Mechanics of the mannitol transporter from Escherichia coli
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Stoichiometry and substrate affinity of the mannitol transporter, EnzymeII\textsuperscript{mtl}, from Escherichia coli

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Abstract

Uptake and consecutive phosphorylation of mannitol in Escherichia coli is catalyzed by the mannitol permease EnzymeII\textsuperscript{mtl}. 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 $K_D$-values in the nano- and micromolar range, respectively. However, reported $K_D$-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 $K_D$-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\textsuperscript{mtl}. Furthermore, the affinity of EnzymeII\textsuperscript{mtl} 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 more than 50% of the binding sites were occupied with a ligand that shows an affinity for EnzymeII\textsuperscript{mtl} in the same range as mannitol.
Introduction

The mannitol permease EnzymeII\textsuperscript{mtl} (EII\textsuperscript{mtl} \textsuperscript{1}) from \textit{E. 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, EnzymeI (EI) and HPr. EII\textsuperscript{mtl} is phosphorylated by HPr at histidine 554 in its IIA-domain, from where the phosphoryl group is transferred to cysteine 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 IIA- and IIB-domains, mannitol is translocated to an inward-facing binding site, either by movement of the mannitol molecule itself or complete binding site reorientation (Lolkema et al., 1990; Lolkema & Robillard, 1992). 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 (Lolkema et al., 1992).

Published results on mannitol binding experiments with wild-type EII\textsuperscript{mtl} reveal large variations in the dissociation constants ($K_D$), ranging from 35 nM up to several micromolars (Boer et al., 1995; Briggs et al., 1992; Grisafi et al., 1989; Lolkema et al., 1990/1993c; Meijberg et al., 1998b; Pas et al., 1988; Saraceni-Richards & Jacobson, 1997a/b; Swaving-Dijkstra et al., 1996a; Veldhuis et al., 2004; Weng & Jacobson, 1993). 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 (Boer et al., 1994; Khandekar & Jacobson, 1989; Lolkema et al., 1993a; Pas et al., 1987; Roossien & Robillard, 1984; Stephan & Jacobson, 1986b). Thus, both subunits may have a different conformation and harbor a mannitol binding site with unequal $K_D$. 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\textsuperscript{mtl} exposes a single binding site to either side of the membrane in an alternating fashion (Elferink et al., 1990; Lolkema et al., 1990/1992). 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\textsuperscript{mtl}, both in the translocation pathway and phosphorylation reactions. Finally, it was proposed that EII\textsuperscript{mtl} contains one mannitol binding site in its native membrane and two in a purified form (Lolkema, 1993).

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\textsuperscript{1} CPM, counts per minute; decylPEG, decyl(poly(ethyleneglycol))300; DTT, dithiothreitol; $E_{\text{tot}}$, total amount of binding sites; EI, Enzyme I from \textit{Escherichia coli}; EII\textsuperscript{mtl}, EnzymeII\textsuperscript{mtl} from \textit{Escherichia coli}; GSH, reduced glutathione; HPr, Heat-stable protein from \textit{Escherichia coli}; ISO, inside-out; $K_D$, dissociation constant, LDH, Lactate Dehydrogenase; Mtl, mannitol; NEM, N-ethylmaleimide; PEP, phosphoenolpyruvate
The reported high-affinity dissociation constants vary approximately 30-fold, from 35 nM up to 1 µM. Since substrate affinity and binding stoichiometry are such crucial parameters for modelling 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 $K_D$ on the concentration of the enzyme. In this paper, we clarify the contradiction existing in the binding data regarding mannitol binding stoichiometry and affinity, using flow dialysis in combination with computer simulations.

**Materials & methods**

**Chemicals & reagents**

D-[1-²H(N)]Mannitol (17.0 Ci/mmol, batch no. 3499-326) was purchased from NEN Research Products. D-[1-¹⁴C]Mannitol (59.0 mCi/mmol, batch no. 78) was purchased from Amersham Biosciences. Radioactivity measurements were performed using Emulsifier Scintillator Plus obtained from Packard. Q-Sepharose Fast Flow was from Amersham Biosciences. Ni-NTA resin was from Qiagen Inc. L-Histidine and N-ethylmaleimide (NEM) were from Fluka. BSA, LDH and β-NADH were from Sigma. Decylpoly(ethylene glycol)₃₀₀ (decylPEG) was obtained from Kwant High Vacuum Oil Recycling and Synthesis (Bedum, The Netherlands). The detergent C₁₀E₅ (decyl pentaethylene glycol ether) was synthesized and purified as described (Swaving-Dijkstra et al., 1996b). 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 harbouring the wild-type *mtlA* gene with a thrombin-cleavable C-terminal his-tag (pMamtla₄₅EI₆his) was transformed and subsequently grown in *E. coli* LGS322 [F thi-1, hisG1, argG6, metB1, tonA2, supE44, rpsL104, lacY1, galT6, gapR49, gapA50, Δ(mtla p), mtldΔ, Δ(gatR MDBA-recA)] as described (Boer et al., 1994; Grisafi et al., 1989). LGS322, not transformed, and thus not expressing the *mtlA* gene was used in control experiments (LGS minus). ISO membrane vesicles were prepared by passage of cells though a French Press at 10,000 Psi, essentially as described (Broos et al., 1999). 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 prior to storage at -80°C. For mannitol binding experiments or purification of EI⁰₆, membrane vesicles were placed at 37°C for quick thawing and thereafter directly placed on ice until further use.

Wild type EI⁰₆ 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 minutes at room temperature. After spinning down the non-solubilized fraction (10 minutes, 250 000 g, 4°C), the supernatant was mixed with washed Ni-NTA resin by stirring for 1 hour 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 10 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 batch-wise elution from Ni-NTA with 80 mM L-Histidine (in 25 mM Tris-HCl, pH 7.6, 0.25% decylPEG, plus 2 mM GSH), 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) as described (Broos et al., 2000). 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 hour 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 100-400 mM in the same buffer.

Activity measurements

The non-vectorial PEP-dependent phosphorylation activity, catalysed by EII<sub>mtl</sub>, was measured as described (Robillard & Blaauw, 1987). Briefly, the assay mixture contained 25 mM Tris-HCl, pH 7.6, 5 mM DTT, 5 mM MgCl₂, 5 mM PEP, 350 nM EI, 17 μM HPt, with or without 0.25% decylPEG, and rate-limiting amounts of EII<sub>mtl</sub> (in nanomolar range). After incubation of the mixture for 5 minutes 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 4 column volumes of H₂O, formed [¹⁴C]-mannitol-1-P was eluted using 2 column volumes of 0.2 N HCl and quantified by liquid scintillation counting.

The number of phosphorylation sites of Ni-NTA purified EII<sub>mtl</sub> was determined by measuring the conversion of PEP into pyruvate, using a lactate dehydrogenase (LDH) linked assay (Gunnewijk & Poolman, 2000). Mixtures of 52.8 Units LDH, 2 μM EI, 4 μM HPt, 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 EII<sub>mtl</sub>. The mixtures were incubated for 2 minutes 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 (ε<sub>NADH</sub> = 6220 L·mol⁻¹·cm⁻¹). After 10 minutes, 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 EII<sub>mtl</sub>, after extrapolation to the point of PEP addition, was used to estimate the number of phosphorylation.
sites, and hence the concentration of EII mtl, assuming two phosphorylation sites per monomer (Pas et al., 1988). To test if all phosphate groups were quantitatively donated to EII mtl, half of the phosphate-accepting groups on EII mtl were made unavailable by quenching Cys384 with NEM. Protein samples were desalted using a Micro Bio-Spin 6 column (Bio-Rad), after which 1 mM NEM was added. After incubation for 10 minutes 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 (Veldhuis et al., 2004). In short, membrane vesicles (or purified protein samples) were suspended in 25 mM Tris-HCl, pH 7.6, 5 mM DTT, and 5 mM MgCl₂, either with or without detergent. The mixture was placed at 25°C for 5 minutes, 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 radiolabelled mannitol stock. All stock solutions (from 25 μM to 1.5 mM) contained 10 μM ³H-mannitol from the radiolabelled stock solution, supplemented with ³H-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 & data analysis
Equilibrium between a ligand (S) and a binding site on a protein (E) is described by

\[ E + S \rightleftharpoons ES \]  

(1)

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

\[ S_{\text{free}} = 0.5 \left( \frac{S_{\text{tot}} - E_{\text{tot}} - K_D}{\sqrt{S_{\text{tot}} - E_{\text{tot}} - K_D}} + 4 \cdot S_{\text{tot}} \cdot K_D \right), \]  

(2)

where \( S_{\text{tot}} \) and \( E_{\text{tot}} \) represent the total ligand concentration and the concentration of the binding sites, respectively. The equilibrium constant \( K_D \) is defined as:

\[ K_D = \frac{E_{\text{tot}} \cdot S_{\text{free}}}{ES} \]  

(3)

With our flow dialysis set-up free, radioactive substrate, added at each titration point \( n \), leaks through a semi-permeable membrane to a fraction collector. The signal \( C_n \) after addition \( n \) (in CPM values) is proportional to \( S_{\text{free}} \) at the specific titration points.
and is defined by:

\[ C^* = \beta \cdot S_{\text{free}} \]  \hspace{1cm} (4)

The parameter \( \beta \) relates the measured radioactivity \( (C^*) \) and the concentration of free ligand \( (S_{\text{free}}) \) in the upper compartment of the flow dialysis system and is measured in an experiment with no binding sites added (Veldhuis et al., 2004). The data \( (C^*) \)-values collected with the mannitol binding experiment, together with the values for \( S_{\text{tot}} \), can now be analysed using Equations 2-4 to calculate the total concentration of binding sites \( (E_{\text{tot}}) \) and the affinity for the substrate \( (K_D) \), on the assumption that the simple binding model (Eq. 1) holds. In case of a three-parameter fitting routine, the data was fitted for \( E_{\text{tot}}, K_D, \) and \( \beta \). The routines were written in the software package Mathematica\textsuperscript{TM} 5.0, using the Levenberg-Marquardt non-linear least-squares minimization algorithm, for fitting of the data. Details for the treatment of systematic and non-systematic errors are described in detail in Veldhuis et al., 2004. Estimates of errors in the binding parameters are based on Monte Carlo simulations of 200 datasets, using a value of 0.08 \( \mu \text{L} \) as the uncertainty in each addition (\( \Sigma \)-value, see Veldhuis et al., 2004), unless stated otherwise. We used computer simulations to investigate the impact of impurities in the radioactive stock or the effect of occupied binding sites (endogenous ligand) on the observed values for \( E_{\text{tot}} \) and \( K_D \). For this purpose, the binding equations were adapted to a one-site (\( E \)) two-ligand \( (S_1 \text{ and } S_2) \) model. With this model virtual datasets (mimicking titration experiments) were generated, and subsequently analysed with the simple one-site one-ligand model (Eq. 2) to solve for \( E_{\text{tot}} \) and \( K_D \). The following equations (5-7) were solved numerically for \( S_{1,\text{free}} \), which is proportional to the measured radioactivity. Several situations were mimicked, whereby the second ligand concentration \( (S_{2,\text{tot}}) \) was kept proportional to either \( S_{1,\text{tot}} \) (the second ligand as a contamination of the ligand stock solution) or to \( E_{\text{tot}} \) (the second ligand as a contamination of the EII\textsuperscript{mtl} stock solution):

\[ S_{1,\text{tot}} = S_{1,\text{free}} + \frac{S_{1,\text{free}} \cdot E_{\text{free}}}{K_{1_D}} \]  \hspace{1cm} (5)

\[ S_{2,\text{tot}} = S_{2,\text{free}} + \frac{S_{2,\text{free}} \cdot E_{\text{free}}}{K_{2_D}} \]  \hspace{1cm} (6)

\[ E_{\text{tot}} = E_{\text{free}} + \frac{S_{1,\text{free}} \cdot E_{\text{free}}}{K_{1_D}^2} + \frac{S_{2,\text{free}} \cdot E_{\text{free}}}{K_{2_D}^2} \]  \hspace{1cm} (7)

\( K_{1_D}^2 \) and \( K_{2_D}^2 \) represent the affinities of \( E \) for \( S_1 \), and \( S_2 \), respectively.
**Results**

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

To investigate the substrate binding properties of EII<sup>mtl</sup>, 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. In order 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 about 5 times the amount of binding sites (Veldhuis et al., 2004). Figure 1 shows some typical titration experiments at $E_{tot}$-concentrations ranging from about 500 nM to 40 μM (corresponding with ~0.2 to ~20 mg·mL<sup>-1</sup> of total membrane protein). The solid lines represent the fitted curves.

![Figure 1](image.png)

Figure 1. Titration curves for different amounts of detergent-solubilized membrane vesicles. The solid lines represent non-linear least-squares curve-fits for $K_D$ and $E_{tot}$. The slope of the dashed line represents the $b$-value, relating measured radioactivity with the free concentration of mannitol in the upper chamber of the flow dialysis system (see Methods & 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. (A) 9 μL membrane vesicles with 25 μM <sup>3</sup>H-mtl stock (B) 64.5 μL membrane vesicles with 300 μM <sup>3</sup>H-mtl stock (C) 200 μL membrane vesicles with 880 μM <sup>3</sup>H-mtl stock (D) 409.5 μL membrane vesicles (different batch) with 2 mM <sup>3</sup>H-mtl stock. Mixture volumes were 450 μL, of which 380 μL was pipetted into the upper chamber of the flow dialysis system.
The best-fit values of the parameters $E_{tot}$ and $K_D$ for all the titration experiments are summarized in Figure 2. Figure 2A 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 Figure 2C (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, $E_{tot} = 40.4$ μM), fitting of the binding data resulted in a $K_D$-value of 2.3 μM (Figure 1D).

Figure 2. Binding parameters plotted against vesicle or protein amount. (A) Observed $E_{tot}$ concentration against the amount of vesicles used for intact (solid circles) and detergent solubilized (open circles) ISO membrane vesicles. The solid lines represent linear fits. (B) Observed $K_D$ against the amount of intact ISO membrane vesicles (solid circles). (C) Observed $K_D$ against the amount of solubilized ISO membrane vesicles (open circles). (D) Observed $K_D$ against the observed $E_{tot}$ for different Ni-NTA purified protein preparations. Error bars were determined using Monte Carlo simulations with $\Sigma = 0.08$ and 500 data sets (Veldhuis et al., 2004).

The decrease in affinity for mannitol at higher membrane vesicle concentrations was not caused by non-specific binding, since addition of membrane vesicles from an ElpG-deficient E. coli LGS strain (LGSmin) at approximately 8 mg·mL$^{-1}$ to an $E_{tot}$ of ~500 nM, did not affect the apparent binding parameters (results not shown).
Accordingly, titration experiments with only LGS minus vesicles did not result in any mannitol binding, as was shown previously (Veldhuis et al., 2004).

**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 $E_{tot}$ of ~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 $E_{tot}$ of 1043 $\pm$ 40 nM. Using 1.0% decylPEG, we obtained $K_D$- and $E_{tot}$-values of 132 $\pm$ 13 nM and 1164 $\pm$ 41 nM, respectively. Finally, at 5.0% decylPEG, the $K_D$- and $E_{tot}$-values were 101 $\pm$ 11 nM 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 $E_{tot}$.

**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 (Lolkema & Robillard, 1990; Pas et al., 1987; Robillard & Blauw, 1987; Roossien et al., 1986). 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. Figure 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 more than 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 $E_{tot}$ 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 (Figure 2D). However, at an $E_{tot}$ of about 1 $\mu$M the observed $K_D$ was two times higher (~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 $E_{\text{tot}}$-values of 1.4 and 6.4 μM, 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$_{mtl}$ was estimated with a number of techniques: protein UV-absorption spectroscopy, Bradford protein detection, and a lactate dehydrogenase (LDH)-coupled spectroscopic assay to determine the number of phosphorylation sites. Determination of the protein concentration by UV-absorption, using an extinction coefficient of 31190 L·mol$^{-1}$·cm$^{-1}$ at 280 nm (calculated according to Pace et al., 1995), resulted in 21.0 μM of total protein. The Bradford protein detection method, using BSA as a standard, resulted in 18.8 ± 0.6 μM total protein ($n=3$). The result of the LDH-coupled assay is shown in Figure 3 and shows that the presence of EII$_{mtl}$ resulted in an additional decrease in absorption ($\Delta$, dotted lines), indicative of PEP-consumption, specifically by EII$_{mtl}$. Extrapolation to the point of PEP addition (see Materials & Methods for details) indicated an EII$_{mtl}$-concentration of the purified protein preparation of 20.7 μM. Subsequent addition of 10 μM mannitol (arrow) resulted in an equivalent PEP-consumption. The correspondence between the numbers from each of the measurements shows that all EII$_{mtl}$ units are functional in phosphorylation. Similar results were obtained for different preparations of purified EII$_{mtl}$.

**Figure 3. Quantitative phosphorylation of EII$_{mtl}$.** The control mixture contained 52.8 Units LDH, 2 μM EI, 4 μM HPr, 100 μM NADH, 20 mM Tris-HCl, pH 7.6, 5 mM MgCl$_2$, 0.25% decylPEG, plus 1 mM DTT. EII$_{mtl}$ was added to a concentration of about 4 μM. The reaction was started by addition of 1 mM PEP, and monitored by following the change in absorbance at 340 nm ($\varepsilon_{\text{NADH}} = 6220$ L·mol$^{-1}$·cm$^{-1}$). After 10 minutes, when the reaction was complete, 10 μM of mannitol was added. The dotted lines represent linear fits through the data points after the reaction was complete. Extrapolation to the point where PEP was added yielded the amount of phosphate-groups donated. The difference between the control (no EII$_{mtl}$) and the sample with EII$_{mtl}$ yielded the amount of phosphorylation sites associated with EII$_{mtl}$ ($\Delta$).
Using a purified protein preparation containing 20.2 ± 1.2 μM (average from the results of the methods mentioned above) of EIIImtl monomeric subunits, we observed 10.3 μM of mannitol binding sites. This indicates that one molecule of mannitol is bound per dimeric EIIImtl. 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 EIIImtl in intact and detergent solubilized membrane vesicles was 1800 and 2000 min⁻¹, respectively. For Ni-NTA purified EIIImtl the specific phosphorylation activity was slightly lower (1600 min⁻¹). Since EIIImtl behaves similarly in purified form and in its native environment regarding binding and phosphorylation (see also Lolkema et al., 1993b), 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 $E_{\text{tot}}$.

The dependence of the apparent affinity constant for mannitol on the concentration of EIIImtl 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 non-empty binding sites in EIIImtl. 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) as 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.

(i) 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 $E_{\text{ex}}$ of ~1 μM, both with a mannitol stock solution of 20 μM. A radioactive mannitol stock solution was prepared with uncut radioactive mannitol, which was adjusted to 20 μM with water and used for the first titration. For the second titration a cold mannitol stock solution of the same concentration (20 μM) was prepared and mixed in a 1:1 ratio with the radioactive stock solution from the first titration. Both titration experiments are shown in Figure 4. Fitting of the data of the uncut $^3$H-mannitol experiment using three-parameter fitting ($K_D$, $E_{\text{coo}}$ and β; Veldhuis et al., 2004) resulted in values for $K_D$, $E_{\text{coo}}$, and β of 117 ± 11 nM, 277 ± 17 nM, and 14.72 ± 0.24
CPM-nM\(^{-1}\), respectively. For the cut-up data set, values for \(K_D\), \(E_{tot}\), and \(\beta\) 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, since 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 more than 5% impurity could be present in our \(^3\)H-mannitol stock solution.

(ii) Non-empty binding sites

Computer simulations were used to examine the possible effect of “non-empty” binding sites (occupied with endogenous ligand) in our titrations. Our model assumes that a non-radioactive second ligand present in the protein sample competes with the same site as mannitol and that equilibrium is established fast on the time scale 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 1-10 \(\mu\)M and the dissociation constant of the second ligand (\(K_{D}^{S}\)) was varied from 1-10 times that of mannitol (\(K_{D}^{S} = 100\) nM). Figure 5 shows the effect of such an endogenous ligand on the apparent binding parameters \(K_{D}^{S}\) (Figure 5A), and
Stoichiometry and substrate affinity of EnzymeII<sub>mtl</sub>

E<sub>tot</sub> (Figure 5B), as a function of the protein concentration. In all cases there was a clear variation of the apparent K<sub>D</sub> (1/S<sub>D</sub>K<sub>D</sub>) with the concentration of protein binding sites (E<sub>tot</sub>): higher protein concentrations resulted in higher K<sub>D</sub>-values, which is similar to the effect observed experimentally (Figure 2B). Of course the effect of a second (endogenous) ligand becomes weaker when its affinity for the protein decreases. The apparent value for E<sub>tot</sub> (observed E<sub>tot</sub>) was slightly lower than the value used for the simulations (added E<sub>tot</sub>), 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 (Figure 2A). Simulations of this type showed that values of K<sub>D</sub> 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 co-purified with EII<sub>mtl</sub>. However, it is not very likely that mannitol would be present in the binding sites, since it is not produced by <i>Escherichia coli</i>. One could argue that mannitol is present in the rich Luria Broth media, but growth of the same <i>E. coli</i> strain LG322 (transformed with wild-type EII<sub>mtl</sub>, pMamtla<sub>P</sub>II<sub>6his</sub>) on M9 minimal medium (Maniatis et al., 1982) 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, since endogenous mannitol (or any ligand that can be phosphorylated) would also act as an acceptor of phosphoryl groups from PEP (see also Figure 3). To ensure that all phosphoryl groups were donated specifically to EII$^{mtl}$, phosphorylation of Cys384 was prevented by quenching with NEM. Again, the LDH-linked assay was performed and the data are shown in Figure 6. Extrapolation of the absorbance change (after 4 minutes) to the point when PEP was added resulted in 4.6 $\mu$M of phosphate donated to reduced EII$^{mtl}$ ($\Delta_1$), and 2.5 $\mu$M of phosphate donated to NEM-quenched EII$^{mtl}$ ($\Delta_2$). From this it could be concluded that either approximately 9% (0.2 $\mu$M) of EII$^{mtl}$ was not labelled 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 (Figure 6, inset). The NEM-quenched sample retained about 15% phosphorylation activity, indicating that not all Cys384 residues were alkylated and explaining 9% extra phosphorylation groups. We therefore conclude that more than 95% of the phosphate groups were specifically donated to EII$^{mtl}$, and that the proposed endogenous ligand cannot be phosphorylated by EII$^{mtl}$.

Figure 6. Quantitative phosphorylation of Cys384-quenched EII$^{mtl}$. For experimental conditions see legend to Figure 3. The dotted lines represent linear fits through the data points from 4-10 minutes. The difference between the control (EI, HPr) and EII$^{mtl}$ (with ($\Delta_2$), or without ($\Delta_1$) NEM) is the amount of phosphorylation sites associated with EII$^{mtl}$. The inset represents the phosphorylation activity of the EII$^{mtl}$ samples, i.e. the amount of mannitol-1-P formed in time.
Dissociation of mannitol from EII<sub>mtl</sub>

The simulations presented in Figure 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<sub>mtl</sub> 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 nmole of EII<sub>mtl</sub> (corresponding with 1.25 nmole of binding sites) in 500 µL buffer A (25 mM Tris-HCl, pH 7.6, 0.25% decylPEG, 2 mM GSH) with a small excess of <sup>3</sup>H-mannitol for 3 minutes at room temperature. Subsequently, the protein was bound to Q-Sepharose (250 µL), the column was washed with 19 column-volumes of buffer A, and the protein was eluted with 9 column-volumes of buffer B (buffer A, supplemented with 300 mM NaCl). Figure 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<sub>mtl</sub>. Assuming all binding sites were occupied with <sup>3</sup>H-mannitol, the difference in the amount of <sup>3</sup>H-mannitol that was bound and the amount that eluted with the protein showed that even after this extensive washing procedure about 39% of the available binding sites were still occupied by <sup>3</sup>H-mannitol.

**Figure 7. Dissociation of mannitol from EII<sub>mtl</sub>**

500 µL of a mixture, with (solid symbols) or without (open symbols) 5 µM EII<sub>mtl</sub> (1.25 nmole 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 <sup>3</sup>H-mannitol (3.34 nmole) for 3 minutes at room temperature and applied onto 250 µL Q-Sepharose. The column was subsequently batch-wise washed with 19 column-volumes of buffer A, and the protein was batch-wise eluted with 9 column-volumes of buffer B (buffer A, supplemented with 300 mM NaCl). Fraction 1 is the flow through, after which washing was started. The arrow indicates the start of elution. All fractions were tested for radioactivity by liquid scintillation counting. The grey triangles with numbers point the fractions for which samples were prepared for SDS-PAGE analysis (inset). Sample 0 represents the starting material, before loaded to the Q-Sepharose column.
Chapter 3

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 Figure 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 behaviour 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 radiolabelled substrate stocks (Ball & Roberts, 1991; Bartlett & Smith, 1995; Drenth & De Zeeuw, 1982; Gu et al., 1995; Honoré, 1987; Merrill & Lewis, 1974; and the references cited in Merrill & Lewis, 1974). On the basis of the manufacturers quality sheets, the compounds were >95% pure. However, analysis of the radiolabelled compounds revealed in some cases purity as low as 30%. Transfer constants for permeation of $^{14}$C-sucrose across the blood-brain barrier in rats where overestimated up to 8 times for certain batches of $^{14}$C-sucrose (Preston et al., 1998). The effects of impurities in radiolabelled stocks on the binding parameters can be dramatic (Builder & Segel, 1978; Lazareno & Birdsall, 2000; Segel, 1994). We excluded the possibility of impurities in the radiolabelled stock solutions with the substrate cut-up control experiment (Figure 4).

The presence of endogenous ligand associated with EII\textsuperscript{mtl} seems a more plausible explanation, as shown by the simulations in Figure 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 (Figure 2D, 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 (Figure 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 (Veldhuis et al., 2004).
For some oligopeptide binding proteins (OppA from *Lactococcus lactis* and *Salmonella typhimurium*, AppA from *Bacillus subtilis*) and for the histidine-binding protein (HisJ) from *Salmonella thyphimurium* endogenous ligands were observed after purification (Ames et al., 1996; Lanfermeijer et al., 1999; Levdkov et al., 2005; Tame et al., 1994). Apparently, some oligopeptides bind with such a strong affinity that purification does not remove the bound ligands. The crystal structure of a mutant of the lactose permease (LacY) from *E. coli* showed an unidentified disaccharide bound at the sugar-binding site (Abramson et al., 2003).

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 of 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 $E_{tot}$, since 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 (Lanfermeijer et al., 1999), 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 (Sanchez, 1998). An enzymatic method to follow the oxidation of mannitol, using mannitol dehydrogenase, was not sensitive enough at the expected concentrations of endogenous ligand (Graefe et al., 2003, and references cited therein). Electrospray mass spectrometry suffered from the high background of detergent, even after removal of more than 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...
EIImtl (Lolkema, 1993). 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 are compatible with this kinetic scheme. Also, for the IICBglc dimer of the glucose permease (EIICB), a model has been proposed with both a high- and a low-affinity binding site (Garcia-Alles et al., 2002), but direct evidence for this is lacking. Although EIImtl and EIICB are not homologous, they behave catalytically in a similar manner and we feel that a re-evaluation of the number of substrate binding sites in EIICB may be necessary.
A theoretical evaluation of ligand removal by column washing

Gertjan Veldhuis, Bert Poolman and Ruud M. Scheek

Introduction

Protein purification is often deliberately performed in the presence of (one of) the native ligand(s), because this might increase the stability of the protein. For subsequent binding studies, this ligand needs to be removed, for example by an extra chromatography step or extensive dialysis. Likewise, purification of EII$^{mtl}$, using Ni-NTA affinity chromatography, has been performed in the presence of mannitol (first described in Broos et al., 2000). It was assumed that an extra purification step without mannitol, using ion-exchange column chromatography, would empty all the mannitol-occupied binding sites.

Suspicion about the presence of residual ligand in the purified protein preparations arose during ligand-binding experiments when a protein concentration-dependent mannitol binding affinity was observed (Chapter 3). The effect was not only observed in (detergent-solubilized) membrane vesicles, but was also significant in purified protein samples. Computer simulations confirmed that endogenous ligand could explain the protein’s concentration-dependent mannitol binding affinity. In this appendix, computer simulations are presented to evaluate the factors that affect the efficiency of column washing procedures to empty the binding sites of a protein preparation.

Theoretical considerations

Consider a procedure in which a protein (E) is immobilized on a column resin and subsequently washed with ligand-free buffer to deplete ligand (S) from its binding sites. The effect of washing of an ES-complex bound to a column can be described by dividing the column into equally sized slices. Figure 1 shows a schematic representation of the column resin divided in five slices. Each of these slices is regarded as a
separate unit with its own values for $S_{tot}$ and $S_{free}$. In this approach it is assumed that $E_{tot}$ is the same for all slices. Furthermore, it is assumed that no protein is leaking from the column and that the $K_D$ is not affected by the immobilisation of the protein on the column. When buffer (containing neither $E$ nor $S$), equal in volume to the volume of slice #1, is applied on top of the column, initially slice #1 will sense no free ligand ($S_{free} = 0$). A new equilibrium is established and the free $S$ from slice #1 ($S_{free}^{1}$) now flows to slice #2, where, again, a new equilibrium is reached. Every slice receives the free ligand from the previous slice ($S_{free}^{n}$) and passes its own $S_{free}^{n}$ onto the next slice. In all slices a new distribution between bound $S$ and free $S$ is attained. In this computational approach the number of slices, the $K_D$ and $E_{tot}$ are investigated as potential parameters that influence the washing efficiency. Note that changing the number of slices in our calculations from 1 upward mimics the transition from a 1-step (batch wise) washing procedure to a more sophisticated chromatographic procedure with a well-packed column and a low elution rate. Note also that equilibration is assumed to be instantaneous in the simulations.

**Results & Discussion**

Column-washing profiles were simulated to test how fast a ligand is removed from the binding sites under different conditions. The experimental conditions of the binding of mannitol-saturated EII$_{mtl}$ on Q-Sepharose were used as a reference point: $E_{tot} = 2.5 \mu$M, $S_{tot} = 0.95 \times E_{tot}$, and $K_D = 250$ nM. The result of washing with 20

![Figure 2. The effect of the number of slices on the depletion of liganded binding sites. Parameters: $E_{tot} = 2.5 \mu$M, $S_{tot} = 0.95 \times E_{tot}$, and $K_D = 250$ nM, using 20 column volumes of washing. The number of slices was varied from 1 - 20.](attachment:figure2.png)
The theoretic approach to column washing column volumes on the degree of occupation of the binding sites is shown in Figure 2 as a function of the numbers of column slices. Apparently, increasing the number of slices above 10 has no significant effect on the speed with which the ligand is removed from the binding sites under these conditions. In the simulations with 10 or more virtual slices, 20 column volumes were sufficient to remove more than 95% of the bound ligand. To test the effect of the $E_{\text{tot}}/K_D$-ratio on the efficiency of washing, similar profiles were simulated using 20 slices, with varying $E_{\text{tot}}/K_D$-ratios (Figure 3). Clearly, the $E_{\text{tot}}/K_D$-ratio is important for the efficiency of washing. Above an $E_{\text{tot}}/K_D$-ratio of 20, a large fraction of binding sites remains occupied with ligand.

The experiment described in Figure 7 (Chapter 3) indicates that 20 column volumes of washing buffer were not sufficient to completely remove all $^3$H-mannitol from EII<sub>mtl</sub>. Under the given circumstances ($E_{\text{tot}}/K_D = 10$), 61% of $^3$H-mannitol was removed from the sites, leaving 39% still associated with EII<sub>mtl</sub>. The results of the simulated column-washing experiments (less than 5% of $E$ still occupied with ligand $S$, Figure 2) do not coincide with the experimental values obtained. Non-uniform distribution of the binding sites - i.e. a higher concentration of the protein in the top layers of the column resin - was found not to explain this difference (simulations not shown). A reason for the discrepancy between experiment and simulation could be that the $K_D$ as determined from the experiments described in Chapter 3, was overestimated. Restricted by the $E_{\text{tot}}/K_D$-ratio for accurate determination of binding parameters (the ideal $E_{\text{tot}}/K_D$-ratio must be between 3-30; see Chapter 2), the lowest protein concentration that can be used in our flow-dialysis setup is around 1 μM. Therefore, the $K_D$-value of 250 nM used in the simulations could well be an overestimation. If the actual affinity of purified EII<sub>mtl</sub> for mannitol is 50 nM, than

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Figure 3. The effect of the $E_{\text{tot}}/K_D$-ratio on the efficiency of ligand removal. Parameters: $E_{\text{tot}} = 2.5$ μM, $S_{\text{tot}} = 0.95 \times E_{\text{tot}}$, and $K_D$ was varied from 1 nM – 2.5 μM. The number of slices was set to 20.
under the simulated conditions ~20% of binding sites remain occupied. Another reason that the simulations do not coincide with the experimental values could be a slow release of mannitol from the binding sites. In our simulations, it is assumed that the flow rate of the column is small enough for equilibrium to be attained in all slices at all times. If the dissociation rate constant $k_{\text{off}}$ is very low, more column volumes of washing will be necessary to deplete all sites.

As described above, it is often assumed that bound ligand is removed from the binding sites of an enzyme by a simple chromatography or dialysis step. This is indeed the case when during this step the concentration of the binding sites is not much larger than the $K_D$ of the protein for its ligand. Given the numerous examples of proteins that have affinities for their ligands in the nanomolar or even sub-nanomolar range, it is clear that residual ligand will still be present in such enzyme preparations after dialysis or column washing of the protein in micromolar concentrations using ligand-free buffers.
Removal of detergents by liquid phase separation

Gertjan Veldhuis, Hjalmar Permentier, Bert Poolman, and Ruud M. Scheek

An attempt was made to identify possible endogenous ligand(s) present in EII\textsuperscript{mtl}, using electrospray mass spectrometry. To detect low levels of endogenous ligands, the excess of detergent had to be removed from the purified protein samples. We anticipated that the endogenous ligand would be a hydrophilic molecule and, like mannitol, would partition in the aqueous phase of water:diethylether or water:hexane mixtures. The detergent C\textsubscript{10}E\textsubscript{5} associated with EII\textsuperscript{mtl}, on the other hand, could form inverted micelles in the apolar solvent and be depleted from the aqueous phase.

In Figure 1, the spectrum of 50 μM C\textsubscript{10}E\textsubscript{5} in 10 mM NH\textsubscript{4}-HCO\textsubscript{3} plus 1 mM LiCl is shown. The peak at m/z 385.2 corresponds to C\textsubscript{10}E\textsubscript{5} in complex with a Li\textsuperscript{+} ion. The signals marked with * are (fragments of) plasticizers (phthalic acid). The estimated monoisotopic mass of C\textsubscript{10}E\textsubscript{5} is 378.2 Da, which is in accordance with the calculated mass of 378.6 Da. To follow the removal of the detergent C\textsubscript{10}E\textsubscript{5} from an aqueous solution, 1 mL of 0.25% (w/v) of the detergent was mixed with tracer amounts of \textsuperscript{14}C-labeled C\textsubscript{12}E\textsubscript{8} (related detergent available in radioactive form). Next, 10 mL of diethylether were added and the mixture was thoroughly mixed. The settled diethylether layer was removed and the amount of residual C\textsubscript{12}E\textsubscript{8} in the aqueous phase was determined by liquid scintillation counting. This procedure resulted in the removal of 99% of C\textsubscript{12}E\textsubscript{8} from the aqueous phase. The efficiency of removal of C\textsubscript{10}E\textsubscript{5} from the aqueous phase was confirmed by the spectrum obtained with electrospray mass spectrometry. Next, \textsuperscript{3}H-labeled mannitol was added to the water phase and subsequently washed with diethylether. This resulted in the recovery of ~60% of the \textsuperscript{3}H-mannitol in the aqueous phase. Apparently, the method is efficient in removing PEG-based detergents from aqueous solutions, whereas most of the alditols, such as mannitol, remain in the water phase.

EII\textsuperscript{mtl} was purified as described (Veldhuis et al., 2005a; Chapter 3). To ensure the presence of “endogenous” ligand, 100 μM mannitol was added to one part of the purified preparation as a control. Another control involved the buffer in which EII\textsuperscript{mtl} was eluted. Excess salt was removed from the three samples, using desalting columns (NAP-10, Amersham Biosciences), equilibrated with 10 mM NH\textsubscript{4}HCO\textsubscript{3}. The samples were then treated with diethylether as described above. Unfortunately, the mass spectra did not reveal possible endogenous ligand(s). We also observed that ionization
of mannitol was very inefficient. In fact, not only alditols but also inositol ionized with low efficiency, making it difficult to identify these compounds when present at nanomole quantities. The purified protein samples (~1 mL) contained ~8 nmol Elifmt.

This means that maximally 4 nmol mannitol was present. Already, with 1 nmol of mannitol or inositol (in ~20 μL) it was difficult to detect these molecules in 10 mM NH₄HCO₃. Together with the decreased sensitivity due to the buffer used, it was impossible to detect nanomole amounts of mannitol, let alone that of an endogenous ligand, of which the ionization efficiency might even be lower.

Figure 1. Mass spectrum of C₁₀E₅