Stoichiometry and Substrate Affinity of the Mannitol Transporter, EnzymeII<sup>mtl</sup>, from *Escherichia coli*

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**ABSTRACT**

Uptake and consecutive phosphorylation of mannitol in *Escherichia coli* is catalyzed by the mannitol permease EnzymeII<sup>mtl</sup>. The substrate is bound at an extracellular-oriented binding site, translocated to an inward-facing site, from where it is phosphorylated, and subsequently released into the cell. Previous studies have shown the presence of both a high- and a low-affinity binding site with *K<sub>D</sub>*-values in the nano- and micromolar range, respectively. However, reported *K<sub>D</sub>*-values in literature are highly variable, which casts doubts about the reliability of the measurements and data analysis. Using an optimized binding measurement system, we investigated the discrepancies reported in literature, regarding both the variability in *K<sub>D</sub>*-values and the binding stoichiometry. By comparing the binding capacity obtained with flow dialysis with different methods to determine the protein concentration (UV-protein absorption, Bradford protein detection, and a LDH-linked protein assay to quantify the number of phosphorylation sites), we proved the existence of only one mannitol binding site per dimeric species of unphosphorylated EnzymeII<sup>mtl</sup>. Furthermore, the affinity of EnzymeII<sup>mtl</sup> for mannitol appeared to be dependent on the protein concentration and seemed to reflect the presence of an endogenous ligand. The dependency could be simulated assuming that &gt;50% of the binding sites were occupied with a ligand that shows an affinity for EnzymeII<sup>mtl</sup> in the same range as mannitol.

**INTRODUCTION**

The mannitol permease EnzymeII<sup>mtl</sup> (EII<sup>mtl</sup>) from *Escherichia coli* binds extracellular mannitol, transports it across the membrane, and releases it inside the cell as mannitol-1-phosphate. The phosphoryl moiety originates from the high-energy donor PEP and is transferred by two cytosolic kinases, Enzyme I (EI) and HPr. EII<sup>mtl</sup> is phosphorylated by HPr at His-554 in its IIA-domain, from where the phosphoryl group is transferred to Cys-384 in the IIB-domain. It was proposed that in the unphosphorylated carrier mannitol resides at an outward-facing binding site, whereas upon phosphorylation of both the A- and B-domains, mannitol is translocated to an inward-facing binding site, either by movement of the mannitol molecule itself or complete binding site reorientation (1,2). The existence of both an outward- and inward-facing binding site was proposed on the basis of association and dissociation kinetics from both sides of the membrane (3).

Published results on mannitol binding experiments with wild-type EII<sup>mtl</sup> reveal large variations in the dissociation constants (*K<sub>D</sub>*) ranging from 35 nM up to several micromolars (1,4–14). The fact that some authors reported both a high and a low mannitol binding affinity suggests two possible mannitol binding sites. It has been shown that the enzyme is dimeric both in the membrane and in the detergent-solubilized state (7,15–19). Thus, both subunits may have a different conformation and harbor a mannitol-binding site with unequal *K<sub>D</sub>*. A second mannitol-binding site would be consistent with translocation of mannitol from an outward- to an inward-facing binding site. Also, it has been proposed that EII<sup>mtl</sup> exposes a single binding site to either side of the membrane in an alternating fashion (1,3,20). Lolkema included a second mannitol binding site into computational models and performed simulations that showed that a second site could explain some of the complex kinetic behavior of EII<sup>mtl</sup>, both in the translocation pathway and phosphorylation reactions. Finally, it was proposed that EII<sup>mtl</sup> contains one mannitol binding site in its native membrane and two in a purified form (21).

The reported high-affinity dissociation constants vary ~30-fold, from 35 nM up to 1 µM. Because substrate affinity and binding stoichiometry are such crucial parameters for modeling the working mechanism of an enzyme, this is an unacceptable situation. Moreover, in our own studies we were confronted with a strong dependency of the apparent *K<sub>D</sub>* on the concentration of the enzyme. In this article, we clarify the contradiction existing in the binding data regarding mannitol binding stoichiometry and affinity, using flow dialysis in combination with computer simulations.

**MATERIALS AND METHODS**

**Chemicals and reagents**

D-[1-<sup>3</sup>H(N)]mannitol (17.0 Ci/mmol, batch no. 3499-326) was purchased from NEN Research Products (Boston, MA). D-[1-<sup>14</sup>C]mannitol (59.0 mCi/
mmol, batch no. 78) was purchased from Amerham Biosciences (Uppsala, Sweden). Radioactivity measurements were performed using Emulsifier Scintillator Plus obtained from Packard (Groningen, The Netherlands). Q-Sepharose Fast Flow was from Amersham Biosciences. Ni-NTA resin was from Qiagen (Valencia, CA); t-histidine and N-ethylmaleimide (NEM) were from Fluka (Milwaukee, WI). BSA, LDH and β-NADH were from Sigma (St. Louis, MO). Decylpoly(ethylene glycol) glycol 300 (decylPEG) was obtained from Kwant High Vacuum Oil Recycling and Synthesis (Bedum, The Netherlands). The detergent C₁₂E₅ (decyl pentaethylene glycol ether) was synthesized and purified as described (22). His-tagged versions of EI and HPr were created, using standard genetic tools as will be described elsewhere. All other chemicals used were analytical grade.

Cell growth, isolation of ISO membrane vesicles, and purification

The plasmid harboring the wild-type mtlA gene with a thrombin-cleavable C-terminal His-tag (pMamlla/EIIhis) was transformed and subsequently grown in E. coli LGS322 (F⁻ thi-I, hisG1, argG6, metB1, tonA2, supE44, rpsL104, lacY1, gatT6, gatR49, gatS50, Δ(mtlA)Δ(3), mtlDΔ(3), gatRΔ(MDBA-recA)) as described (6,19). LGS322, not transformed, and thus not expressing the mtlA gene was used in control experiments (LGS3mmw).

ISO membrane vesicles were prepared by passage of cells through a French press at 10,000 PSI, essentially as described (23). The membrane vesicles were washed once in 25 mM Tris-HCl, pH 7.6, 5 mM DTT and 1 mM NaN₃, and quickly frozen in small aliquots in liquid nitrogen before storage at −80°C. For mannitol binding experiments or purification of EImut, membrane vesicles were placed at 37°C for quick thawing and thereafter directly placed on ice until further use.

Wild-type EImut was purified by Ni-NTA affinity chromatography. Briefly, membrane vesicles at ~2 mg protein/mL were solubilized in 25 mM Tris-HCl, pH 8.5, 50 mM NaCl, 10 mM 2-mercaptoethanol, 2% (v/v) decylPEG, and 25 mM imidazole for 10 min at room temperature. After spinning down the nonsolubilized fraction (10 min, 250,000× g, 4°C), the supernatant was mixed with washed Ni-NTA resin by stirring for 1 h at 4°C. After draining the flow-through, the column was subsequently washed with 10 column volumes buffer A (25 mM Tris-HCl, pH 8.5, 300 mM NaCl, 0.3% decylPEG, 2 mM GSH, plus 2 mM imidazole), and 10 column volumes buffer B (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.3% decylPEG, plus 2 mM GSH). After batchwise elution from Ni-NTA with 80 mM l-histidine (in 25 mM Tris-HCl, pH 7.6, 0.25% decylPEG, plus 2 mM GSH), 2 mg protein/mL were solubilized in 25 mM NaCl, 100 mM NaCl was added to the samples. Protein purity of the samples was confirmed with SDS-PAGE analysis.

If required, further purification of the protein samples was performed on a MonoQ HR 5/5 column (Pharmacia Biotech, Piscatway, NJ) as described (24). The Ni-NTA purified protein samples were −4–5 times diluted in 25 mM Tris-HCl, pH 7.6, 0.25% decylPEG, plus 2 mM GSH before loading. The column was washed for 1 h at 0.25 mL min⁻¹ with the same buffer supplemented with 100 mM NaCl. Elution of the proteins was started by applying a linear NaCl gradient from 100 to 400 mM in the same buffer.

Activity measurements

The nonvectorial PEP-dependent phosphorylation activity, catalyzed by EImut, was measured as described (25). Briefly, the assay mixture contained 25 mM Tris-HCl, pH 7.6, 5 mM DTT, 5 mM MgCl₂, 5 mM PEP, 350 mM EL, 17 μM HPr, with or without 0.25% decylPEG, and rate-limiting amounts of EImut (in nanomolar range). After incubation of the mixture for 5 min at 30°C, the reaction was started by adding 1 mM [14C]-mannitol. The reaction was quenched at given time intervals by loading the samples on Dowex AG1-X2 columns. After washing the column with four column volumes of H₂O, formed [14C]-mannitol-I-P was eluted using two column volumes of 0.2 N HCl and quantified by liquid scintillation counting.

The number of phosphorylation sites of Ni-NTA purified EImut was determined by measuring the conversion of PEP into pyruvate, using a lactate dehydrogenase linked assay (26). Mixtures of 52.8 units LDH, 2 μM EL, 4 μM HPr, 100 μM NADH, 20 mM Tris-HCl, pH 7.6, 5 mM MgCl₂, 0.25% decylPEG, plus 1 mM DTT were prepared, with or without EImut. The mixtures were incubated for 2 min at 30°C, after which the reaction was started by the addition of 1 mM PEP. The oxidation of NADH was monitored by following the absorbance at 340 nm (εNADH = 6220 L mol⁻¹ cm⁻¹). After 10 min, when the reaction was complete, 10 μM mannitol was added. The difference in the extent of NADH oxidation between the control and the reaction with EImut, after extrapolation to the point of PEP addition, was used to estimate the number of phosphorylation sites, and hence the concentration of EImut, assuming two phosphorylation sites per monomer (9). To test if all phosphate groups were quantitatively donated to EImut, half of the phosphate-accepting groups on EImut were made unavailable by quenching Cys-384 with NEM. Protein samples were desalted using a Micro Bio-Spin 6 column (Bio-Rad, Hercules, CA), after which 1 mM NEM was added. After incubation for 10 min at room temperature, the reaction was stopped by adding 5 mM DTT. The LDH assay was carried out as described above.

Mannitol binding experiments

Mannitol binding was quantified with the flow dialysis technique (13). In short, membrane vesicles (or purified protein samples) were suspended in 25 mM Tris-HCl, pH 7.6, 5 mM DTT, 5 mM MgCl₂, either with or without detergent. The mixture was placed at 25°C for 5 min, after which the sample was loaded into the upper chamber of the flow dialysis cell. Titration was performed by several small additions from a concentrated radiolabeled mannitol stock. All stock solutions (from 25 μM to 1.5 mM) contained 10 μM [3H]-mannitol from the radiolabeled stock solution, supplemented with [14C]-mannitol up to the desired concentration. Each stock solution thus contained identical amounts of radioactivity. The data analysis is described in the next section.

Theoretical considerations and data analysis

Equilibrium between a ligand (S) and a binding site on a protein (E) is described by

\[ E + S \leftrightarrow ES. \]  

(1)

The concentration of unbound, free ligand (Sfree) is obtained from the general binding equation

\[ S_{\text{free}} = 0.5 \times (S_{\text{tot}} - E_{\text{tot}} - K_D + \sqrt{(S_{\text{tot}} - E_{\text{tot}} - K_D)^2 + 4 \times S_{\text{tot}} \times K_D}), \]  

(2)

where Sₜₜ and Eₜₜ represent the total ligand concentration and the concentration of the binding sites, respectively. The equilibrium constant K_D is defined as:

\[ K_D = \frac{E_{\text{free}} \times S_{\text{free}}}{ES}. \]  

(3)

With our flow dialysis setup free, radioactive substrate, added at each titration point n, leaks through a semipermeable membrane to a fraction collector. The signal Cn after addition n (in CPM values) is proportional to Sfree at the specific titration points and is defined by:

\[ C_n = \beta \times S_{\text{free}}. \]  

(4)

The parameter β relates the measured radioactivity (Cₙ) and the concentration of free ligand (Sfree) in the upper compartment of the flow dialysis system and is measured in an experiment with no binding sites added (13). The data (Cₙ-values) collected with the mannitol binding
Mannitol Binding to EII<sub>mtl</sub>

RESULTS

The affinity of EII<sub>mtl</sub> for mannitol in detergent-solubilized ISO membrane vesicles

To investigate the substrate binding properties of EII<sub>mtl</sub>, the flow dialysis technique was used. The detergent decylPEG was added to solubilize the ISO membrane vesicles, thereby making all binding sites accessible to mannitol. To obtain reliable $E_{tot}$- and $K_D$-values, the $E_{tot}/K_D$-ratio was kept between 3 and 30 at all membrane vesicle concentrations and the total amount of substrate at the end of the titrations was ~5 times the amount of binding sites (13). Fig. 1 shows some typical titration experiments at $E_{tot}$-concentrations ranging from ~500 nM to 40 μM (corresponding with ~0.2–~20 mg/mL of total membrane protein). The solid lines represent the fitted curves. The best-fit values of the parameters $E_{tot}$ and $K_D$ for all the titration experiments are summarized in Fig. 2. Fig. 2 A shows that, as anticipated, the apparent concentration of binding sites ($E_{tot}$) increased linearly with the concentration of detergent-solubilized membrane vesicles used (open circles). Surprisingly, we found that the apparent affinity constant for mannitol varied strongly with the amount of detergent-solubilized membrane vesicles, as shown in Fig. 2 C (open circles): the affinity decreased from ~120 nM at the lowest (~0.5 μM sites) to ~1 μM at the highest vesicle concentrations that could be used for this batch (~15 μM sites). When an even higher enzyme concentration was used (from a vesicle batch with a higher expression level, $E_{tot} = 40.4 \mu$M), fitting of the binding data resulted in a $K_D$-value of 2.3 μM (Fig. 1 D).

![Graphs showing titration curves for different amounts of detergent-solubilized membrane vesicles. The solid lines represent nonlinear least-squares curve fits for $K_D$ and $E_{tot}$. The slope of the dashed line represents the $\beta$-value, relating measured radioactivity with the free concentration of mannitol in the upper chamber of the flow dialysis system (see Methods and Materials). The dotted line is a computer simulated curve obtained with a $K_D$ of 100 nM and the corresponding value of $E_{tot}$ from the actual experiment.](image)

![Graphs showing titration curves for different amounts of detergent-solubilized membrane vesicles. The solid lines represent nonlinear least-squares curve fits for $K_D$ and $E_{tot}$. The slope of the dashed line represents the $\beta$-value, relating measured radioactivity with the free concentration of mannitol in the upper chamber of the flow dialysis system (see Methods and Materials). The dotted line is a computer simulated curve obtained with a $K_D$ of 100 nM and the corresponding value of $E_{tot}$ from the actual experiment.](image)
The decrease in affinity for mannitol at higher membrane vesicle concentrations was not caused by nonspecific binding, because addition of membrane vesicles from an EII\textsuperscript{mtl-} deficient E. coli LGS strain (LGS\textsuperscript{minus}) at \(8\) mg/mL to an \(E_{\text{tot}}\) of \(500\) nM, did not affect the apparent binding parameters (results not shown). Accordingly, titration experiments with only LGS\textsuperscript{minus} vesicles did not result in any mannitol binding, as was shown previously (13).

Effect of decylPEG on the binding parameters

In the titration experiments with detergent solubilized-membrane vesicles, the ratio decylPEG/EII\textsuperscript{mtl} was not constant: the concentration of decylPEG varied from 0.25\% at the lowest to 1.0\% at the highest membrane vesicle concentration. To check for an effect of the amount of decylPEG on the binding parameters, titrations were performed at a fixed vesicle concentration (corresponding to \(E_{\text{tot}}\) of \(\sim 1\) \(\mu\)M), over a 20-fold range of decylPEG (from 0.25 to 5.0\%, final concentration). At 0.25\% decylPEG, fitting of the binding data resulted in a \(K_D\) of 89 \(\pm\) 9 nM and an \(E_{\text{tot}}\) of 1043 \(\pm\) 40 nM. Using 1.0\% decylPEG, we obtained \(K_D\)- and \(E_{\text{tot}}\)-values of 132 \(\pm\) 13 and 1164 \(\pm\) 41 nM, respectively. Finally, at 5.0\% decylPEG, the \(K_D\)- and \(E_{\text{tot}}\)-values were 101 \(\pm\) 11 and 913 \(\pm\) 41 nM, respectively. These results show that the \(K_D\) was not affected by increasing amounts of decylPEG, i.e., increasing decylPEG/EII\textsuperscript{mtl} ratios. We conclude that variations in the decylPEG/EII\textsuperscript{mtl} ratios cannot explain the observed variation of the \(K_D\) with \(E_{\text{tot}}\).

The affinity of EII\textsuperscript{mtl} for mannitol in intact ISO membrane vesicles

The association state of EII\textsuperscript{mtl} has been studied extensively both in the purified and membrane-solubilized form (17,25,27,28). From this work it is most probable that with the concentrations used in the titrations, solubilized EII\textsuperscript{mtl} is predominantly dimeric. To test whether a monomer/dimer oligomerization process could explain the observed variations in \(K_D\), the titrations were repeated with intact ISO membrane vesicles, where the amount of vesicles is not expected to influence the association equilibrium. Fig. 2 shows that also for intact membrane vesicles the affinity for mannitol varied strongly with the concentration of membrane vesicles used. We conclude that variations in the oligomerization state can be excluded as a cause of the dependence of the affinity for mannitol on the concentration of EII\textsuperscript{mtl}.

The affinity of purified EII\textsuperscript{mtl} for mannitol

EII\textsuperscript{mtl} was purified using Ni-NTA chromatography, and the protein samples were >95\% pure, as judged from SDS-PAGE analysis (data not shown). Similar titrations as described above were performed with these purified protein samples. In all cases the observed \(E_{\text{tot}}\) increased linearly with the amount of purified protein used. The \(K_D\) showed a similar, albeit less steep, dependence on the protein concentration as observed for the (detergent-solubilized) membrane vesicles (Fig. 2 D). However, at an \(E_{\text{tot}}\) of \(\sim 1\) \(\mu\)M the observed \(K_D\) was two times higher (\(\sim 250\) nM) than in the detergent-solubilized membrane vesicles. Further
purification of the Ni-NTA purified protein, using MonoQ ion-exchange chromatography, did not yield significantly different results: at $E_{tot}$ values of 1.4 and 6.4 $\mu$M, the $K_D$-values were 303 and 594 nM, respectively.

**Binding stoichiometry**

To determine the stoichiometry of substrate binding, it is important to know the concentration of functional protein with high accuracy. The concentration of Ni-NTA purified EII$^{\text{mtl}}$ was estimated with a number of techniques: protein UV-absorption spectroscopy, Bradford protein detection, and a LDH-coupled spectroscopic assay to determine the number of phosphorylation sites. Determination of the protein concentration by UV absorption, using an extinction coefficient of 31,190 L mol$^{-1}$ cm$^{-1}$ at 280 nm (calculated according to Pace et al. (29)), resulted in 21.0 $\mu$M of total protein. The Bradford protein detection method, using BSA as a standard, resulted in 18.8 ± 0.6 $\mu$M total protein ($n = 3$). The result of the LDH-coupled assay is shown in Fig. 3 and shows that the presence of EII$^{\text{mtl}}$ resulted in an additional decrease in absorption ($\Delta$, dotted lines), indicative of PEP consumption, specifically by EII$^{\text{mtl}}$. Extrapolation to the point of PEP addition (see Materials and Methods for details) indicated an EII$^{\text{mtl}}$ concentration of the purified protein preparation of 20.7 $\mu$M. Subsequent addition of 10 $\mu$M mannitol (arrow) resulted in an equivalent PEP consumption. The correspondence between the numbers from each of the measurements shows that all EII$^{\text{mtl}}$ units are functional in phosphorylation. Similar results were obtained for different preparations of purified EII$^{\text{mtl}}$.

Using a purified protein preparation containing 20.2 ± 1.2 $\mu$M (average from the results of the methods mentioned above) of EII$^{\text{mtl}}$ monomeric subunits, we observed 10.3 $\mu$M of mannitol binding sites. This indicates that one molecule of mannitol is bound per dimeric EII$^{\text{mtl}}$. From this it must be concluded that the putative second mannitol binding site observed earlier does not exist, at least in the purified enzyme. The specific mannitol phosphorylation activity of EII$^{\text{mtl}}$ in intact and detergent-solubilized membrane vesicles was 1.8 and 2.0 $\mu$M mtl-1-P/min/nM EII$^{\text{mtl}}$, respectively. For Ni-NTA purified EII$^{\text{mtl}}$ the specific activity was slightly lower (1.6 $\mu$M mtl-1-P/min/nM EII$^{\text{mtl}}$). Because EII$^{\text{mtl}}$ behaves similarly in purified form and in its native environment regarding binding and phosphorylation (see also Lolkema et al. (30)), it is fair to conclude that also embedded in the membrane the transporter has only one binding site. Furthermore, for the experiments performed with membrane vesicles, a linear relation was observed between the amount of membrane vesicles and $E_{tot}$.

![FIGURE 3](image)

**The dependence of the apparent affinity constant for mannitol on the concentration of EII$^{\text{mtl}}$ explained by a second ligand**

The affinity for mannitol appeared to be highly dependent on the amount of protein material used (intact or detergent-solubilized membrane vesicles, and purified protein). To explain this dependency we considered several possibilities: incorrect radioligand concentration, impurities in the $^3$H-mannitol stock, or nonempty binding sites in EII$^{\text{mtl}}$. We used computer simulations to investigate the effect of an unidentified second ligand, competing with radioactive mannitol for the same binding site. It could be present as: i), a contamination of the radioactive mannitol stock solution, or ii), a contamination of the protein stock solution, implying that the mannitol binding sites may not be empty at the start of our titrations, as is usually assumed.

**Impurities in the $^3$H-mannitol stock**

The most straightforward way to check for impurities or an incorrect radioligand stock concentration is to perform titration experiments at a fixed protein concentration, using fixed substrate stock concentrations, and different ratios between unlabeled and radiolabeled species. We performed two titration experiments at an $E_{tot}$ of ~1 $\mu$M, both with a mannitol stock solution of 20 $\mu$M. A radioactive mannitol stock solution was prepared with uncut radioactive mannitol, which was adjusted to 20 $\mu$M with water and used for the first titration. For the second titration a cold mannitol stock solution of the same concentration (20 $\mu$M) was prepared.
and mixed in a 1:1 ratio with the radioactive stock solution from the first titration. Both titration experiments are shown in Fig. 4. Fitting of the data of the uncut $^3$H-mannitol experiment using three-parameter fitting ($K_D$, $E_{tot}$, and $\beta$) resulted in values for the $K_D$, $E_{tot}$, and $\beta$-value of 117 ± 11 nM, 277 ± 17 nM, and 14.72 ± 0.24 CPM nM$^{-1}$, respectively. For the cut-up data set, values for the $K_D$, $E_{tot}$, and $\beta$-value of 109 ± 10 nM, 284 ± 18 nM, and 7.59 ± 0.14 CPM nM$^{-1}$, respectively, were obtained. The $\beta$-value for the cut-up experiment was found to be two times lower than the $\beta$-value for the uncut experiment, because half of the radioactivity was used. The similarity between the two binding isotherms and the fitting results, demonstrated that the concentration of $^3$H-radiolabeled mannitol, as given by the manufacturer, was correct. We estimated that not >5% impurity could be present in our $^3$H-mannitol stock solution.

**Nonempty binding sites**

Computer simulations were used to examine the possible effect of "nonempty" binding sites (occupied with endogenous ligand) on our titrations. Our model assumes that a nonradioactive second ligand present in the protein sample competes with the same site as mannitol and that equilibrium is established fast on the timescale of our titrations. In one set of simulations we assumed the endogenous ligand to be present at a concentration equal to 75% of the concentration of binding sites employed. $E_{tot}$ was varied from 1 to 10 $\mu$M and the dissociation constant of the second ligand ($K_D^{S2}$) was varied from 1 to 10 times that of mannitol ($K_D^{S1} = 100$ nM).

Fig. 5 shows the effect of such an endogenous ligand on the apparent binding parameters $K_D^{S1}$ (Fig. 5 A), and $E_{tot}$ (Fig. 5 B), as a function of the protein concentration. In all cases there was a clear variation of the apparent $K_D$ ($K_D^{S1}$) with the concentration of protein binding sites ($E_{tot}$): higher protein concentrations resulted in higher $K_D$-values, which is similar to the effect observed experimentally (Fig. 2 B). Of course the effect of a second (endogenous) ligand becomes weaker when its affinity for the protein decreases. The apparent value for $E_{tot}$ (observed $E_{tot}$) was slightly lower than the value used for the simulations (added $E_{tot}$), but this effect was more or less the same at all protein concentrations used, resulting in linear relationships between the observed and "actual" concentration of binding sites, again precisely as was found experimentally (Fig. 2 A). Simulations of this type showed that values of $K_D^{S2}$ in the range of 100–500 nM may well explain our experimental results. This leaves mannitol itself as a possible candidate for the unidentified second ligand, apparently copurified with EII$^{amt}$. However, it is not very likely that mannitol would be present in the binding sites, because it is not produced by *E. coli*. One could argue that mannitol is present in the rich Luria Broth media, but growth of the same *E. coli* strain LGS322 (transformed with wild-type EII$^{amt}$, pMamtlAP,EII6his) on M9 minimal medium (31) with glucose as sole carbon source, showed a similar dependency of the $K_D$ on the protein concentration (data not shown).

Furthermore, the LDH-linked assay should have resulted in a higher number of phosphoryl groups accepted from PEP, because endogenous mannitol (or any ligand that can be phosphorylated) would also act as an acceptor of phosphoryl groups from PEP (see also Fig. 3). To ensure that all
phosphoryl groups were donated specifically to EII\textsuperscript{mtl}, phosphorylation of Cys-384 was prevented by quenching with NEM. Again, the LDH-linked assay was performed and the data are shown in Fig. 6. Extrapolation of the absorbance change (after 4 min) to the point when PEP was added resulted in 4.6 $\mu$M of phosphate donated to reduced EII\textsuperscript{mtl} ($\Delta 1$), and 2.5 $\mu$M of phosphate donated to NEM-quenched EII\textsuperscript{mtl} ($\Delta 2$). From this it could be concluded that either $\sim 9\%$ (0.2 $\mu$M) of EII\textsuperscript{mtl} was not labeled by NEM, or that there is ligand present that could be phosphorylated, causing the extra $9\%$ of donated phosphate groups. To discriminate between these two possibilities, we determined the phosphorylation activity of both preparations (Fig. 6, inset). The NEM-quenched sample (circles) retained $\sim 15\%$ phosphorylation activity compared with the untreated sample (squares), indicating that not all Cys-384 residues were alkylated and explaining the $9\%$ extra phosphorylation groups. We therefore conclude that $>95\%$ of the phosphate groups were specifically donated to EII\textsuperscript{mtl}, and that the proposed endogenous ligand cannot be phosphorylated by EII\textsuperscript{mtl}.

**Dissociation of mannitol from EII\textsuperscript{mtl}**

The simulations presented in Fig. 5, indicated that an endogenous ligand could explain the experimental results, but only if present in considerable amounts with an affinity that is comparable to the affinity of EII\textsuperscript{mtl} for mannitol itself. This implies that much of the endogenous ligand must remain bound during purification of the protein. To test whether this is a plausible possibility, we incubated 2.5 nmol of EII\textsuperscript{mtl} (corresponding with 1.25 nmol of binding sites) in 500 $\mu$L buffer A (25 mM Tris-HCl, pH 7.6, 0.25% decylPEG, 2 mM GSH) with a small excess of $^3$H-mannitol for 3 min at room temperature. Subsequently, the protein was bound to Q-Sepharose (250 $\mu$L), the column was washed with 19 column volumes of buffer A, and the protein was eluted with nine column volumes of buffer B (buffer A, supplemented with 300 mM NaCl). Fig. 7 shows the time course of the radioactivity appearing in the eluate during the washing and elution steps, with (solid symbols) and without (open symbols) EII\textsuperscript{mtl}. Assuming all binding sites were occupied with $^3$H-mannitol, the difference in the amount of $^3$H-mannitol that was bound and the amount that eluted with the protein showed that even after this extensive washing procedure $\sim 39\%$ of the available binding sites were still occupied by $^3$H-mannitol.

**DISCUSSION**

We have characterized the substrate binding properties of EII\textsuperscript{mtl}, using flow dialysis, and conclude that the unphosphorylated protein has a single high-affinity mannitol binding site per dimeric EII\textsuperscript{mtl}. Binding isotherms generated at different protein concentrations did not reveal a low-affinity binding site even at the highest protein concentrations. In our binding experiments we observed a significant dependence of the dissociation constant for mannitol on the protein concentration: an increase in protein concentration resulted in a significant increase in the apparent dissociation constant. As shown in Fig. 1 the binding isotherms could be fitted appropriately with a simple one-site one-ligand model: the $\chi^2$-values did not indicate that this model was inadequate.

Computer simulations showed that such binding behavior could be expected when competing ligands are present in either the substrate stock or in the protein preparation. Several authors have reported either chemical and/or radiochemical impurities in their radiolabeled substrate stocks...
A mixture (500 μL), with (solid symbols) or without (open symbols) 5 μM EII mtl (1.25 nmol binding sites in buffer A, 25 mM Tris-HCl, pH 7.6, 0.25% decylPEG, plus 2 mM GSH), was incubated with 6.67 μM 3H-mannitol (3.34 nmol) for 3 min at room temperature and applied onto 250 μL Q-Sepharose. The column was subsequently batchwise washed with 19 column volumes of buffer A, and the protein was batchwise eluted with 9 column volumes of buffer B (buffer A, supplemented with 300 mM NaCl). Fraction 1 is the flow through, and the protein was batchwise eluted with 9 column volumes of buffer B. Inset: Sample 0 represents the starting material, before loaded to the Q-Sepharose column.

The presence of endogenous ligand associated with EII mtl seems a more plausible explanation, as shown by the simulations in Fig. 5. The simulations further show that, to explain the observed effects, a considerable fraction of the sites should be occupied with an endogenous ligand that has an affinity similar to that of mannitol itself. In agreement with this model is the observation that the dependence is less strong in the purified protein samples (Fig. 2 D, diamonds), where more of the endogenous ligand appears to have been removed during purification. Indeed, we showed that only very extensive washing will remove endogenous mannitol from the binding sites (Fig. 7). If this explanation is correct, our best estimate for the $K_D$ would be that observed at the lowest protein concentrations, where the effect of an endogenous ligand is smallest. Reliable binding measurements at even lower protein concentrations are not possible with the flow dialysis technique (13).

For some oligopeptide binding proteins (OppA from Lactococcus lactis and Salmonella typhimurium, AppA from Bacillus subtilis) and for the histidine-binding protein (HisJ) from S. typhimurium endogenous ligands were observed after purification (42–45). Apparently, some oligopeptides bind with such a strong affinity that purification does not remove the bound ligands. In the crystal structure of a mutant of the lactose permease (LacY) from E. coli showed an unidentified disaccharide bound at the sugar-binding site (46).

With flow dialysis a radioactive tracer is used to measure the binding parameters. In these experiments any unlabeled endogenous ligand will be exchanged with the tracer in the course a binding experiment. Therefore, the concentration of binding sites appears unaffected but their affinity appears to decrease at increasing concentrations. When techniques like fluorescence are used to measure ligand binding, endogenous ligand will affect most the apparent value for $E_{tot}$, because endogenous ligand will typically diminish the total change in fluorescence observable during a titration. This is indeed what we observed for a single-tryptophan mutant (EII mtl-W30, data not shown): different purified preparations showed different degrees of fluorescence increase upon addition of mannitol.

The next logical step would be to remove the endogenous ligand or discover its nature. For the OppA oligopeptide binding protein (43), endogenous ligands could be removed by unfolding, washing, and subsequent refolding. However, this is not possible for membrane proteins like EII mtl. Despite several attempts, we were unable to identify the proposed competing ligand. A colorimetric assay to determine the content of alditols was hampered by a high background (47). An enzymatic method to follow the oxidation of mannitol, using mannitol dehydrogenase, was not sensitive enough at the expected concentrations of endogenous ligand (48). Electron spray mass spectrometry suffered from the high background of detergent, even after removal of $>99\%$ of the detergent by water/diethylether liquid phase separation (data not shown). Also, extensive dialysis of membrane vesicles did not (completely) abolish the $K_D$ dependence on the enzyme concentration. Further research is necessary to elucidate the nature of the proposed endogenous ligand.

In conclusion, we demonstrated that dimeric, unphosphorylated EII mtl has only a single high-affinity binding site. This new insight into the substrate-binding mechanism will have consequences for models of the phosphotransferase system in general. The “restricted access model”, describing the mannitol-concentration-dependent translocation of binding sites, explains most of the kinetic properties of EII mtl (21). Numerical analysis of the kinetic scheme, however, provided no clues regarding coupling of binding sites within each monomer and our conclusion that each dimer has only one high-affinity binding site is compatible with this kinetic scheme. Also, for the IICB$^{E_k}$ dimer of the glucose permease.
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(Elm), a model has been proposed with both a high- and a low-affinity binding site (49), but direct evidence for this is lacking. Although Elm and Elm are not homologous, they behave catalytically in a similar manner and we feel that a reevaluation of the number of substrate binding sites in Elm may be necessary.

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