d$ | 75% | 90% | 50% | 75% | 90% |
| MBP-WT       | 11 ± 1.2 | 1.4 | 3.8 | 21 | 1.2 | 4.0 | 22 |
| MBP-Ala      | 20 ± 4.4 | 8.5 | 24 | 44 | 4.5 | 11 | 29 |
| MBP-Del      | 17 ± 5.2 | 6.6 | 16 | 33 | 2.2 | 12 | 31 |
| MBP-DM       | 21 ± 3.0 | 51 | >180 | >180 | 33 | 120 |

$^a$ Measured by linear least-squares fit to the first 3–5 data points after injection.

$^b$ Average ± S.D. for six measurements ranging in protein concentration from 63 nM to 4 μM.

$^c$ Time required for RU to reach the indicated percentage of the RU at the beginning of the dissociative phase.

$^d$ RU at the beginning of the dissociative phase, obtained using 250 nM MBP-WT, 63 nM MBP-Ala and MBP-Del, and 31 nM MBP-DM.

$^e$ RU at the beginning of the dissociative phase, obtained using 2000 nM for MBP-WT, 500 nM MBP-Ala and MBP-Del, and 125 nM MBP-DM.

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**Fig. 5.** Comparison of off-rates by SPR. Binding to an amylose-coupled sensor chip was measured as in Fig. 3, except that the proteins were at different concentrations so that their response maximum was the same, 40 units for panels A and C, and 230 units for panels B and D. Note that panels C and D are enlargements of panels A and B, respectively, in the region where dissociation first takes place. MBP-WT (light gray curves), MBP-Del (dark gray curves), and MBP-DM (black curves) were at concentrations of 250, 63, and 32 nM, respectively, for panels A and C; and 2000, 500, and 125 nM for panels B and D. The running buffer for these experiments included 100 μM maltose to abrogate rebinding effects during the dissociation phase.

genecity in the binding sites, there are clear differences in the rate of dissociation when MBP-WT is compared with the three mutants. In particular, MBP-DM dissociates from the chip surface up to 40 times more slowly than MBP-WT (Table III). Thus, the increases in affinity brought about by the mutations are largely because of decreases in $k_{\text{OFF}}$. 
To analyze further the binding reaction between the MBP molecules and maltodextrins, we coupled pullulan to a sensor chip. Pullulan is a highly soluble, linear polymer comprised of repeating maltotriose and maltotetraose units (27). Protein concentrations in the micromolar range were required to observe significant binding to the pullulan-coupled sensor chip; examples of the sensorgrams obtained using the pullulan-coupled chip are given in Fig. 6A. The on- and off-rates for binding to the pullulan chip were too fast to measure, but these fast rates meant that the system reached steady state for all of the protein concentrations tested. The trend observed with the amylose-coupled chip is also present with the pullulan-coupled chip; that is, at any given protein concentration, MBP-DM exhibited the highest response factor at steady state, MBP-WT the lowest, and MBP-Del was midway between the two. We plotted the response factor at steady state for MBP-WT and MBP-DM (Fig. 6B); however, we were unable to saturate the surface of the chip, and therefore cannot obtain a reliable fit of these data to the equation for steady-state binding to obtain a dissociation constant. Nevertheless, these results show that the balancing interface mutations have a general and additive effect on the affinity of MBP for a variety of maltodextrins.

Conformation of MBP in Solution—The engineered MBP mutants, especially MBP-DM, have a dramatically increased affinity for maltodextrins. The mutations were intended to destabilize the open conformation of the binding protein and shift the open-closed equilibrium toward the closed conformation (12). This raises the possibility that, even in the absence of ligand, there is a significant population of the engineered MBP molecules in the closed conformation. We used SAXS to analyze directly the solution conformation of MBP-WT and the three mutants in the ligand-free state, and MBP-DM (Fig. 6B); however, we were unable to saturate the surface of the chip, and therefore cannot obtain a reliable fit of these data to the equation for steady-state binding to obtain a dissociation constant. Nevertheless, these results show that the balancing interface mutations have a general and additive effect on the affinity of MBP for a variety of maltodextrins.

**TABLE IV**

| Protein  | Ligand-free Radius of gyration | 1 mM Maltose Radius of gyration |
|----------|-------------------------------|-------------------------------|
| MBP-WT   | 22.3 Å                       | 21.5 Å                       |
| MBP-Ala  | 22.6 Å                       | 21.7 Å                       |
| MBP-Del  | 22.5 Å                       | 21.6 Å                       |
| MBP-DM   | 22.4 Å                       | 21.3 Å                       |

*Calculated using the indirect transform method implemented in the program Gnom (38).*

**Fig. 7.** The solution conformation of unliganded MBP-DM resembles that of MBP-WT. The SAXS curves for MBP-DM (solid black curve) and MBP-WT (dashed gray curve), both in the absence of maltose, have been scaled to yield identical I (0) values. The expanded lower angle region is illustrated in the inset. Data were collected at BioCAT (beamline ID18) at APS, using sample-to-detector distances of 2780 (with 5 mg/ml protein) and 255 mm (with 8 mg/ml protein); these data were scaled and merged in the region of Q = 1.5 nm<sup>-1</sup> to yield the continuous curves illustrated here.

**Fig. 6.** SPR analysis of MBP-pullulan interactions. A, the general form of the sensorgrams obtained for MBP molecules binding to a pullulan-coupled sensor chip is illustrated. The flow rate across the chip was 25 µl/min; the injections were 30 µl of MBP-WT at 16 µM (light gray curve), 40 µl of MBP-Del at 15 µM (dark gray curve), or 40 µl of MBP-DM at 12 µM (black curve). B, the maximum response factor obtained at steady state for MBP-WT (circles) or MBP-DM (squares) is plotted at various protein concentrations.
found no significant differences between the four proteins (Table IV); in the presence of 1 mM maltose, the \( R_g \) values decreased by \(-1\) Å in all cases.

The \( R_g \) value is determined primarily by the very low angle region of the scattering curve, but the conformational change in going from open to closed will affect the whole scattering curve. Close inspection of the entire scattering curves for the three engineered proteins revealed no significant difference between them and MBP-WT in either the presence or absence of maltose. The results for MBP-DM in the absence of maltose are presented in Fig. 7, in which the curves for unliganded MBP-WT and unliganded MBP-DM are superimposed, and it is clear that the curves for the two proteins are essentially identical. To summarize, the SAXS analysis shows that the mutations have not significantly affected the solution conformation of the protein in either the presence or absence of maltose.

We were surprised that the mutations had no effect on the conformation of unliganded MBP. Previously, it had been shown that the open conformation of MBP in solution corresponded reasonably well with the crystal structure of the open conformation (28). However, the exceptional quality of the SAXS data obtained at APS for MBP-WT and MBP-DM, combined with the program Crysol (29) allowed us to carry out a careful comparison between the crystal structures of the closed and open conformations of MBP (9, 10), and the solution conformations of MBP-DM and MBP-WT.

The program Crysol (29) was used to calculate the theoretical scattering from the crystal structures of unliganded, open MBP (9, 10) and liganded closed MBP (9, 10). When these theoretical scattering curves are plotted (Fig. 8 panels A and B), the difference in conformation between unliganded and liganded MBP is quite obvious. These calculated scattering curves were then used to compare the crystal structures with the experimental scattering curves obtained for the proteins in solution. In the presence of 10 mM maltose, the conformation of MBP in solution (either MBP-WT or MBP-DM) exactly matched that of the closed, ligand-bound conformation in the crystal (9) (Fig. 8C). On the other hand, the open, unliganded conformation of MBP seen in the crystal (10) failed to give a perfect match to scattering data for either unliganded MBP-WT (not shown) or MBP-DM (Fig. 8D). In qualitative terms, the solution scattering from the unliganded proteins suggested the presence of the closed conformation. We used a linear least-squares analysis to find a combination of the open and closed conformations that would match the solution scattering for unliganded MBP. For both MBP-WT and MBP-DM, a mixture consisting of 85% open conformation and 15% closed conformation yielded a perfect match to our solution scattering data (Fig. 8E). Note that prior to the SAXS analysis, the proteins had been denatured and passed through a gel filtration column to remove any residual maltose, and then refolded and passed through a second gel filtration column.

Fig. 8. Unliganded MBP-DM contains a mixture of open and closed conformations. The crystal structures of MBP are compared with the solution conformations in the presence and absence of maltose. In each of the panels A–E, two scattering curves are illustrated, and the difference between them is given at the top of each panel. A, crystal structures of MBP (9, 10) were used to calculate theoretical SAXS curves for MBP in the closed, ligand-bound conformation (dashed curve) and the open, ligand-free conformation (dotted curve). B is a Kratky plot of the same theoretical curves in panel A; the Kratky plot highlights differences in the “medium angle” region of momentum transfer (i.e. \( 0.5 < Q < 1.5 \text{ nm}^{-1} \)). Panels C and D illustrate the expected differences in solution scattering caused by the two conformations of MBP. Panels C–E are Kratky plots comparing experimental SAXS data with the theoretical curves for the open and closed conformations of MBP. C, SAXS data for ligand-bound MBP-DM (solid curve) are compared with the theoretical SAXS curve for the closed conformation (dashed curve). D, SAXS data for unliganded MBP-DM (solid curve) are compared with the theoretical SAXS curve for the open conformation (dotted curve). E, SAXS data for unliganded MBP-DM (solid curve) are compared with the theoretical SAXS curve for a mixture of 85% open conformation and 15% closed conformation.
structure and interactions of the truncated loop in both MBP-
compared with room temperature for the wild-type protein. The
crystallographic data for the mutants were collected at 100 K,
type protein, but we expect this is because of the fact that the
34564
MBP-DM are both slightly more
Data Bank code 1ANF; Ref. 9) with a root mean square devia-
and MBP-DM superimpose over those of MBP-WT (Protein
ligand, but the crystals were badly twinned and the structure
crystals and diffraction data from MBP-Ala in the absence of
absence of maltose (Tables V and IV, and Fig. 9). We obtained
High resolution crystal structure analysis was used to investi-
gate the structural effects of the mutations. Structures were
closed conformations, even though they do have a profound
effect on the equilibrium between the unliganded open and
unliganded MBP exists to a significant degree in the closed
—
—
we are confident that the proteins were absolutely free of maltose. Therefore, it appears that
unliganded MBP exists to a significant degree in the closed
conformation of MBP-WT (Protein Data Band code 1OMP; Ref.
2) with a root mean square deviation of
High Resolution Analysis of Structural Changes in MBP-Del
and MBP-DM—We believe that the mutations introduced into
the balancing interface increase the affinity of MBP for its
ligands by selectively destabilizing the open conformation. High resolution crystal structure analysis was used to investigate the structural effects of the mutations. Structures were solved for MBP-Del and MBP-DM in both the presence and absence of maltose (Tables V and IV, and Fig. 9). We obtained crystals and diffraction data from MBP-Ala in the absence of ligand, but the crystals were badly twinned and the structure could not be solved.

For the maltose-bound structures, the CA atoms of MBP-Del and MBP-DM are different from MBP-WT, but in all other respects, the proteins appear to be identical. There are no significant differences in the fold or in side chain conformations anywhere in the protein. In the closed, liganded conformation, the balancing interface is “broken” and the residues on each domain that normally make contact in the open conformation are solvent exposed and too far apart to make interdomain contacts. Therefore, in the closed, liganded conformation, the mutations have not caused any changes in interdomain contacts.

In the case of the unliganded proteins, the CA atoms of MBP-Del and MBP-DM superimpose over those of the open conformation of MBP-WT (Protein Data Bank code 1OMP; Ref. 10) with a root mean square deviation of 0.5 Å in both cases. Except for the truncated loop, there are no significant changes in the structure of the mutant proteins when compared with wild-type. However, the mutations have resulted in the removal of contacts in the balancing interface. Referring to Fig. 9, A and B, it can be seen that the truncation of the loop results in a loss of interdomain contacts that are normally present in the open conformation of MBP. In Fig. 9C, the molecular surface of MBP-DM in the open, unliganded conformation, is shown, illustrating the cavity formed by mutation of Met-321 and Gin-325 to alanine; included in this figure are the side chains of Met-321 and Gin-325 from the open, unliganded structure of MBP-Del. In the open conformation of MBP-Del and MBP-WT, the side chains of Met-321 and Gin-325 make contact with the opposite domain, and these contacts are removed in the open conformation of MBP-DM. To summarize, the mutations have not had an observable effect on the overall structure of the
proteins when compared with MBP-WT, but they have removed contacts in the balancing interface that are normally present only in the open conformation.

**DISCUSSION**

We have demonstrated that removal of interactions in the balancing interface of MBP results in a general increase in affinity for maltodextrins. The increase in affinity is due primarily to a decrease in the rate of dissociation between MBP and its ligand. The introduction of larger, bulky groups into the balancing interface has been used previously to engineer MBP and its ligand. The figures in panels A and B were made with SwissPDBViewer (39), whereas the figure in panel C was made with SPOCK (40) and Raster3D (41).

**Fig. 9. Structural analysis of the open and closed conformations.** For panels A and B, open, unliganded structures of MBP are illustrated on the left, whereas liganded and closed structures of MBP are illustrated on the right. Panel A shows the backbone structures of MBP-WT (9, 10), with residues 171 to 178 highlighted in black. Panel B shows the backbone structures of MBP-Del, in exactly the same orientation as the wild-type protein. Note that for MBP-Del, the truncated loop cannot make contact with the N-terminal domain in either the open or closed conformations. Panel C illustrates the cavity formed in the balancing interface of the open conformation when Met-321 and Gln-325 are mutated to alanine. Here, the molecular surface of open, unliganded MBP-DM is shown, with the side chains of Met-321 and Gln-325 from the structure of open, unliganded MBP-Del. The figures in panels A and B were made with SwissPDBViewer (39), whereas the figure in panel C was made with SPOCK (40) and Raster3D (41).

**High Affinity MBP Mutants**

In a thermodynamic sense, the effect of the mutations can be easily understood with the simple binding equilibrium illustrated in Fig. 1. Here, the removal of interactions (this study) or the introduction of bulky groups (12) in the balancing interface will destabilize the open conformation, and, in the presence of maltose, this will shift the overall equilibrium further toward the closed, liganded conformation (Fig. 1). The dependence of ligand binding affinity on conformational equilibria has been termed “conformational coupling” (12).

Conformational coupling is not only an important consideration for protein engineering (31), but is likely a major factor in the evolution of proteins. For example, the function of the loop comprising residues 171–177 is not obvious. Whereas the loop is conserved in MBP molecules from a number of different bacteria, there are certain species for which residues in the loop have been deleted. In the crystal structure of MBP from *P. furinosis* (32), the loop is truncated and the structure resembles that of our MBP-Del mutant. Interestingly, *Pyrococcus furinosis* MBP binds maltotriose with a 20-fold greater affinity than *E. coli* MBP (32). The results from the present study suggest that this loop may have evolved specifically to function as a modulator of ligand binding affinity: mutations in the loop, which are relatively easy to accommodate, alter the affinity of the protein for its ligand by changing its conformational equilibria, and the loop therefore provides a mechanism for natural selection to alter ligand binding affinity without affecting specificity (33).

The precise mechanism by which the mutations exert their
effect requires consideration of ligand binding kinetics, and here the situation becomes somewhat more complicated than the simple equilibria presented in Fig. 1. The mutated residues make no interdomain contacts in the closed, ligand-bound conformation, for either the wild-type or mutant proteins, and therefore in the simplest case the mutations will affect only the open conformation of the binding protein (Fig. 1B and Fig. 9); that is, the mutations should affect only the rate of closing of the open conformation. On this basis, the SPR results are counterintuitive because they indicate that the most pronounced effect of the mutations is to decrease the rate of dissociation (k
OFF
), a process that involves opening of the already closed binding protein (Fig. 1). However, the binding process can be expanded further to include equilibria between the open and closed conformations, in both the presence and absence of bound maltose (Fig. 10). In this case, the
K
OFF
expressed as a ratio of the off and on rates, is further broken down into individual kinetic steps.

\[ K_{OFF} = \frac{k_{on}}{k_{off}} = \frac{k_{b\cdot k_{3}}}{k_{c\cdot k_{3}}} \]  
(Eq. 3)

The maltose binding site is not accessible in the closed conformation, and therefore maltose is shown binding to, and dissociating from, only the fully open conformation (Fig. 10).

The existence of the open, liganded species provides a mechanism for the observed decrease in k
OFF
: the mutations have shifted the open-closed equilibrium toward the closed conformation by increasing k
b
, the rate of closing of the open conformation, thereby decreasing the concentration of open, liganded MBP and the rate of maltose dissociation.

While this analysis provides a reasonably satisfying explanation for the mechanism by which the mutations increase the affinity for maltdextrins, there are two surprising results from our study that are not easily rationalized. The first is that, whereas the mutations have apparently shifted the conformational equilibrium of the liganded binding protein from the open to the closed, they have had no observable effect on the conformation of the unliganded binding protein, as measured using SAXS. Referring to Fig. 10, it could be that the amount of closed, unliganded binding protein is so small (for example, below 0.5%) that the change in equilibrium brought by the mutations is not observable by SAXS. The almost complete absence of the closed, unliganded conformation is an intriguing possibility because it has been shown that unliganded MBP is able to promote ATP hydrolysis by MalFGK
K
(3) and it has been generally assumed that the closed conformation, or something close to it, was responsible. If, in the absence of ligand, the closed conformation is present only in very small quantities, it is difficult to understand how it could cause a significant activation of the MalFGK
ATPase. In fact, our SAXS measurements are consistent with unliganded MBP existing in a closed: open ratio of ~1:9, and therefore our observation that the mutations have no significant effect on the conformational equilibrium of the unliganded protein defies easy explanation.

The second surprising result is the change in the fluorescence properties of the mutants. As discussed under “Results,” the tryptophan responsible for the ligand-induced attenuation of fluorescence is probably Trp-158, which is in the balancing interface and has interactions that are altered when the protein changes conformation. Our structural studies, both crystalllographic and in solution by SAXS, indicate that there are no changes caused by the mutations that can explain their effect on the fluorescence properties. Nevertheless, the mutations produce a general and additive reduction in ligand-induced fluorescence attenuation. The lack of any observable structural change suggests that the decrease in fluorescence quenching is because of a change in the dynamic structure of MBP; that is, the molecular dynamics of MBP, rather than the average structure, are affected by the balancing interface mutations.

A dynamic structure for unliganded MBP is supported by a time-resolved tryptophan fluorescence study in which it was found that unliganded MBP, but not liganded MBP, undergoes large internal fluctuations on a 15 ns time scale (i.e. corresponding to rates of \(7 \times 10^4 \text{ s}^{-1}\)), a relaxation process that was attributed to motion of the individual domains (34). The unliganded protein can exist in a number of different conformations, as evidenced by our SAXS results showing that the unliganded conformation in solution does not exactly match the unliganded crystal structure of MBP. NMR spectroscopy has also been used to measure the conformation of MBP in solution, and, consistent with our SAXS results, it was found that the liganded conformation was almost perfect for the crystal structure, but that the unliganded conformation in solution differed slightly from that in the crystal (35). In fact, the glucose/galactose receptor from Salmonella typhimurium was crystallized in the closed conformation in the absence of ligand (36), indicating that the closed, unliganded conformation of this class of binding proteins is fully accessible in solution.

We have accomplished our goal of engineering the balancing interface to bring about a general increase in the affinity of MBP for its ligands. Crystal structure analysis shows that the mutations remove interdomain contacts made across the balancing interface, consistent with the idea that they selectively destabilize the open conformation. However, the balancing interface mutations appear to increase ligand affinity without altering the open-closed equilibrium of the unliganded protein, and they have a general effect on the tryptophan fluorescence of MBP. These results suggest that the balancing interface mutations work by altering the molecular dynamics of the open conformation.

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