Ligands that are transported by the maltose transport system of *Escherichia coli* must first bind to the periplasmic maltose-binding protein (MBP). However, binding of a ligand does not always lead to its transport. As reported earlier, reduced or oxidized maltodextrins bind tightly to MBP but are not transported; some mutant MBPs, such as MalE254, bind maltodextrins tightly but cannot produce their transport. In this study, UV differential spectroscopy and fluorescence emission spectroscopy were used to study the modes by which various ligands bind to MBP. Maltose binding produced a red shift in the fluorescence emission spectrum of wild type MBP and a sharp hypochromic trend below 256 nm in its UV spectrum (R mode (for red)). On the other hand, binding of reduced, oxidized, or cyclic maltodextrins produced a pronounced blue shift in the fluorescence emission spectrum of wild type MBP and a peak at about 250 nm in its UV difference spectrum (B mode (for blue)). Binding of reducing maltodextrins to wild type MBP produced spectral changes that seemed to be a mixture of predominantly R mode binding and some B mode binding, whereas their binding to mutant MBP MalE254 produced changes indicative of pure B mode binding. Thus, the ligands that are bound exclusively via the B mode to either the wild type or MalE254 MBP are not transported.

Maltose and its higher homologs, the maltodextrins, are actively transported across the cytoplasmic membrane of *Escherichia coli* via a high affinity, periplasmic binding protein-dependent transport system (reviewed in Ref. 1). The periplasmic component of this transport system, the maltose-binding protein (MBP), is unusual in that it recognizes and tightly binds not only a disaccharide maltose, but also longer, linear maltodextrins (1). Ferenci et al. (4) showed that when the reducing glucose unit of linear maltodextrins are reduced, oxidized, or substituted, they were not transported into the cells, although they bound to MBP with good affinity. Similarly, “maltodextrin-negative” mutants of MBP show only a marginal decrease in affinity toward maltodextrins, yet they do not support the transport of maltodextrins in whole cells (5).

Because the ligand-binding site of MBP is exceptionally rich in aromatic amino acid residues (6), binding induces major changes in both the fluorescence (2) and UV absorption (7) spectra of MBP. Interestingly, it has long been known that maltose binding produces a red shift of the fluorescence emission spectrum of MBP, whereas binding of the cyclic maltodextrin (β-cyclodextrin) produces a pronounced blue shift (2). Similarly, binding of linear and cyclic maltodextrins produced different changes in the UV absorption spectra of MBP (7). In this study, we examined the binding of various ligands to MBP by fluorescence and UV differential absorbance spectroscopy, to understand why certain ligands are bound tightly to MBP but are not transported. The results show that there are two ways in which a ligand can bind to MBP, one causing a red shift of fluorescence emission spectrum of MBP and a hypochromic change in the region below 265 nm of its UV absorption spectrum (R mode, for red) and another causing a blue shift of the fluorescence emission spectrum of MBP and a peak around 250 nm of its UV difference spectrum (B mode, for blue). We found that those ligands that bound to MBP exclusively via the B mode all failed to be transported.

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

**CORRELATION WITH THE STRUCTURE OF LIGANDS AND THE STRUCTURE OF BINDING PROTEIN**

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**EXPERIMENTAL PROCEDURES**

Cloning and Sequencing—The entire coding region of the *malE254* allele was sequenced. It was amplified by polymerase chain reaction using genomic DNA of strain pop1153 (K-12 Hfr his malE254) (5) as template and two 20-mer oligonucleotide primers that bound to sites just outside the unique *Stu*I and *Eco*RI restriction sites. Three independently derived polymerase chain reaction products were then subcloned into pBluescript S/K (+ and −) (Stratagene Cloning Systems) by ligation between the unique *Hinc*II and *Eco*RI sites. Single-stranded DNA was obtained by using M13KO7 helper phage (Stratagene Cloning Systems) and used as template for sequencing using the Sequenase version 2.0 DNA sequencing kit (U.S. Biochemical Corp.). One of the recombinant plasmids, pEH1 (pBluescript S/K + with *malE254* insert), was used for subsequent *malE254* MBP expression.

Preparation of MBP—Wild type and *malE254* MBPs were prepared from strain HS2019 (K-12 F *araD13 lacU169 rpsL thi DmalE444*) (8) containing *pFD1* (*malE* blb+) (9) and from strain pop1153 or strain HS2019 containing *pEH1*, respectively. The strains were grown at 37 °C in LB broth (1% Bacto-tryptone, 0.5% Bacto-yeast extract, and 0.5% NaCl) containing 0.4% maltose and 100 μg/ml ampicillin when needed. MBP was isolated from osmotic shock fluids by affinity chromatography (10). Wild type MBP and *malE254* MBP were eluted from the column by the addition of 10 and 500 mM maltose, respectively. MBP, precipitated with (NH₄)₂SO₄ (90% saturation), was then dialyzed against 10 mM potassium phosphate buffer, pH 7.0, and bound maltose was removed either by extensive dialysis (11) or by denaturation in 6 M guanidine-HCl followed by renaturation through dialysis. Protein concentrations were calculated using the UV extinction coefficient of MBP of 1.7 (ε₉₅₀ nm) at 280 nm (12).
Sugars and Sugar Analogs—Maltose (99% pure, purchased from Calbiochem), maltitol, maltotriose, maltotetraose, maltohexaose, and $\beta$-cyclodextrin (purchased from Sigma) were used without further purification. Maltotetraose and maltohexaose were converted to their glucitol derivatives via borohydride reduction (13). Maltohexaonic acid was prepared by the oxidation of maltohexaose (14). Modifications were apparently complete as judged by the absence of reducing maltooligosaccharides on thin layer chromatography and the inability of the modified products to reduce ferricyanide reagent (15).

**RESULTS**

**Fluorescence Emission Spectra of MBP**—The emission maximum of wild type MBP occurs at 348 nm. The addition of various ligands altered the intrinsic fluorescence of this protein. Maltose induced a 2.5-nm red shift, whereas $\beta$-cyclodextrin caused a 6-nm blue shift (Fig. 1A), as reported earlier (2). These changes can be taken as signatures for the two modes of ligand binding, the R and B modes. Maltoheptaose induces a slight blue shift (2). We found that both maltotetraose and maltohexaose produced very small blue shifts (0.5–1 nm), while maltotriose caused a slight red shift (0.5 nm) (Fig. 1B and data not shown). These data suggest that both the R and B binding modes are utilized by the maltodextrins and that the R mode was important with shorter maltodextrins such as maltotetraose and under our experimental conditions that included the presence of excess ligands (see Ref. 12). All ligands also induced a 5–10% quenching of the fluorescence as well.

Nonreducing derivatives of linear maltodextrins, i.e. maltotetraitol, maltohexaitol, and maltohexaonic acid, caused marked blue shifts (2, 4, and 5 nm, respectively) and induced a 5–10% quenching (Fig. 1B), a result suggesting that these derivatives bind to MBP predominantly via the B mode. Maltitol produced only a small blue shift (0.5 nm) and a 3% quenching (data not shown).

The *malE254* allele was isolated during a search for mutants that were able to grow on maltose but not on longer maltodextrins (5). This and other “maltodextrin-negative” *malE* mutant(s), producing altered MBP, are able to grow on maltose but not on maltodextrins, although their MBPs have poor affinity.

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2 J. A. Hall, A. K. Ganesan, and H. Nikaido, unpublished results.
for maltose and good affinity for maltodextrins. With the malE254 MBP, $K_D$ values for maltodextrins are between 10 and 40 $\mu M$, while those for maltotriose and maltose are approximately 300 $\mu M$ and 2.0 $\mu M$, respectively (5). This phenotype has remained an enigma until now. The emission spectrum of the malE254 MBP had a maximum at 346 nm (Fig. 1C), showing a 2-nm blue shift in comparison with wild type MBP. As was the case with the wild type protein, the malE254 MBP emission spectrum was red-shifted by 2 nm by maltose and blue-shifted by approximately 3.5 nm by $\beta$-cyclodextrin (Fig. 1C). The non-reducing derivatives of maltodextrin (maltotetraitol, maltohexaose, maltohexaitol, and maltohexaonic acid) similarly produced marked blue shifts (ranging from 3 to 4 nm) (Fig. 1D and data not shown). However, unmodified maltodextrins (maltotriose, maltotetraose, and maltohexaose), which caused only marginal blue shifts in the wild type MBP (Fig. 1B), produced large blue shifts with the mutant MBP (Fig. 1D and results not shown). These observations suggest that the mutant MBP binds both reducing and nonreducing maltodextrin derivatives only in the B mode. Maltitol caused a slight red shift of 0.5 nm but caused no observable fluorescence quenching (data not shown), in contrast to all other ligands that produced approximately 5% quenching.

**UV Difference Spectra of Wild Type MBP**—The UV absorbance spectrum of wild type MBP was altered upon binding of maltose, maltotriose, and $\beta$-cyclodextrin (Fig. 2A). The difference spectra were very similar to those reported previously (7). The first derivatives were also calculated (Fig. 2B). The spectra differed mainly in two regions: below 265 nm and between 310 and 280 nm.

In the region below 265 nm, both maltose and maltotriose (Fig. 2A) caused sharp hypochromatic trends. In contrast, the difference spectrum of $\beta$-cyclodextrin lacked this hypochromatic trend and instead had a peak at 250 nm (Fig. 2A). This feature was also seen clearly in the first derivative spectra (Fig. 2B) and resulted in the curve crossing the x axis at 250 nm in the spectrum of $\beta$-cyclodextrin. In this region, difference curves generated in the presence of maltodextrin derivatives (maltotetraitol, maltohexaitol, and maltohexaonic acid) were drastically different from those of unmodified maltodextrins, showing a peak around 255 nm (or a crossing of the x axis near 255 nm in the first derivatives (Fig. 2B)) much like that found with $\beta$-cyclodextrin.

The region between 310 and 280 nm is quite complex and is best compared in the first derivative spectra. With maltose, the first derivative had peaks at 310, 294, and 286 nm (Fig. 2B). The latter two peaks appeared to have been blue-shifted in the presence of maltotriose to 291 and 282 nm (Fig. 2B). With $\beta$-cyclodextrin, the 294-nm peak was blue-shifted even more (to 290 nm), and a new peak appeared at 296 nm. There was also a marked flattening (and an apparent red shift) of the peak located close to 310 nm (Fig. 2B). Maltotetraitol, maltohexaitol, and maltohexaonic acid produced first derivative curves that had many of the signatures of the $\beta$-cyclodextrin spectrum, such as the prominent peak around 290–291 nm, the additional shoulder around 296 nm, and also a characteristic flattening of the 310-nm peak (Fig. 2B).

The binding of ligands thus caused characteristic changes in the UV difference spectra. Those ligands that bind predominantly via the R mode, such as maltose and maltodextrins, produced hypochromatic changes in the region below 265 nm, and also characteristic changes in the region between 280 and 310 nm. In contrast, ligands that bind exclusively via the B mode, such as reduced, oxidized, or cyclic maltodextrins, produced a peak around 250–255 nm without the hypochromatic changes in the region below 265 nm, as well as another set of characteristic changes in the region between 280 and 310 nm.

The addition of maltitol to MBP produced a difference curve that was, for the most part, featureless (data not shown). A possible reason may be that maltitol cannot interact effectively with MBP either via the R or the B mode. Indeed, the $K_D$ of maltitol is $\sim$50 $\mu M$, while that of maltose is 1 $\mu M$ (4).

**UV Difference Spectra of malE254 MBP**—The difference spectra generated for maltose and $\beta$-cyclodextrin bound to this mutant MBP were similar to those with the wild type MBP (Fig. 3A), although in the region between 310 and 290 nm the peaks were slightly blue-shifted (Fig. 3B). When the nonreducing derivatives of maltodextrins were added, the changes were similar to those seen with the wild type MBP and included the 282- and 290-nm peaks as well as the 296-nm shoulder and the flattening of 307 nm peak, the latter two features reminiscent of the $\beta$-cyclodextrin spectrum (Fig. 3B). However, in contrast to the situation with wild type MBP, practically the same spectra of B type binding were produced by the addition of reducing maltodextrins, such as maltotriose (Fig. 3). These results suggest that the malE254 MBP binds reducing maltodextrins exclusively in the B mode, although it can bind maltose (at high concentrations) in the R mode.
Two Ligand-binding Modes of E. coli Maltose-binding Protein

Fig. 3. UV difference spectra of malE254 MBP. Ultraviolet absorbance spectra of malE254 MBP bound to various ligands were determined, and the spectrum of the free protein was subtracted from them to generate the difference spectra. A, difference spectra generated by the binding of maltose (---), maltohexaose (- - -), and β-cyclodextrin (——). B, in the center, the first derivatives of the difference spectra for maltose, maltohexaose, and β-cyclodextrin are shown, with the line types identical to those used in A. The first derivatives of the difference spectra generated by the addition of maltobiose (top) and maltotetraose (bottom) are also shown, with vertical displacements to avoid cluttering. For other conditions, see the legend to Fig. 2.

Sequence of the malE254 Allele—DNA sequencing showed that the malE254 allele contained a single base substitution, a G-C to A-T transition, which replaced an aspartic acid at position 65 of the mature protein with an asparagine (GAC to AAC). Asp65 lies within the binding pocket of MBP and makes direct contacts with both maltose and β-cyclodextrin (6, 17). In the case of maltose, Asp65 makes strong hydrogen bond contacts with the hydroxyl groups at the 2- and 3-positions of the nonreducing glucose unit (18). The phenotype of the MalE254 MBP will be examined in more detail under "Discussion."

DISCUSSION

An interesting feature of the maltose transport system is that it transports not only maltose, a disaccharide, but also maltodextrins at least up to maltoheptaose (1). Previous studies showed that the binding of various ligands to MBP resulted in different changes in its intrinsic fluorescence emission spectra (2) and UV absorption spectra (7). As described in the Introduction, we define the binding mode that produces a red shift in the fluorescence emission spectrum and a hypochromatic effect in the UV absorption spectrum below 265 nm as the "R mode," and the one producing a blue shift and a 250-nm peak, respectively, as the "B mode." In this study we examined the binding of those ligands that are not transported into the cytoplasm. Previously it was observed that nonreducing derivatives of maltodextrins, such as reduced or oxidized maltodextrins, or methyl glycosides, were often bound by MBP with high affinity but were not transported (4). Since these derivatives were recognized by LamB, the outer membrane channel for maltodextrins, and inhibited the transport of maltose (4), they apparently had little difficulty in reaching the periplasm. When we examined the mode of binding of these derivatives, we found that both reduced and oxidized derivatives of maltodextrins bound to MBP nearly exclusively via the B mode. Thus, both the UV difference and fluorescence spectra of wild type MBP bound to these derivatives were very similar to those of the β-cyclodextrin-MBP complex. In contrast, binding of unmodified maltodextrins to wild type MBP produced spectra between those of MBP-maltose and MBP-β-cyclodextrin complexes, suggesting that they bound partly via the R mode and partly via the B mode (Figs. 1 and 2). Especially with UV difference spectra, the R mode seemed to predominate, since there were only very small differences between the spectra of maltose-MBP and maltohexaose-MBP complexes in the region below 265 nm (Fig. 2A). These results thus suggest that those substrates that are successfully transported bind to MBP at least partly via the R mode, whereas substrates that bind exclusively by the B mode are not transported. The latter is thus a physiologically inactive mode of binding.

This conclusion was reinforced by the study of the malE254 mutant MBP. Mutants of this class (5) can transport maltose but not maltodextrins. Yet this mutant MBP binds maltose rather poorly and paradoxically binds maltodextrins with good affinity (5). These results can now be explained if the mutant MBP binds maltodextrins always in the inactive, B mode. We found indeed that the malE254 MBP bound maltodextrins (and their derivatives) exclusively by the B mode (Figs. 1D and 3). The mutant MBP, however, appeared to bind maltose in the R mode when maltose was present at high concentrations (Fig. 3). The present results thus solve the long-standing puzzle that this mutant MBP was incapable of producing an active transport of maltodextrins despite its high affinity to them (5); the overall binding affinity of ligands to MBP may be misleading, since what matters is only the binding through the R mode.

We have so far defined the R and B modes of binding operationally. However, two modes of ligand binding to MBP have been described also by a method that allows some insight into the molecular details. Thus, 3H NMR studies using ligands tritiated on the anomeric carbon of the reducing glucose residue showed that maltose binds to MBP only in a way causing a large upfield shift of the anomeric tritium resonance, whereas maltodextrins bind to MBP in this way as well as in a different manner causing only a small upfield shift of this resonance (12). Furthermore, only the former mode has a strong preference for α-anomers. Thus, the former mode seems to involve strong interaction of the anomeric group of the reducing glucose with a group at the binding site, whereas such an interaction is not important in the latter mode. These two binding modes were therefore called "end-on" and "middle" binding modes (12). (However, "end-on" does not mean that nonreducing sugar moieties do not interact strongly with the binding site, since the only atom that was observed by NMR was the tritium on anomeric carbon). Maltose thus uses the end-on mode exclusively. In contrast, at low ligand:protein ratios, after all of the α-maltodextrin molecules have become bound through the high affinity, end-on binding, the remaining unliganded MBP molecules bind β-anomers through the middle mode (12). The use of these two modes by maltose and maltodextrins
leading to an active site substitution of Asp 65 for Asn. In the
sembling those of
ably the middle) mode exclusively (see above), produced both
and oxidized maltodextrins, which utilize the B (and presum-
to prevent the closing of the two domains (17). Since reduced
mains close together (6, 19); these are ligands that preferen-
tively, closed and open forms of the MBP-ligand complex.

ty.3 It also interacts with one of the glucose residues (called g3)
in the MBP-β-cyclodextrin complex (17). If we assume, as de-
cribed above, that the R and B modes correspond to closed and
open global conformations of MBP, the behavior of this muta-
tion will thus hinder the end-on binding severely, without
affecting much the middle binding, precisely the result that has
been observed earlier (5) as well as in this study.

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3 Although the high resolution coordinates of maltose-ligated wild type MBP are not accessible, those of maltose-ligated MalE175 mut-
tant MBP, which has mutations far away from the binding site and has
essentially unaltered binding and transport properties, are available
(1MDP, Brookhaven Protein Data Bank).