Two Modes of Ligand Binding in Maltose-binding Protein of Escherichia coli

ELECTRON PARAMAGNETIC RESONANCE STUDY OF LIGAND-INDUCED GLOBAL CONFORMATIONAL CHANGES BY SITE-DIRECTED SPIN LABELING

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Binding of ligands to the maltose-binding protein (MBP) of Escherichia coli often causes a global conformational change involving the closure of its two lobes. We have introduced a cysteine residue onto each of these lobes by site-directed mutagenesis and modified these residues with spin labels. Using EPR spectroscopy, we examined the changes, caused by the ligand binding, in distance between the two spin labels, hence between the two lobes. The binding of both maltose and maltotetraose induced a considerable closure of the N- and C-terminal lobes of MBP. Little closure occurred upon the binding of maltotetraol or β-cyclodextrin. Previous study by fluorescence and UV differential absorbance spectroscopy (Hall, J. A., Gehring, K., and Nikaido, H. (1997) J. Biol. Chem. 272, 17605–17609) showed that maltose and a large portion of maltotetraose bound to MBP via one mode (R mode or “end-on” mode), which is physiologically active and leads to the subsequent transport of the ligands across the cytoplasmic membrane. In contrast, maltotetraol and β-cyclodextrin bound to MBP via a different mode (B mode or “middle” mode), which is physiologically inactive. The present work suggests that the B mode is nonproductive because ligands binding in this manner prevent the closure of the two domains of MBP, and, as a result, the resulting ligand-MBP complex is incapable of interacting properly with the inner membrane-associated transporter complex.

The maltose-binding protein (MBP) of E. coli is absolutely required for maltose and maltodextrin transport across the cytoplasmic membrane (see Ref. 1 and references therein). Various ligands bind tightly to MBP. However, the specific binding to MBP does not necessarily lead to the subsequent transport of the ligand via the inner membrane-associated transporter complex, which is composed of one copy of MalF, one copy of MalG, and two copies of MalK (2). In the preceding paper (1) we used fluorescence and UV differential absorbance spectroscopy to characterize the modes of binding of various ligands to MBP. Maltose bound exclusively via one mode that involved a red shift of the intrinsic fluorescence emission spectrum of MBP as well as other characteristic changes in UV absorption spectrum (R mode, for red shift). Maltodextrins were also shown to bind largely through the R mode when added in excess. In contrast, β-cyclodextrin as well as reduced or oxidized maltodextrins bound to MBP exclusively through another mode, which caused a blue shift of the fluorescence emission spectrum and other signature alterations in UV absorption spectrum (B mode, for blue shift). A mutant MBP, MalE254, bound even unmodified maltodextrins exclusively via the B mode. We found that whenever a ligand was bound to MBP via the R mode, it was transported. In contrast, when it bound to MBP purely through the B mode, it failed to be transported through a wild type transporter complex, even when the binding to MBP occurred with very high affinity. We have also argued (1) that the R and B modes most likely correspond to the “end-on” and “middle” modes of binding, previously defined on the basis of NMR chemical shift of the 3H atom on the anomeric carbon of the ligand molecules (3).

The binding of R mode ligands, such as maltose and maltotriose, was earlier shown to produce a large scale conformational change in MBP (4) involving the closing of its two lobes (5, 6). In contrast, one of the B mode ligands, β-cyclodextrin, binds to MBP without causing the closing (6). This correlation suggests that the R and B modes may produce closing and nonclosing of the two lobes of MBP, respectively. However, because fluorescence, UV absorption, and chemical shift of the anomeric 3H are all influenced mainly by the local environment surrounding the ligand, these results did not provide concrete data on the global conformation of MBP.

In the present work we have used electron paramagnetic resonance (EPR) spectroscopy to examine the global conformational changes of MBP, caused by the R and B modes of binding. EPR spectroscopy is a sensitive method for examining protein structure and dynamics and has become widely applicable due to the development of site-directed spin labeling (for reviews see Refs. 7 and 8). Spin labeling EPR has previously been used to estimate interspin separations in proteins (9, 10). Recently, this approach has been systematically extended to measure distances between two site-specifically placed spin labels and calibrated using well known α-helical peptides (11). Using this approach, we have spin-labeled two residues of MBP, one in the N-terminal lobe and one in the C-terminal lobe, and examined the extent of closure of these lobes occurring upon the binding of various ligands. Our results indicated that those ligands that bind to MBP solely by the B mode caused little change in the global conformation of MBP upon binding. In contrast, those ligands that complex with MBP
primarily via the R mode caused the two domains of MBP to come together considerably.

EXPERIMENTAL PROCEDURES

Mutagenesis, Cloning, and Sequencing—Strain XL1-Blue MRF (ΔmalE) was used for all cloning steps, and plasmid pLWY (malE' bla) was utilized as the template for all mutagenesis reactions. This plasmid was constructed by ligating the EcoRI-NdeI fragment of malE' allele from the plasmid pPD1 (malE' bla) (12) with the large EcoRI-NdeI fragment of plasmid pUC19. The ligated DNA was transformed into strain XL1-Blue MRF. DNA from these transformants was analyzed, and a plasmid that could be cut with both HindIII and BglII was retained as pLWY.

Site-directed mutagenesis was performed by using sequential PCR steps (13). To produce the S211C mutation, the mutant primer 5'-GATTACTGCACTCCGAAAGTC-3' and an external primer immediately outside the BlpI site unique to pLWY were used in the first PCR amplification. The resulting blunt-end fragments were isolated on a 0.8% agarose gel, purified using the GeneCleanII kit (BIO 101), and used in the second PCR amplification. The two blunt-ended fragments were here amplified with the two aforementioned external primers to generate the full-length fragment containing the S211C mutation. This full-length product was then digested with both BlpI and BglII. The resultant fragment was isolated and purified as above and finally ligated into BlpI- and BglII-digested pLWY to produce pH1. This procedure was also used to produce the D41C mutation except that, in the first PCR amplification, the mutant primers 5'-CATCGGTA-AACTGGAAAGAG-3' and 5'-CAGTTTACACGGATGCTCAAC-3' were used with external primers immediately outside the unique BsiWI and PsvII sites, respectively. The full-length product was digested with BsiWI and PsvII, and the resultant fragment ligated into pLWY to produce plasmid pH2. Both pH1 and pH2 were sequenced across the respective PCR-amplified regions using the Sequenase version 2.0 DNA sequencing kit (U.S. Biochemical Corp.) to ensure that no other mutations occurred.

A plasmid that contains both the S211C and D41C mutations in the malE gene was constructed by ligating the small fragment derived from digestion of pH2 with BglII and EcoRI with the large fragment derived from digestion of pH1 with the same two restriction enzymes. After ligation and subsequent transformation of strain XL1-Blue MRF, plasmid DNA was isolated, and was digested with both BglII and EcoRI to ensure that they exhibited the correct restriction patterns. The plasmid DNA was further sequenced across both mutated regions. One plasmid, pH3, was used for subsequent expression of D41C/S211C MBP.

Sugar and Sugar Analogs—These were described in the preceding paper (1).

Purification of MBP—Wild type MBP and D41C, S211C, and D41C/S211C mutant MBPs were prepared from strain HS2019 (K-12 F+ araD139 Δ(lacU169 rpsL thi) Δ(galE44) (14) containing the appropriate plasmid and purified as described (1), except for the following changes. All mutant MBP proteins were eluted from cross-linked amylose columns with 100 mM maltose, and bound maltose was removed from all MBP proteins by denaturation in 8 M guanidine HCl followed by renaturation through dialysis. Purified MBP proteins were shown to be at least 95% pure by staining of SDS-polyacrylamide gels.

Fluorescence Spectroscopy—The binding of various ligands to wild type and mutant MBP proteins, unlabeled or labeled with spin label, was determined using fluorescence emission spectroscopy (1). For the determination of the mode of binding, ligand was added to MBP such that greater than 90% of all MBP present would be in the ligand-complexed form.

Proteoliposome Assays of ATP Hydrolysis and Maltose Transport—The inner membrane transport complex (MalFGK2) was prepared from strain H7N41 (K-12 argB his rplL1 malEΔ Δ(malB13 ΔuncBC [le::Tn10/F lacI72]) (15) containing pMR1 (malK cat) and pMG23 (malH malI malL malM malN malP malT malU malF QSIW), and used for subsequent transformation of strain HS2019 (ΔmalE) (15). Cells were grown, and envelope fraction was constructed by incubating 15 μM protein with 150 μM MTSSL for 2 h at room temperature in 20 mM DTT, 10 mM KPO4, pH 8.0. DTt was then removed via extensive dialysis against 10 mM KPO4, pH 8.0, at 4 °C. Spin labeling of MBPs was accomplished by incubating 15 μM protein with 150 μM MTSSL for (D41C and S211C mutants) or 300 μM MTSSL for (D41C/S211C wild type MBP proteins) for 2 h at room temperature in 10 mM KPO4, pH 8.0. Wild type MBP was “mock” spin labeled to ensure that the spin labeling procedure had no significant effect on the activity of the MBP. After the MTSSL labeling, the solutions were dialyzed extensively against 10 mM KPO4, pH 8.0, to remove excess free MTSSL. After dialysis, MBP proteins were placed in 10 mM KPO4, pH 8.0, 15% glycerol, concentrated using Microcon 10 filter units (Amicon, Inc.), and used immediately for EPR experiments or substrate uptake assays. Final protein concentrations were calculated using the UV extinction coefficient of MBP of 1.7 (ε 280) at 280 nm (3) and were between 250 and 400 μM.

EPR Spectroscopy and Spectral Analysis—EPR spectra were collected using a Bruker ESP 300 E spectrometer equipped with a loop-gap resonator (Medical Advances, Milwaukee, WI) and a low noise amplifier (Miteq, Hauppauge, NY). Room temperature spectra were collected using 1-milliwatt microwave power and a modulation amplitude of 1 gauss. Low temperature spectra were collected at 140 K using 8-milli- watt microwave power and a modulation amplitude of 2 gauss. Spin concentrations were determined by comparing doubly integrated spectra to that of a TEMPO standard solution. The labeling percentages were 90 ± 10% for both of the single mutants and 80 ± 10% for the double mutant.

EPR spin-spin interactions between two spin labels lead to EPR spectral broadening in the case of interspin distances less than about 25 Å. However, quantitative analysis of data is difficult. Alternatively, use of motionally frozen, unoriented samples near liquid nitrogen temperature allows us to obtain interspin distances directly from spectral analysis (11). This technique provides accurate estimates for interspin distances in the range of 8–25 Å, and its application to a model system (a series of spin-labeled a-helical peptides) has been described (11).

RESULTS

Construction and Function of Mutant MBP—Our objective was to examine the global conformational change that MBP undergoes upon binding various ligands, through alteration of the distance between its N- and C-terminal domains. We constructed a MBP double mutant that had both Asp41 and Ser211 mutations (5, 6). These residues are located on the close proximity of the ligand binding site (Fig. 1). These residues are also on the surface of MBP and, therefore, are presumably more accessible to the labeling agent, MTSSL. The distance between these residues changes from 24 to 15 Å (measured between a-carbons) when MBP binds maltose (Fig. 1).

The properties of wild type MBP were examined to make certain that the site-directed mutations and the introduction of spin type and mutant MBP, this ensures that >90% of all MBP inside the ligand-complexed form. The hydrolysis of ATP by the MalFGK2 transport complex was measured as described (15), except that all reactions were stopped after 5 min. All values were corrected by subtracting ATP hydrolysis by liposomes prepared without the MalFGK2 transport complex and MBP.

When the transport of maltose into proteoliposomes was examined, the dilution mixture contained 20 mM KPO4, pH 6.2, 5 mM ATP, and 1 mM DTT. The proteoliposomes were resuspended to a final concentration of 0.45 mg/ml protein in 20 mM KPO4, pH 6.2, 3 mM MgCl2, with or without 1.0 μM MBP, and incubated with 10 μM [14C]maltose at room temperature for 5 min. The proteoliposomes were then diluted 1:10 with 50 mM KPO4, pH 6.2, 3 mM MgCl2, filtered through a Millipore filter (0.22-μm GSWP), and washed with 5 ml of 50 mM LiCl. Filters were then air-dried and counted in a liquid scintillation counter using Ecolume (ICN) as scintillant.

Spin Labeling—MBPs were labeled with (1-oxo-2,2,5,5-tetramethylylpyrrrolidin-3-yl) methyl methanethiosulfonate spin label (MTSSL) obtained from Renal (Budapest, Hungary). MBP (15 μM) was first incubated for 1 h at room temperature in 20 mM DTT, 10 mM KPO4, pH 8.0. DTT was then removed via extensive dialysis against 10 mM KPO4, pH 8.0, at 4 °C. Spin labeling of MBPs was accomplished by incubating 15 μM protein with 150 μM MTSSL (for D41C and S211C mutants) or 300 μM MTSSL (for D41C/S211C wild type MBP proteins) for 2 h at room temperature in 10 mM KPO4, pH 8.0. Wild type MBP was “mock” spin labeled to ensure that the spin labeling procedure had no significant effect on the activity of the MBP. After the MTSSL labeling, the solutions were dialyzed extensively against 10 mM KPO4, pH 8.0, to remove excess free MTSSL. After dialysis, MBP proteins were placed in 10 mM KPO4, pH 8.0, 15% glycerol, concentrated using Microcon 10 filter units (Amicon, Inc.), and used immediately for EPR experiments or substrate uptake assays. Final protein concentrations were calculated using the UV extinction coefficient of MBP of 1.7 (ε 280) at 280 nm (3) and were between 250 and 400 μM.
expressing either wild type or D41C/S211C MBP grew on both maltose and maltotetraose at approximately the same rate, but neither strain grew on maltotetraitol (data not shown).

**EPR Spectroscopy—**Fig. 2 (left column) shows the EPR spectra, normalized to spin concentration, for unligated and various liganded forms of spin-labeled D41C/S211C double mutant MBP. The increased spin-spin interactions in maltose- or maltotetraose-ligated MBP are seen to have broadened EPR lines, resulting in a reduction in the amplitudes of the first derivative spectra in comparison with that of the unliganded MBP (Fig. 2, a and c). This spectral broadening is more clearly seen in the integrated spectra, which are shown in the right column of Fig. 2 (see a and c). In contrast, the addition of β-cyclodextrin produced little effect (Fig. 2b), and maltotetraol produced an almost identical spectra (Fig. 2d). All of the spectra contained a fast motional component that was less than 5% of the total spin population and was most likely caused by nonspecific spin labeling of impurities in the MBP protein preparation.

Ligand binding did not affect mobilities of the nitroxide side chains, since the addition of maltose, maltotetraose, β-cyclodextrin, or maltotetraitol produced no changes in the EPR spectra for either of the spin-labeled single mutant MBPs (not shown). Therefore, the spectral broadening that occurred upon the addition of maltose and maltotetraose to the spin-labeled double mutant MBP must be the result of an increase in spin-spin interactions.

At room temperature, broadening due to spin-spin interactions is the result of a complex interplay of exchange interactions, dynamic and static magnetic dipolar interactions, and molecular tumbling and is difficult to analyze quantitatively, although several methods aimed at estimating interspin distances at ambient temperatures have been proposed (10, 18, 19). Analysis of the room temperature EPR spectra was also made difficult by the fact that labeling of the MBP was not quite quantitative. The spin-labeled double mutant MBP contained 1.6 ± 0.1 spin labels/protein, and the observed changes in the EPR spectra were due to changes in spin-spin interactions in the fraction (approximately 60%) that was labeled at both sites.

Because of these complications, we did not attempt to analyze the room temperature spectra in a quantitative manner, but the data of Fig. 2 nevertheless suggest the following: 1) binding of maltose and maltotetraose produces an identical EPR change consistent with a significant reduction in interspin distance; 2) binding of maltotetraol and β-cyclodextrin produces EPR changes of a different type, consistent with little reduction in interspin distance.

To determine quantitatively the change in distance between the nitroxides attached to two sites in the D41C/S211C mutant MBP, the EPR spectra at low temperature (140 K) were measured and analyzed using a Fourier deconvolution technique described previously (11). Recording the spectra in frozen so-

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**Table I**

| Kd | Maltose | Maltotetraose | Maltotetraitol | β-Cyclodextrin |
|----|---------|---------------|----------------|---------------|
| μM |
| Wild type | 1 | 1.6 | 7.6 | 1 |
| D41C/S211C | 4 | 4 | 20 | 5 |
| Wild type (MTSSL)* | 1 | ND* | ND | ND |
| D41C (MTSSL) | 1 | ND | ND | ND |
| S211C (MTSSL) | 1 | ND | ND | ND |
| D41C/S211C (MTSSL) | 1 | ND | 15 | <10 |

* Treated with MTSSL.  
* ND, not determined.

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The EPR spectra at low temperature (140 K) were measured and analyzed using a Fourier deconvolution technique described previously (11). Recording the spectra in frozen so-
EPR Study of Maltose-binding Protein Conformation

**Fig. 2. Room temperature EPR spectra of D41C/S211C mutant of MBP with and without ligands.** EPR spectra for the spin-labeled double mutant in the presence of no ligand (thin line) and maltose (thick line) (a), no ligand (thin line) and β-cyclodextrin (thick line) (b), maltotetraose (thin line) and maltose (thick line) (c), and maltodextrin (thin line) and β-cyclodextrin (thick line) (d) are shown. The first derivative spectra are on the left, and the corresponding integrated (absorbance) spectra are on the right. The spectra are normalized to represent the same number of spins. The sweep width is 150 gauss.

**Fig. 3. Low temperature EPR spectra for spin-labeled mutant MBP proteins.** Low temperature spectra for the double mutant in the presence of maltose (thick line) and no ligand (thin line) (a), maltotetraose (thin line) and maltose (thick line) (b), and β-cyclodextrin (thin line), maltodextrin and no ligand (thick lines) (c) are shown. The dashed line in a is the average of the EPR spectra for the spin-labeled single mutants. All of the spectra are normalized to represent the same number of spins.

**Fig. 4. Fourier deconvolution analysis.** A, the low temperature absorbance (integrated) spectrum for the spin-labeled double mutant in the presence of maltose (thick line) and the average of the absorbance spectra for the spin-labeled single mutants (thin line). B, the real part of the broadening function (in Fourier space) obtained when the spectra in A are used as the interacting and noninteracting spectra, respectively. C, same as in A except that the interacting spectrum (thick line) has been modified by subtracting the monoradical component before the deconvolution. D, the real part of the broadening function (in Fourier space) when the spectra in C are used as input. From the dipolar broadening function in B it can be seen that the monoradical fraction is about 40%, as expected from the spin labeling ratio determined by comparing the spin count at room temperature to the protein concentration from UV measurements. The Fourier deconvolution technique has been described (11).

**DISCUSSION**

Intrinsic fluorescence spectra of MBP suggested the possibility that the conformation of MBP is altered in different ways by different ligands (1, 20). This possibility is supported also by UV differential absorbance spectroscopic studies (1, 21). It has thus become clear that maltose and excess linear maltodextrins bind in a way that produce a significant red shift of fluorescence emission spectra and other signature changes in UV absorbance spectra (R mode), whereas reduced, oxidized, or cyclic maltodextrins bind exclusively in a way that produces large blue shifts in fluorescence emission spectra (B mode) (1). Previous 3H NMR study, using maltose and maltodextrins 3H-labeled on the anomeric carbon of the reducing glucose moiety (3), showed the existence of two ligand binding modes, "end-on" and "middle," and we have indicated already the reasons why these two modes are likely to correspond to the R and B modes, respectively (1). Thus the end-on mode, which involves a tight interaction of the anomeric hydrogen with the binding site (3), is utilized only by ligands with the reducing
glucose moiety. In contrast, ligands without reducing glucose residue, and therefore without anomeric hydrogen, such as reduced, oxidized, or cyclic maltodextrins, must use an alternative, middle (or B) binding mode.

These spectroscopic studies clearly showed that ligands can complex with MBP in different ways, but they could not provide any information on the global conformation of MBP. On the other hand, x-ray crystallographic studies have shown that maltose binding causes the N- and C-terminal domains of MBP to come closer together (5, 22). β-Cycloexatin, in contrast, does not cause this conformational change (6). As described in the preceding paper (1), we can hypothesize that R and B modes of binding may generate local conformational alterations of the type seen with the binding of maltose and β-cycloexatin, respectively, a hypothesis substantiated by the present study using EPR spectroscopy.

We measured the distance between the two lobes of MBP. Using a doubly MTSSL-modified D41C/S211C MBP, which was spin-labeled in both the N- and C-terminal domains, we have found that only those ligands that complexed via the physiologically active R mode caused a marked broadening in the EPR spectra (Figs. 2 and 3). This broadening effect is most likely due to stronger spin-spin interactions between the two spin labels and indicates that the two spin labels come closer to each other when MBP binds ligands by this mode. Low temperature EPR measured the change in distance to be from 16.5 to 10.5 Å. Although the crystal structures predict the distance to change from 24 to 15 Å, much of the difference between these two sets of figures is likely to be caused by the fact that the crystallographic distance was measured between α-carbons, whereas the EPR method measured the distance between paramagnetic centers of the spin label, which is located several angstroms away from the α-carbon. We note that in both sets of numbers the maltose-induced decrease was of a similar relative magnitude (36 and 38%). One could argue that the observed broadening and the speculated change in distance are due only to physical perturbation of the spin labels due to ligand binding. However, the fact that neither of the singly MTSSL-labeled MBPs (D41C and S211C) exhibited broadening upon maltose binding argues against this interpretation. It should also be emphasized that the doubly spin-labeled MBP exhibited binding and transport properties very similar to those of the wild type MBP (see Table I and “Results”).

Ligands that bound solely via the physiologically inactive B mode (maltotetraol and β-cycloexatin) caused little additional broadening in the EPR spectra of the doubly labeled MBPs (Figs. 2 and 3), a result suggesting that they did not cause the two spin labels and, therefore, the two domains of MBP to close together. In other words, maltotetraol-bound MBP is in a global conformation similar to the β-cycloexatin-bound MBP, whose structure has been defined by x-ray crystallography (6). However, maltotetraol appeared to cause a very slight broadening in the EPR spectra in comparison with β-cycloexatin (Fig. 3c); it may therefore cause the two lobes of MBP to come together a little. This is consistent with the observation that maltotetraol alters the fluorescence emission and UV differential spectra of MBP in a direction caused by β-cycloexatin, but not as extensively (1). However, we cannot exclude the possibility that these slight changes observed were due to the presence of small amounts of unmodified maltotetraol in the maltotetraol preparation.

This study has made it possible to explain why certain ligands, although they bind to MBP with high affinity, are incapable of being transported across the cytoplasmic membrane. Ligands that bind exclusively by the B or middle mode do not induce the closing of the two lobes of MBP. Possibly only the MBP in the closed conformation can induce, when interacting with the periplasmic surface of the MalFGK2 transporter complex, the signals that allow the MalK ATPase to become active and hydrolyze ATP (15). This possibility is examined in the following paper (23).

The knowledge that the middle mode binding does not cause the closing of the lobes of MBP explains much of the difference observed by fluorescence and UV differential absorbance spectroscopy between various ligands. X-ray crystallographic studies (5, 6, 22) showed that several Trp and Tyr residues enter into stacking interactions with pyranose rings of the ligands. Both β-cycloexatin and maltose interact almost identically with Trp 340 and Tyr 155 residues of MBP (6, 24). However, Trp 62 and Trp 230 interact very differently with ligands, depending on whether the closure of the two lobes takes place. It therefore seems likely that differences in fluorescence emission and UV absorption between the two binding modes are ultimately caused by the different degrees of quenching of these two Trp residues.

Why does the middle mode binding result in MBP remaining in an “open” conformation? Decisive answers to this question obviously require crystallographic studies. However, a possible scenario can be proposed. In the β-cycloexatin-MBP complex, three consecutive glucose residues of the ligand, labeled as g1, g2, and g3 in Fig. 1 of Ref. 5, interact tightly with the binding site of MBP. It is thus clear that there are at least three monosaccharide-binding sites, which we may call sites 1–3. Maltotetraol, for example, may be expected to bind so that the three glucose residues of this ligand bind, respectively, to sites 1–3 to maximize the stabilization. This, however, will leave the linear, glucitol moiety unbound at the head, which may produce steric hindrance for the closing of the two lobes, just as the rest of the β-cycloexatin molecule, including the g7 residue presumably at the position corresponding to glucitol in maltotetraol, may act in preventing the closing of the lobes (6).

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