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UV difference spectroscopy of ligand binding to maltose-binding protein

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We have used UV absorbance spectroscopy to study the binding of linear and circular maltodextrins to maltose-binding protein (MBP). Titrations with maltose yield three isosbestic points in the difference spectrum of MBP, consistent with two protein conformations: ligand-free and ligand-bound. In contrast, titrations with maltotetraose reveal three conformations: ligand-free, a low-affinity liganded state, and a high affinity liganded state. These results confirm and extend the results from tritium NMR spectroscopy, namely, that MBP can bind maltodextrin either by the sugar's anomeric end (high affinity) or by the middle of the maltodextrin chain (low affinity).

Maltose; Maltodextrin; UV difference spectroscopy; Anomer specificity; Periplasmic binding protein

1. INTRODUCTION

Maltose-binding protein (MBP) is a periplasmic protein from *Escherichia coli* involved in transport and chemotaxis towards maltose and maltodextrins [1]. Purified MBP binds both linear and circular maltodextrin molecules (α(1,4)-glucose polymers) with high affinity (K_D = ~ 1 μM) and a stoichiometry of one ligand per MBP [2]. The 2.4 Å X-ray crystal structure of MBP shows two folded domains connected by a hinge region with the substrate binding site located between the two domains [3].

Upon ligand binding, MBP undergoes a conformational change. This change was first observed by fluorescence emission spectroscopy [4] but definitive proof required fluorescence energy transfer experiments with fluorescently derivatized protein [5]. The nature of this conformational change was partially clarified by small-angle neutron scattering experiments with arabinose binding protein (a similarly structured protein) that suggested that the two domains come together during ligand binding in what was colorfully termed the "Venus flytrap" model [6,7].

Tritium NMR spectroscopy with labeled maltodextrins has shown that MBP recognizes the ligand's anomeric configuration, binding α-anomers with much higher affinity than β-anomers [8]. The degree of anomeric specificity depends on the length of the maltodextrin. In addition, two distinct tritium NMR resonances were detected for intermediate length β-maltodextrins bound to MBP. These were interpreted to arise from sugar–protein complexes with the ligand bound either by its reducing end ('end-on binding') or by its middle ('middle binding').

We confirm these conclusions by using UV absorbance difference spectroscopy. Linear and circular maltodextrins, upon addition to MBP, induce specific and characteristic changes in the protein's near UV absorption spectrum that can be used to distinguish between the two types of ligand–MBP complexes. We find that addition of maltose (two glucose residues) to MBP results in only one ligand–MBP complex while addition of maltotetraose (four glucose residues) results in two complexes: end-on-binding and middle-binding.

2. MATERIALS AND METHODS

Maltodextrins, linear α(1,4) glucopyranose polymers ranging from maltose (two glucose residues) to maltoheptaose (seven glucose residues) and circular (β-cyclodextrin, seven glucose residues) were purchased from Sigma Chemical Co. and used without further purification. MBP (maltose-binding protein) was purified as described [8] using the cross-linked amylose affinity column of Ferenci and Klotz [9]. To remove bound maltose, MBP was extensively dialyzed as described by Silhavy et al. [10]. The UV extinction coefficient of MBP used to calculate protein concentrations was 1.7 ($\varepsilon^{1\%}_{1\,cm}$) at 280 nm [8].

UV spectroscopy was carried out on a Perkin-Elmer Lambda 2 spectrometer and a Shimadzu UV-2000 spectrometer. For difference spectra, MBP (OD ~ 1.280 nm) in 20 mM potassium phosphate buffer, pH 7.4, was added to both sample and reference cuvettes and the baseline correction procedure of the spectrometer run. Titrations were made by successively adding 5 μl of a maltodextrin solution (1 mM) containing MBP to the sample cuvette (1 ml). The presence of MBP in the stock maltodextrin solution eliminated any dilution of MBP in

Abbreviations: MBP, maltose-binding protein; NMR, nuclear magnetic resonance; UV, ultraviolet.

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the sample cuvette. Titrations were always started by a blank (no addition) spectrum that was subtracted from subsequent spectra. Spectra were acquired at ambient temperature with the slowest possible scan speeds (20 nm/min, Perkin-Elmer; ≈ 12.5 nm/min, Shimadzu) and either 1 or 2 nm monochromator slit widths.

The computer fitting of the maltotetraose titration was carried out by a series of Fortran programs on a Silicon Graphics Personal Iris workstation. Optimization of the fitting parameters was achieved by minimizing an error function using a descending simplex algorithm [11]. The error function was calculated by comparing the six observed spectra with simulated spectra based on the calculated concentrations of bound α- and β-maltotetraose and on model spectra for the α- and β-maltotetraose–MBP complexes. The observed difference spectra were digitized every 0.5 nm between 240.5 and 340 nm with a limiting resolution of 5×10^{-5} absorbance units.

In the simplest fitting, 10 parameters were varied: a constant (frequency independent) offset for each observed spectrum (6 parameters), and the composition of the two model spectra (4 parameters). Each model spectrum was calculated as a linear combination of two observed spectra: one before saturation of the available MBP sites (15 μM addition) and one after complete saturation (30 μM addition). The amount of bound α- and β-maltotetraose was calculated analytically from the amount of maltodextrin added, the equilibrium solution (input) α/β ratio, the concentration of MBP, and the dissociation constants for α- and β-maltotetraose. In later fittings, more parameters were added to the minimization. The input α/β ratio, the MBP concentration, and the dissociation constants were allowed to vary; a linear (frequency dependent) offset for each observed spectrum was added (to account for non-specific scatter). And, finally, the model spectra were calculated as linear combinations of all observed spectra in order to reduce the effects of noise or other bias in the individual spectra.

3. RESULTS

3.1. *Difference spectra of MBP*

The UV absorbance spectrum of maltose-binding protein (MBP) showed a beautiful set of difference curves when maltodextrins were added (Fig. 1). The absorbance changes were saturable and highly reproducible (Fig. 2). Comparison of the amount of maltodextrin added and of the amount of MBP present was consistent with a one-to-one stoichiometry of binding (Fig. 2).

For the purpose of comparing the spectra, it was convenient to divide the spectra into three regions: above 280 nm, 280 nm to 265 nm, and below 265 nm. The most dramatic differences between the spectra obtained with three substrates, maltose, maltoheptaose, and cyclic maltoheptoase, occurred above 280 nm (Fig. 1). As discussed below, the fractional changes relative to absolute absorbance were also greatest in this region. It is possible that the movement of individual chromophores may be responsible for the numerous peaks and valleys observed. MBP contains 8 tryptophan residues that are responsible for the majority of the protein absorbance, particularly above 290 nm. Between 265 nm and 280 nm, addition of the three substrates generated essentially identical difference spectra. Maltoheptaose produced a small vertical offset but like the other maltodextrins showed an identical pattern of three small peaks at 278 nm, 273 nm, and at 265 nm.

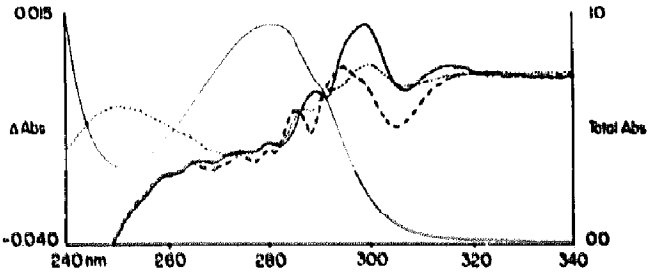

Fig. 1. UV difference spectra of maltose-binding protein (MBP). The ultraviolet absorbance spectrum of MBP (16 μM, thin line, right hand scale) is compared to the difference spectra after addition of 60 μM maltose (heavy line), maltoheptaose (dashed line) or β-cyclodextrin (cyclomaltoheptaose, dotted line). The difference spectra were aligned (zeroed) at 320 and expanded 19-fold (left hand scale). Buffer: 10 mM Tris·Cl, pH 7.5, 20°C.

In the third region, below 265 nm, the spectrum of MBP in the presence of cyclic maltodextrin departs strongly from that in the presence of linear maltodextrins. There was little evidence for fine structure in this region of the spectrum.

The difference spectra of saturating concentrations of intermediate length maltodextrins between maltose and maltoheptaose were also examined (Fig. 3 and data not shown). The difference curves for maltotetraose and maltopentaose were very similar to the difference curve for maltoheptaose. The difference curve for maltotriose was intermediate between maltose and maltoheptaose but more similar to maltoheptaose. This situation is similar to changes in the fluorescence emission spectrum of MBP measured upon the binding of intermediate length maltodextrins [4].

One of the most notable features of these difference spectra was the large relative amplitudes between 300 and 320 nm. The maltose difference spectrum shows a small peak of hyperchromicity at 316 nm where the MBP absorbance spectrum has less than 2% of its peak 280 nm absorbance. The change at this wavelength corresponds to 50% increase in absorbance. This most likely represents a red shift in the absorption spectrum of the most red-shifted tryptophan. Movement of an aromatic residue into a more hydrophobic environment can lead to a red shift of its absorption spectrum [12,13]. Similarly, the maltoheptaose difference spectrum shows a peak of hypochromicity at 304–305 nm where MBP has only 7% of its peak 280 nm absorbance. The change at this wavelength corresponds to almost a 20% decrease in absorbance, possibly due to movement of a tryptophan into a more hydrophilic environment.

At shorter wavelengths, all the linear maltodextrins generated, upon addition to MBP, a marked hypochromicity around 250 nm. For maltose, this corresponded to a decrease in relative absorbance of 14% at 247 nm. As is clear from the vertical axis in Fig. 1, the total absorbance change of MBP (measured between 340 and

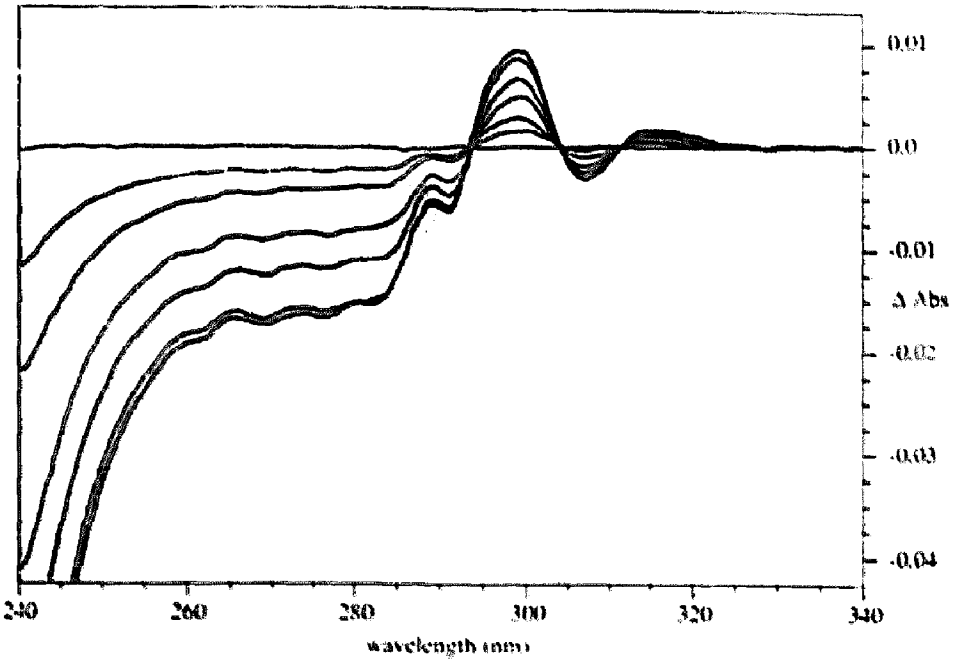

Fig. 2. Titration of the UV difference spectra by maltose additions to maltose-binding protein. Isosbestic points are present at 311, 304 and 294 nm. Additions to MBP (13 μM) were carried out as described in Section 2 to obtain final concentrations of 2, 4, 8, 12, 24 and 44 μM maltose. Small offsets (−0.5 to −1.3 mAbs) were added to align the spectra at 340 nm and a blank spectrum (difference of two no-addition spectra) shown for reference. Buffer: 20 mM K_2PO_4, pH 7.4, 20°C.

240 nm) was not conserved. In all cases, the addition of ligand caused a general decrease in absorption.

3.2. *Titration of maltose*

Titration of maltose into MBP yielded three isosbestic points consistent with a titration between two conformations: maltose-free and maltose-liganded MBP (Fig. 2). The difference spectrum was saturable and additions after saturation had no measurable effect. In principle, both the dissociation constant and protein-ligand stoichiometry can be calculated from the titration curves (see e.g. [14]). In practice, however, the high affinity of maltose (relative to the MBP concentration) makes determination of the dissociation constant relatively inaccurate. This problem might be overcome by the use of 10 cm path length cuvettes.

3.3. *Titration of maltotetraose*

Titration of maltotetraose did not yield any isosbestic points. By eye, the spectra appear to represent a mixture of several absorbing species in varying ratios (Fig. 3). The nature of this mixture became clear after NMR experiments showed that α- and β-maltodextrins bind with very different affinities and that they form different types of bound complexes. [8]. Since linear maltodextrins exist in solution as two anomers, α and β, a titration with maltotetraose necessarily contains three components: ligand free MBP, α-maltodextrin–MBP, and β-maltodextrin–MBP. In the case of maltose, because the dissociation constants of α- and β-maltose are similar, the ratio of α and β complexes remains constant throughout the titration. (It is also probable that difference spectra of the two complexes, α- and β-maltose–MBP, are similar.)

In contrast, because the dissociation constants of α- and β-maltotetraose differ by a factor of at least 10 [8], the ratio of the α and β complexes does not remain constant throughout the titration and three components are required to fit the observed spectra. In order to extract the two difference spectra of α-maltodextrin–MBP and β-maltodextrin–MBP, a Fortran computer program was written to fit the observed spectra. As described in Section 2, a descending simplex method was used. The results of a representative fitting are shown in Fig. 4. In the upper panel the two model difference spectra are shown α- and β-maltotetraose bound to MBP along with the 10-fold expanded residual differences between the observed and simulated spectra.

A large number of different simulations were carried out in order to determine the robustness and uniqueness of the fitting. Different degrees of freedom were included (e.g. fixed or variable dissociation constants, fixed or variable input α/β ratios, etc) to assess each parameter's relative importance and to avoid problems with local minima. From these studies, we conclude that: (i) certain parameters and the general shape of the model spectra were well determined (identical in all fittings), (ii) certain parameters were inter-related which

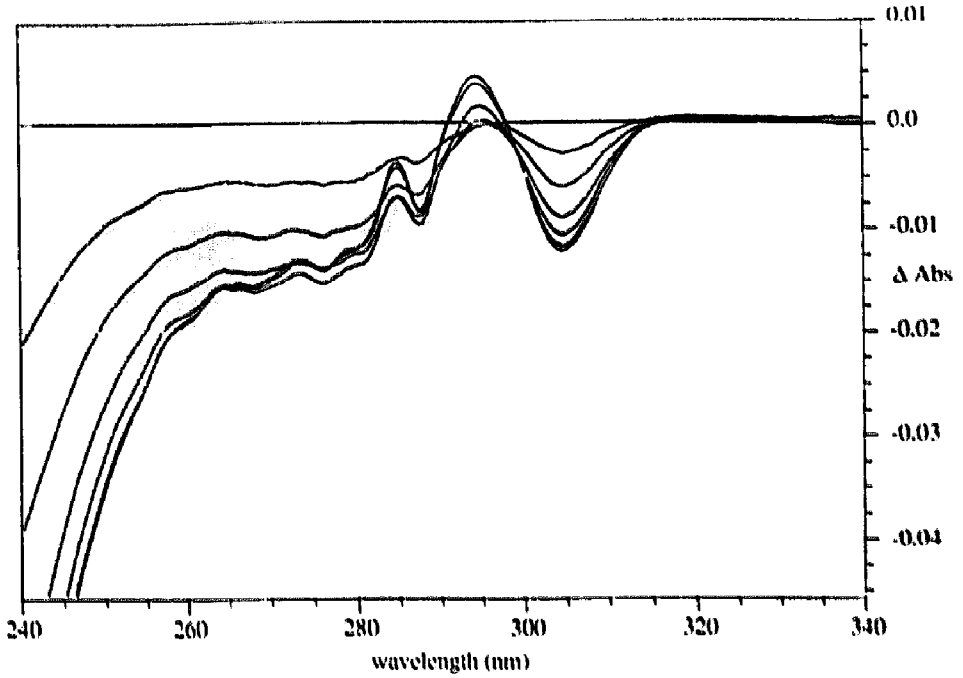

Fig. 3. Titration of the UV difference spectra by maltotetraose additions to maltose-binding protein. Additions were made to MBP (16.4 μM) to obtain final concentrations of 5, 10, 15, 20, 25 and 30 μM maltotetraose. The spectra were aligned according to the computer fit in Fig. 4 and a line at zero absorbance drawn for reference. Buffer: 20 mM K_2PO_4, pH 7.4, 20°C.

precluded their simultaneous fitting, and (iii) other parameters were under-determined. As an example of the last category, the binding affinities of α- and β-maltotetraose were poorly defined because of the high MBP concentration (see Section 3.2). On the other hand, the same zero offsets of the spectra were consistently found in all fittings. Of particular interest were the parameters that were inter-related. For example, the relative amplitude of the model beta spectrum depended on the MBP concentration. In the majority of fittings, the MBP concentration was fixed at 16.4 μM.

The most serious limitation of the computer fitting was the inability to account for anomeric inter-conversion during acquisition of the spectra. Because of the higher affinity of MBP for α-maltotetraose, the presence of MBP shifts the equilibrium α/β ratio from its solution value of ≈ 0.5 to values of 5 or more [8]. Using D-glucose as a guide, we estimate that the rate constant for inter-conversion of maltodextrins in our experimental conditions to be approximately 40 min. This was reflected in the titration fitting by a shift in the free input α/β ratio from 0.5 to 2.0 in the best fittings. Fixing the input α/β ratio to 0.5 did not significantly change the shape of the model difference spectra but did almost double the root-mean-squared sum of error residuals.

Comparison of the model spectra with those of Fig. 1, reveals a striking similarity between the model β-maltotetraose–MBP spectrum and the β-cyclodextrin–MBP (cyclomaltoheptaose) spectrum. Between 270 and 310 nm, both spectra show an identical pattern of rounded peaks descending to a broad valley (negative peak) at 275 nm. The similarity is even more pronounced around 250 nm where both spectra present a broad peak. The presence of this peak is the most characteristic difference between the difference spectra of the cyclic and linear maltodextrins.

The model α-maltotetraose–MBP spectrum resembles the last experimental curve in the titration with maltotetraose (Fig. 3), i.e. the curve obtained with a large excess of ligand. This is a natural consequence of the high affinity of the α anomer since late in the titration, addition of free α-maltotetraose displaces any bound β-maltotetraose. As described above, the maltotetraose and maltoheptaose difference curves at saturating concentrations are quite similar.

4. DISCUSSION

We have studied changes in the UV absorbance spectrum of MBP upon ligand binding. Our results are a strong confirmation of the model of maltodextrin binding to MBP presented previously [8]. The conclusion of this NMR study was that maltodextrins longer than maltose could bind to MBP in two different fashions: either with the maltodextrin reducing end in the MBP binding pocket or with the middle glucose residue(s) occupying this site. The balance between these two binding modes was seen to depend on the length of the maltodextrin and on the configuration of the reducing end (α or β). While intermediate length

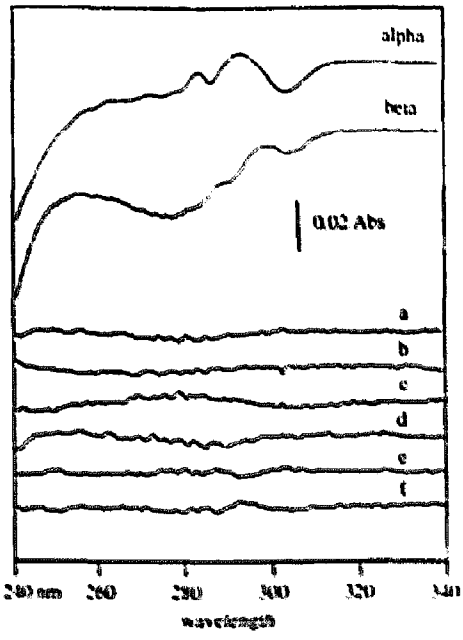

Fig. 4. Calculated UV difference spectra for α-maltotetraose and β-maltotetraose. Computer fitting of the maltotetraose titration shown in Fig. 3 yielded two model spectra: one for a high affinity form (*alpha*) and one for a low affinity form (*beta*). The remaining error residuals after fitting of observed spectra are shown below the model spectra in order of increasing maltotetraose concentration (a–f). Spectra are plotted on the same vertical scale (enlarged 10-fold for the residual spectra) and offset for comparison. The computer fitting calculated a K_D of 0.005 μM for α-maltotetraose, 3.17 μM for β-maltotetraose, and an input α/β ratio of 2.0. The MBP concentration was fixed at 16.4 μM.

β-maltodextrins bound in both modes. α-maltodextrins up to maltohexaose bound exclusively 'end-on' due to the much higher affinity of MBP for such complexes.

In complement to the NMR study which observed ^{1}H-labeled ligands, in the present study, we have studied MBP-ligand complexes by observing the endogenous aromatic reporter groups in MBP. As first observed by NMR, we confirm that the complex titration behavior of maltotetraose is due to the presence of two components: a low-affinity and high-affinity ligand. Based on the NMR work, we assign these to β- and α-maltotetraose, respectively. It was previously proposed that cyclic maltodextrins must bind by the 'middle' binding mode because they lack a terminal residue for 'end-on' binding [8]. The clear similarity between the β-maltotetraose and β-cyclodextrin difference spectra confirms this hypothesis.

The model β-maltotetraose difference spectrum should, in fact, represent contributions from both 'end-on' and 'middle' binding. NMR of maltotetraose–MBP complexes measured approximately a 2:1 ratio of 'middle' to 'end-on' binding for the β anomer. Some of the minor differences between the β-maltotetraose and β-cyclodextrin difference spectra may be due to the contribution from 'end-on' binding. We note also that the form of the β-maltotetraose difference spectrum is completely different from the maltose difference spectrum which rules out the presence of endogenous maltose as a trivial explanation for the complex maltotetraose titration behavior.

Despite the richness of UV absorbance difference spectroscopy compared to fluorescence emission spectroscopy, it has not been frequently used in the study of periplasmic binding proteins (see however [15,16]). In particular, UV absorbance difference spectroscopy should have a clear advantage in the study of the MBP tryptophan mutant proteins [17]. Such a study would allow better insight into the roles of the different tryptophan residues in ligand binding and might allow the assignment of some of the transitions observed. In this regard, the additional dimension provided by circular dichroism spectroscopy might be helpful.

Lastly, one wonders about the physiological relevance for the two modes of maltodextrin binding. Does middle binding occur with the presumed conformational changes in MBP that permit it to interact with the other protein components in maltose transport and chemotaxis? This question could be answered to certain degree if cyclodextrins were found to be active or inactive in vitro systems (or other systems devoid of the outer membrane permeability barrier) [18,19].

We may also be in a position to explain a class of MBP mutants, described in 1982, that have very unusual transport properties in whole cells [20]. These mutants bind both maltose and maltodextrins, support transport maltose, but do not transport maltodextrins in whole cells. The unusual aspect is that, while maltose transport by wild-type MBP is subject to competitive inhibition by maltodextrins, transport by the mutant MBP's is only slightly inhibited [20]. A similar enigma exists in the case of maltodextrin derivatives (e.g. *p*-nitrophenyl-O-maltohexaoside) that bind to MBP with good affinity but are not competitive inhibitors of maltose transport [21,22]. The fact that these derivatives are altered at the sugars' reducing end gives us the essential hint that in both cases (mutant MBP or altered ligand) maltodextrin is unable to bind in the physiologically active end-on conformation. Middle binding is unaffected but physiologically inactive. Since MBP is present in wild-type cells in large excess (roughly 5-fold) [23], no competitive inhibition of transport activity in whole cells is observed until high concentrations of the inactive substrate are added.

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