Ogden, D. C., & Colquhoun, D. (1983) *Pfluegers Arch. 399*, 246-248.
Ogden, D. C., & Colquhoun, D. (1985) *Proc. R. Soc. London, B 225*, 329-355.
Olson, E. N., Glaser, L., Merlie, J. P., Sebanne, R., & Lindstrom, J. (1983) *J. Biol. Chem. 258*, 13946-13953.
Papke, R., & Oswald, R. (1989) *J. Gen. Physiol. 93*, 785-811.
Papke, R. L., Millhauser, G., Lieberman, Z., & Oswald, R. E. (1988) *Biophys. J. 53*, 1-10.
Pasquale, E. B., Takeyasu, K., Udgaonkar, J. B., Cash, D. J., Severski, M. C., & Hess, G. P. (1983) *Biochemistry 22*, 5967-5973.
Sakmann, B., & Adams, P. R. (1979) *Adv. Pharmacol. Ther. 1*, 81-90.
Sakmann, B., & Neher, E. (1984) *Am. Rev. Physiol. 46*, 455-472.
Sakmann, B., Patlak, J., & Neher, E. (1980) *Nature 286*, 71-73.
Schubert, D., Harris, A. J., Devine, E. E., & Heinemann, S. (1974) *J. Cell Biol. 61*, 398-402.
Shiono, S., Takeyasu, K., Udgaonkar, J. B., Delcour, A. H., Fujita, N., & Hess, G. P. (1984) *Biochemistry 23*, 6889-6893.
Sigworth, F. J. (1983) in *Single-Channel Recording* (Sakmann, B., & Neher, E., Eds.) pp 3-35, Plenum, New York.
Sine, S. M., & Taylor, P. (1979) *J. Biol. Chem. 254*, 3315-3325.
Sine, S. M., & Steinbach, J. H. (1984) *Biophys. J. 46*, 277-284.
Sine, S. M., & Steinbach, J. H. (1986) *J. Physiol. (London) 373*, 129-162.
Sine, S. M., & Steinbach, J. H. (1987) *J. Physiol. (London) 835*, 325-359.
Steinbach, J. H., Covarrubias, M., Sine, S. M., & Steele, J. (1986) in *Nicotinic Acetylcholine Receptor: Structure and Function*, pp 219-231, Springer-Verlag, New York.
Stroud, R. M., McCarthy, M. P., & Schuster, M. (1990) *Biochemistry 29*, 11009-11023.
Takeda, K., & Trautmann, A. (1984) *J. Physiol. (London) 349*, 353-374.
Takeyasu, K., Shiono, S., Udgaonkar, J. B., Fujita, N., & Hess, G. P. (1986) *Biochemistry 25*, 1770-1776.
Udgaonkar, J. B., & Hess, G. P. (1986) *J. Membr. Biol. 93*, 93-109.
Udgaonkar, J. B., & Hess, G. P. (1987a) *Biophys. J.*, 873-882.
Udgaonkar, J. B., & Hess, G. P. (1987b) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8758-8762.
Unwin, N., Toyoshami, C., & Kubalek, E. (1988) *J. Cell Biol. 107*, 1123-1138.
Walker, J. W., McCray, J. A., & Hess, G. P. (1986) *Biochemistry 25*, 1799-1805.
Wilcox, M., Viola, R. W., Johnson, K. W., Billington, A. P., Carpenter, B. K., McCray, J. A., Guzikowski, A. P., & Hess, G. P. (1990) *J. Org. Chem. 55*, 1585-1589.

Mechanics of Solute Translocation Catalyzed by Enzyme II^{mtl} of the Phosphoenolpyruvate-Dependent Phosphotransferase System of *Escherichia coli*†

Juke S. Lolkema,* Dolf Swaving Dijkstra, and George T. Robillard
Department of Biochemistry and Institute BIOSON, University of Groningen, Nijenborgh 4, 9714 AG Groningen, The Netherlands

Received October 3, 1991; Revised Manuscript Received April 3, 1992

ABSTRACT: The kinetics of binding of mannitol to enzyme II^{mtl} embedded in the membrane of vesicles with an inside-out or a right-side-out orientation were analyzed at 4 °C in the absence of the phosphoryl group donor, P-HPr. The binding to the right-side-out oriented vesicles equilibrated too fast to be monitored by the flow dialysis technique. On the other hand, with the inside-out oriented membrane vesicles two conformational changes of the enzyme could be detected kinetically. One change involved a recruitment of binding sites from a state of the enzyme where the binding sites were inaccessible from the cytoplasmic volume. The second change involved a conformational change of the enzyme that followed upon the initial binding to the cytoplasmic-facing binding site leading to a state with a higher affinity for mannitol. Equilibrium binding to the inside-out and right-side-out oriented membrane vesicles at 4 °C indicated that the two transitions did not represent the translocation of the binding site, free and with mannitol bound to it, to the other side of the membrane. Instead, a model is proposed in which the conformational changes represent transitions from states with the binding pocket opened to the cytoplasmic side of the membrane to occluded states of the enzyme in which the binding sites, with or without mannitol bound, are not accessible to either side of the membrane.

Bacterial solute transporters may be classified according to the way energy is provided for the accumulation of the substrate. Accumulation may be achieved through the coupling of the transport to the downhill movement of an ion (H^+ or Na^+) in the secondary transport proteins, to the hydrolysis of ATP in the carriers that use periplasmic binding proteins or the ATPase type carriers, and finally, to the concomitant phosphorylation of the substrate in the case of the P-enolpyruvate-dependent phosphotransferase system. Though these different types of energy coupling to the transport may result in significantly different enzymes, they all have one particular

† This work was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for Scientific Research (NWO).
* To whom correspondence should be addressed.

0006-2960/92/0431-5514$03.00/0

step in common: the translocation of the solute. Nature may have developed different molecular mechanisms for the translocation steps along with the different ways of energy coupling, but it is more likely that they, at least to some extent, resemble one another. As a minimum requirement, a binding site for the solute has to be opened up to one side of the membrane at one point in the catalytic cycle and to the other side of the membrane at some other point. Little is known about the mechanical movements of the protein or the movement of the solute in between these two states.

In general, steady-state kinetics is not very informative about isomeric transitions within the whole set of other transitions that determine the kinetic behavior. Individual transitions have to be isolated from the rest of the kinetic scheme through single-turnover or transient kinetics. This is often difficult because of the rate of these single steps unless one can make use of the specifics of the coupled reaction to trap the enzyme in intermediate states. Our recent understanding of the mechanism of the coupling between transport and phosphorylation catalyzed by the mannitol transport protein of *Escherichia coli* allows us to study transitions related to the translocation of mannitol at a time scale of minutes (Lolkema et al., 1991a, 1990).

The mannitol transport protein of *E. coli* is a member of the P-enolpyruvate-dependent phosphotransferase system (Robillard & Lolkema, 1988). It is termed enzyme II^{mtl}.[1] In the overall reaction, the transport of mannitol into the cell is coupled to its phosphorylation. The phosphoryl group originates from P-enolpyruvate and is donated to the incoming sugar through the sequential phosphorylation and dephosphorylation of a number of proteins, the last one being the carrier protein itself. The direct phosphoryl group donor to enzyme II^{mtl} is a small protein termed P-HPr. Enzyme II^{mtl} is a 68-kDa polypeptide that is believed to function as a dimer (Roossien & Robillard, 1984; Stephan & Jacobson, 1986; Roossien et al., 1986; Pas et al., 1987; Robillard & Blaauw, 1987; Khandekar & Jacobson, 1989; Lolkema & Robillard, 1990). It consists of three domains which according to the nomenclature of Saier and Reizer (1991) are termed IIA, IIB, and IIC (Grisafi et al., 1989; White & Jacobson, 1990; van Weeghel et al., 1991a,b). The former two protrude into the cytoplasm whereas the latter constitutes the transmembrane part of the enzyme. The most C-terminal A domain is phosphorylated by P-Hpr, after which the phosphoryl group is transferred to the B domain. Mannitol accepts the phosphoryl group directly from the B domain.

Recently we have proposed a model for the mechanism by which phosphorylation of the sugar is coupled to the transport of the sugar (Lolkema et al., 1991a). In the model, coupling between phosphorylation and translocation of mannitol is not obligatory, but both partial activities use the inwardly directed mannitol binding site on the translocator C domain. Nevertheless, phosphorylation plays an important role in the transport of mannitol in the sense that phosphorylation of the B domain highly activates the translocation activity of the C domain. Independent evidence has been presented for the functional interaction between the B and C domains involving the phosphorylation site on the former (Lolkema et al., 1991b) and for the translocation activity of the C domain (Lolkema et al., 1990). In this view enzyme II^{mtl} is a facilitated diffusion enzyme whose activity is regulated by the state of phosphorylation of the B domain. This offers the opportunity to study the properties of the translocator domain in the deactivated state.

In a previous study, performed at room temperature, dissociation of mannitol from the translocator domain in the absence of P-HPr was shown to occur on a time scale of minutes (Lolkema et al., 1990). In the present study we will analyze the association kinetics of the binding of mannitol to enzyme II^{mtl} embedded in membrane vesicles under the same conditions but at 4 °C, which slows down the processes even more. The study shows that isomeric transitions of the translocator domain, both with and without mannitol bound to it, can be detected kinetically.

Materials and Methods

Materials. [1-^{3}H(N)]-D-Mannitol (706.7 GBq/mmol) was purchased from NEN Research Products. Decylpoly(ethylene glycol) 300 (decylPEG) was synthesized by B. Kwant in our laboratory.

Cell Growth and Membrane Preparation. E. coli strain ML308-225 was grown at 37 °C in medium 63 (Saier et al., 1976) containing 0.5% mannitol as the carbon source. Cells were grown as described (Lolkema et al., 1991a). ISO membrane vesicles and RSO membrane vesicles were prepared according to Reenstra et al. (1980) and Kaback (1971), respectively. RSO membranes were purified on a sucrose gradient (Lolkema & Walz, 1990; Lolkema et al., 1990). Membranes were stored in 25 mM Tris, pH 7.6, 1 mM DTT, and 1 mM NaN_3 in liquid nitrogen. Membrane protein was measured according to Bradford (1976). Aliquots were thawed rapidly before use, and each sample was used only one time.

Flow Dialysis. Binding of [^{3}H]mannitol to enzyme II^{mtl} was measured with the flow dialysis technique (Colowick & Womack, 1969) as described (Lolkema et al., 1990). All experiments were performed in a buffer containing 25 mM Tris, pH 7.6, 5 mM DTT, and 5 mM $MgCl_2$ and at 4 °C unless otherwise indicated. For the experiments at 4 °C the whole setup was placed in the cold room and the buffer flowing through the lower compartment was preequilibrated at 4 °C as well. The volume of the sample was 400 μL unless otherwise stated. The half-time of the system response was 18 s at 4 °C and 10 s at room temperature and was determined as described (Lolkema et al., 1990). The leak of label from the upper compartment to the dialysate amounted to 1% every 9 min. Since no sample was left in the upper compartment for longer than 7 min, the reduction of the concentration of mannitol due to this leak was neglected. Bound [^{3}H]mannitol was displaced from the binding sites by addition of 100 μM unlabeled mannitol. The exchange rate was analyzed as described (Lolkema et al., 1990).

Results

Kinetics of Binding to Inside-Out (ISO) and Right-Side-Out (RSO) Membrane Vesicles. Binding of mannitol to enzyme II^{mtl} embedded in cytoplasmic membrane vesicles derived from cells that were induced for the mannitol transport system was measured with the flow dialysis technique (Colowick & Womack, 1969; Lolkema et al., 1990). This technique measures, after equilibration, the free substrate concentration in the upper compartment by the radioactivity in the dialysate. Throughout this paper the latter is expressed as the equivalent of the free concentration of mannitol in the upper compartment when the flow dialysis system is at equilibrium. The response of the system to a stepwise change in the free concentration is rather slow with a half-time of about 10 s, at best. Consequently, the time course of the binding of substrate to the

[1] Abbreviations: ISO, inside out; RSO, right side out; DTT, dithiothreitol; decylPEG, decylpoly(ethylene glycol) 300; mtl, mannitol; pts, phosphotransferase system.

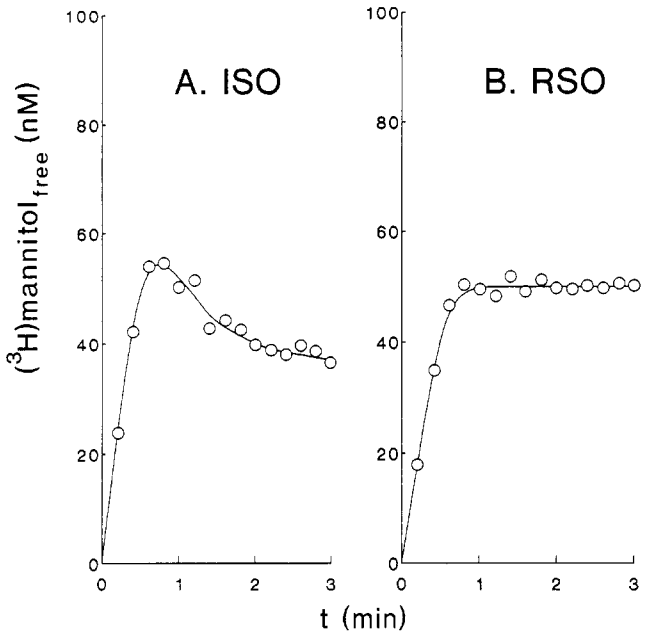


FIGURE 1: Kinetics of binding of [^{3}H]mannitol to ISO and RSO membranes at 4 °C. The response of the flow dialysis system was monitored after addition of 100 nM [^{3}H]mannitol to suspensions of ISO or RSO membrane vesicles. Membrane protein concentrations were 0.38 and 0.56 mg/mL for the ISO and RSO vesicles, respectively.

enzyme should not be much faster than this response in order to be monitored by the system.

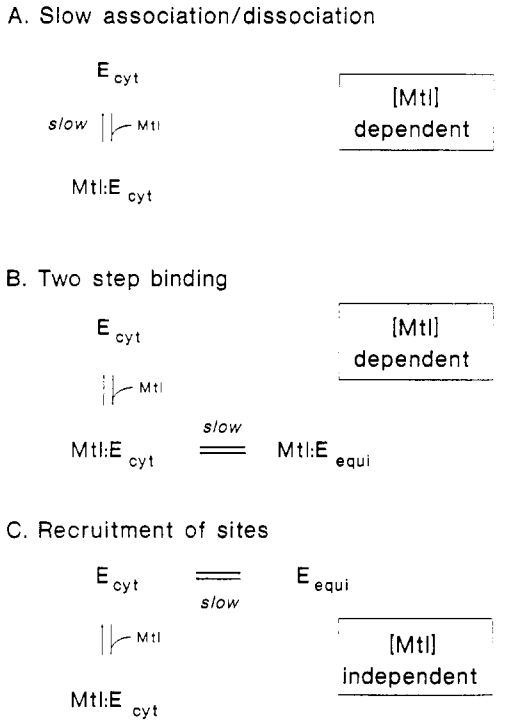
Panels A and B of Figure 1 show the response of the system when 100 nM [^{3}H]mannitol is added to ISO and RSO membrane vesicles, respectively, equilibrated at 4 °C. The free concentration in the upper compartment is 100 nM, initially, and subsequently drops to the equilibrium concentration, which is 49 nM with the RSO vesicles (Figure 1B). Upon the addition of the labeled mannitol to the upper compartment the radioactivity in the dialysate will increase and respond with a certain sluggishness to the changing free concentration during the binding process. In fact, with the RSO vesicles (Figure 1B) the same overall response would have been seen if 49 nM [^{3}H]mannitol had been added in the absence of membranes. This indicates that the binding of mannitol to enzyme II^{mtl} embedded in membrane vesicles with a right-side-out orientation is a process that is much faster than the response time of the flow dialysis system. In contrast, with the ISO membranes the system senses for a significant period of time a higher free concentration than the equilibrium concentration (Figure 1A). Apparently, binding of mannitol to enzyme II^{mtl} embedded in membrane vesicles with an inside-out orientation is a process that is slow enough to be picked up by the flow dialysis system.

The analytical function describing the response with the ISO vesicles would be a combination of the response function of the system (see Materials and Methods) and the second-order binding kinetics. This, in combination with the scatter in the data, does not allow for a quantitative analysis of the data in terms of the rate constants for the binding equilibrium. In the following treatment the rate of the binding process will be qualitatively evaluated by the relative height of the overshoot in the response of the system.

Analysis of the Kinetics of Binding to ISO Membranes. A number of different mechanisms pointed out in Scheme I may contribute to the slow time course of the binding of mannitol to ISO membranes.

(*A*) *Slow Association/Dissociation Equilibrium.* The on and off rate constants of the binding equilibrium would be

Scheme I: Mechanisms To Explain the Slow Binding Kinetics of Mannitol to Enzyme II^{mtl} Embedded in the Membrane of an ISO Vesicle[a]



[a] Capital E indicates enzyme II^{mtl}. The subscripts to E indicate the orientation of the mannitol binding site on the enzyme. This notation for the states of the enzyme will be used throughout this paper. In state E_{cyt} the binding site is accessible from the cytoplasmic phase of the membrane. In state E_{equi} the precise orientation of the binding site is not known, but it is *not* directly accessible from the cytoplasmic volume. In the boxes on the right is indicated whether or not the rate of equilibration of the binding would depend on the mannitol concentration.

small numbers. Since the approach to equilibrium depends upon the product of k_{on} and the free mannitol concentration, increasing the [^{3}H]mannitol concentration increases the rate of binding and, consequently, the overshoot would be reduced.

(*B*) *Two-Step Binding.* Initially, the binding would be to a state with a lower affinity than the equilibrium affinity. The transition of this state (Mtl:E_{cyt}) to a second state with higher affinity (Mtl:E_{equi}) would be the slow step. The latter would only be accessible through the former. The overshoot would depend on the [^{3}H]mannitol concentration since, eventually, higher concentrations would saturate the lower affinity site (E_{cyt}). The transition to the high-affinity site would be silent once all sites are occupied.

(*C*) *Recruitment of Sites.* A fraction of the binding sites would be in a state where they would not be accessible to mannitol at the cytoplasmic side of the membrane. In Scheme I this state (E_{equi}) is labeled with the subscript "equi" in analogy with state Mtl:E_{equi} under mechanism B. These empty sites would slowly recruit to a state were binding can take place (E_{cyt}). The transient would be independent of the [^{3}H]-mannitol concentration.

The first two mechanisms would predict a reduction of the overshoot at higher mannitol concentrations, whereas the third mechanism predicts no effect of the mannitol concentration. A number of experiments were performed to analyze the response in this respect. Different concentrations of [^{3}H]-mannitol were added to ISO membranes equilibrated at 4 °C at such a membrane protein concentration that the bound concentration of mannitol was more or less a constant fraction of the total concentration of mannitol after equilibration

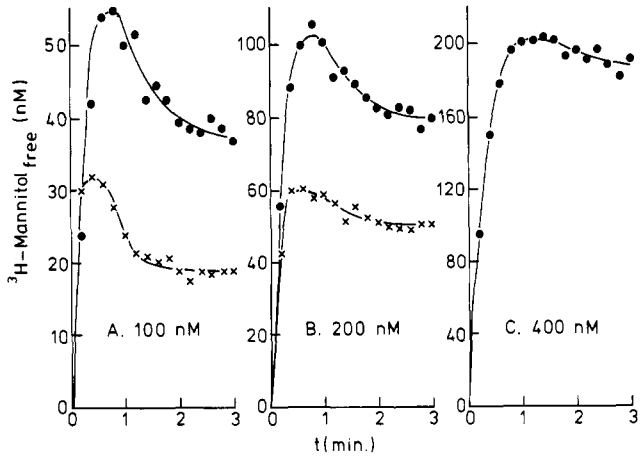


FIGURE 2: Kinetics of binding of [^{3}H]mannitol to ISO membranes at different mannitol concentrations. ISO membranes at membrane protein concentrations of 0.38 mg/mL (A), 0.5 mg/mL (B), or 0.75 mg/mL (C) were equilibrated either at 4 °C (●) or at room temperature (×). At t = 0 the suspensions were made 100 nM (A), 200 nM (B), or 400 nM (C) in [^{3}H]mannitol. The dead time of the system response is left out of the figure.

(Figure 2, ●). Clearly, the pronounced overshoot of the response with 100 nM [^{3}H]mannitol is diminished significantly when the total concentration is raised to 200 nM [^{3}H]mannitol and has almost disappeared at 400 nM [^{3}H]mannitol (filled symbols in Figure 2, panels A, B, and C, respectively). Experiments in which the response was followed for more than 1 h showed, however, that after this mannitol-dependent overshoot had relaxed, there was still a slow increase in binding until equilibrium was reached. The plateau marked by (×) in Figure 2A,B is the final equilibrium level. The time course of this second phase turned out to be independent of the mannitol concentration (see below). Therefore, the kinetics of binding of mannitol to ISO membranes is characterized by a mannitol concentration-dependent component that relaxes within a couple of minutes and a mannitol concentration-independent component that takes much longer to equilibrate.

The experiments with 100 and 200 nM [^{3}H]mannitol were repeated at room temperature (Figure 2, ×). The overshoot seen at 100 nM [^{3}H]mannitol relaxes more rapidly than observed at 4 °C and has almost disappeared when the concentration is raised to 200 nM. The binding has equilibrated after only 2 min. No second slower component could be detected.

The Mannitol Concentration-Dependent Overshoot. The mannitol-dependent overshoot in the binding kinetics to ISO membranes is a manifestation of either the slow binding step to the cytoplasmic binding site or a two-step binding process (Scheme I, parts A and B, respectively). We can discriminate between these two possibilities by analyzing the dissociation process rather than the association process. Addition of a large excess of unlabeled mannitol after complete equilibration of [^{3}H]mannitol and ISO membranes at 4 °C is followed by a slow displacement of labeled mannitol from the binding sites (Figure 3, ○). This exchange of free [^{1}H]mannitol and bound [^{3}H]mannitol measures the dissociation rate of the latter pool from the binding sites (Lolkema et al., 1990). In the case that the mannitol-dependent overshoot would be caused by a slow association/dissociation equilibrium, bound mannitol would dissociate with this rate constant even when the excess of unlabeled mannitol would be added at any time before equilibration of the binding. On the other hand, in the case that the binding would proceed through a lower affinity state (Mtl:E_{cyt}) to a second higher affinity state (Mtl:E_{equi}), a

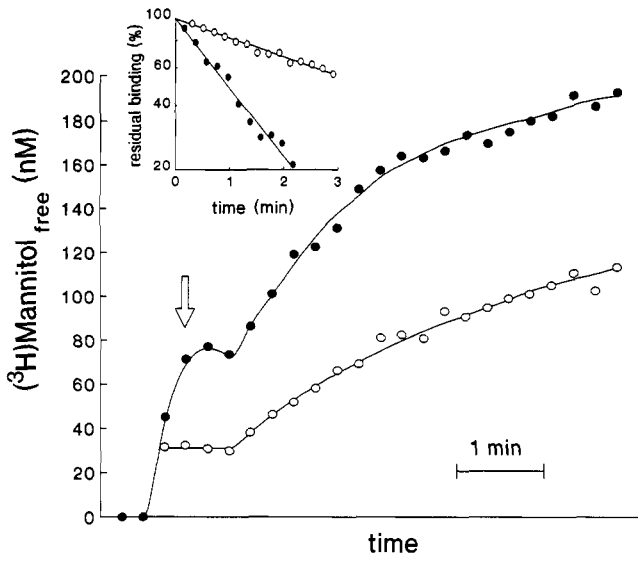


FIGURE 3: The rate of exchange of free [^{1}H]mannitol and [^{3}H]-mannitol bound to ISO membranes before and after equilibration of the binding. The flow dialysis cell equilibrated at 4 °C was loaded with 840 μL of ISO membranes (0.75 mg/mL) and made 200 nM in [^{3}H]mannitol at time zero. Immediately, 440 μL was removed and kept outside the cell at 4 °C. To the remaining 400-μL suspension was added 100 μM unlabeled mannitol at t = 48 s to start the exchange reaction. Samples were collected for 5 min (●). The membranes removed before were left at 4 °C for 90 min to allow the binding to equilibrate. After this period 400 μL of the mixture was reloaded in the flow dialysis cell. Unlabeled mannitol was added (at the arrow) after enough samples were collected to determine the free concentration of mannitol in equilibrium with the bound concentration (○). In the figure the tract indicated by the open symbols is back-shifted 90 min with respect to the track indicated by the closed symbols. In both cases the final concentration of [^{3}H]mannitol was determined by addition of 0.5% of the detergent decylPEG 4 min after the start of the exchange process. This released essentially all label immediately. The data are analyzed in the insert. Plotted is the residual binding after addition of unlabeled mannitol on a logarithmic scale. The amount of binding at the start of the exchange process was calculated from the free concentration of [^{3}H]mannitol at 24 s (or two data points) after the addition of unlabeled mannitol. The half-times of the exchange processes after correction for the response time of the system were 39 s (●) and 191 s (○).

fraction of the bound mannitol molecules may still be present in the low-affinity state and dissociate much more rapidly (see Scheme IB). Addition of excess [^{1}H]mannitol at the top of the overshoot showed a significantly faster exchange, indicating that the step leading to the slow exchange had yet to take place (Figure 3, ●). This experiment shows that the binding of mannitol to ISO vesicles is a two-step phenomenon.

The displacement of bound mannitol after complete equilibration follows a single exponential, indicating that, eventually, all bound mannitol molecules end up in state Mtl:E_{equi}. The half-time for dissociation from this state to the cytoplasm equals 191 s (insert of Figure 3, ○). The exchange rate observed after addition of unlabeled mannitol at the top of the overshoot would represent the dissociation rate constant of mannitol bound to the cytoplasmic-facing binding site. The residual binding versus time gives a reasonable fit to an exponential in the first 2 min, indicating that the fraction of bound mannitol molecules in the Mtl:E_{equi} state in this period is still small (insert of Figure 3, ●). The dissociation rate constant equals 39 s. The rapid decrease of the overshoot in going from 100 to 400 nM [^{3}H]mannitol (Figure 2, panels A–C) indicates that the binding constant of mannitol to the cytoplasm-facing site on E_{cyt} is well below 400 nM. Therefore, at the concentrations of mannitol used in these experiments, the pseudo-first-order association rate constant, k_{on}[Mtl], will

Table I: Analysis of the Kinetics of Binding of Mannitol to ISO Membranes at High Mannitol Concentrations[a]

	$[[^3H]mannitol]_{tot}$	
	800 nM	2.4 μM
$[[^3H]mannitol]_{bound}$ (t = 0) (pmol/mg)	253	265
$[[^3H]mannitol]_{bound}$ (t = ∞) (pmol/mg)	357	392
k (min^{-1})	0.147	0.122

[a] Experiments were performed as described in the legend to Figure 4 and analyzed as explained in the text. The membrane protein concentrations in the experiments with 800 nM and 2.4 μM mannitol were 1 and 2.8 mg/mL, respectively.

be in the same order of magnitude as the dissociation constant. Consequently, the overshoot in the free concentration is caused both by the slow association of mannitol to state E_{cyt} and by the slow transition to state $Mtl:E_{equi}$.

The two steps in the binding of mannitol to ISO membranes were also demonstrated at room temperature. The exchange of mannitol bound to ISO vesicles with free mannitol at room temperature has been analyzed before and was shown to have a half-time of about 60 s (Lolkema et al., 1990). Addition of excess unlabeled mannitol at the top of the overshoot at room temperature (see Figure 2A, ×) resulted in a biphasic dissociation behavior, indicating that a smaller fraction of the bound mannitol molecules could be trapped in state $Mtl:E_{cyt}$ (not shown).

The Mannitol Concentration-Independent Overshoot. The recruitment of binding sites to the cytoplasmic face of the membrane at 4 °C is analyzed in Figure 4 and Table I. At a total mannitol concentration of 800 nM the mannitol-dependent contribution to the equilibration of the binding is invisible. Nevertheless, after addition of 800 nM [^{3}H]mannitol to the ISO membranes, the initial rise of the response of the flow dialysis system is followed by a slow decrease in the free concentration (Figure 4, ●). Samples were taken during the first 7 min, after which the mixture was removed from the cell and left on the bench at 4 °C for 90 min. This was done to minimize the leak of mannitol out of the flow dialysis system (see Materials and Methods). Sampling was resumed after reloading of the sample after 90 min. No change in the free concentration was observed, indicating a concentration of bound mannitol of 441 nM at equilibrium (Figure 4, ○). The data were analyzed by plotting the difference between the free concentration at the indicated time points and the free concentration after equilibration in a semilogarithmic plot (Figure 4, insert). The data fit a single exponential with a slope of 0.139 min^{-1}. This exponential is a good approximation of the kinetics of the recruitment of sites since it is considerably slower than the response time of the flow dialysis system and the binding of mannitol to the recruited site. Back-extrapolation of the exponential to time t = 0 indicates that at the beginning of the experiment 274 nM or 62% of the sites were accessible from the cytoplasmic side of the membrane.

Two similar experiments were performed with a different membrane preparation at total concentrations of mannitol of 800 nM and 2.4 μM. Both the increase in binding over a period of 90 min and the rate constant for the increase were not significantly different in the two experiments (Table I). Therefore, the increase of binding is consistent with a recruitment of binding sites to the cytoplasmic phase of the membrane. The level of binding represents the total concentration of binding sites since the binding is essentially saturated.

The equilibrium constant between the two unloaded states of the enzyme may be calculated from the increase in the binding in these experiments. Averaging over the three shown experiments results in a value for $K(E_{equi} \leftrightarrow E_{cyt})$ of 2.1 ± 0.5.

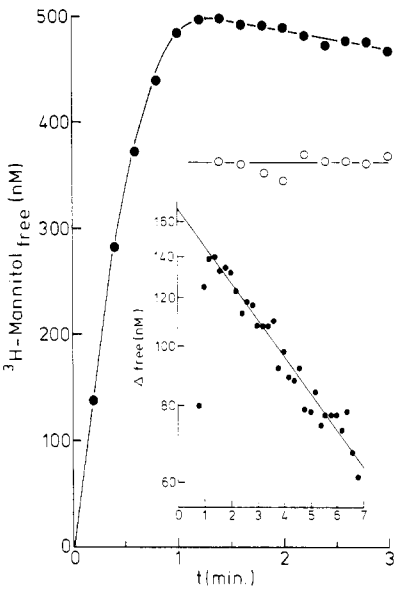


FIGURE 4: Kinetics of binding of [^{3}H]mannitol at high concentration to ISO membranes at 4 °C. A suspension of ISO membranes at a protein concentration of 1.0 mg/mL was made 800 nM in [^{3}H]-mannitol at time zero. Samples were collected for 7 min. Then the membranes were removed from the cell and left for 90 min at 4 °C. After this time the membranes were loaded again and additional samples were collected. Main plot. The response of the flow dialysis system during the first 3 min (●) and after 90 min (○). The dead time of the system response is left out of the figure. Insert. The decline in the free concentration during the first 7 min on a logarithmic scale. The data points indicate the difference between the free concentration of mannitol (●, main plot) minus the constant free concentration measured after 90 min (○, main plot). The slope of the line equals 0.139 min^{-1}.

The back and forward rate constants would be $k(E_{equi} \rightarrow E_{cyt})$ = 0.136 ± 0.012 min^{-1} and k ($E_{cyt} \rightarrow E_{equi}$) = 0.06 ± 0.02 min^{-1}, respectively.

Equilibrium Binding to RSO and ISO Vesicles. A plausible explanation for the physical meaning of states E_{equi} and $Mtl:E_{equi}$ would be that they represent those states of the enzyme with the binding site accessible from the periplasmic side of the membrane which is inside the ISO vesicle (see Scheme IIB). The transitions between E_{cyt} and E_{equi} on the one hand and $Mtl:E_{cyt}$ and $Mtl:E_{equi}$ on the other hand would represent the translocation of the unloaded and loaded binding site, respectively. Consequently, binding of mannitol to ISO or RSO membrane vesicles would end up in exactly the same situation once the binding has equilibrated. Furthermore, the equilibrium distribution of states E_{cyt} and E_{equi} together with the rate constant for the transition $E_{cyt} \rightarrow E_{equi}$ of 0.087 min^{-1} would predict a slow increase ($t_{1/2}$ = 8 min) in the binding to RSO vesicles after the immediate binding to 38% of the sites that would be accessible from the periplasmic volume at t = 0. The experiment in Figure 5 compares the binding of mannitol to ISO and RSO vesicles.

The membrane protein concentration of the RSO and ISO vesicles used in the experiments described in Figure 5 was chosen such that the enzyme II^{mtl} concentration was about the same. This was checked by solubilization of the membranes which resulted in approximately the same level of binding in the two preparations (Figure 5, open symbols). Before the addition of decylPEG to the ISO vesicles the response of the flow dialysis system went through all the stages described in the previous paragraphs to end up at an equilibrium distribution of 45 nM free and 155 nM bound [^{3}H]mannitol (Figure 5, ●). The decrease in the binding upon addition of the detergent decylPEG is due to a change in the affinity constant

Scheme II: The Physical Meaning of State E_{equi}[a]

A. Induced fit

$$\begin{array}{ccc} E_{cyt} & = & {}^{*}E_{cyt} \\ \| \text{-Mtl} & & \| \text{-Mtl} \\ Mtl{:}E_{cyt} & = & Mtl{:}{}^{*}E_{cyt} \end{array}$$

B. Translocation

$$\begin{array}{ccc} E_{cyt} & = & E_{per} \\ \| \text{-Mtl} & & \| \text{-Mtl} \\ Mtl{:}E_{cyt} & = & Mtl{:}E_{per} \end{array}$$

C. Occlusion

$$\begin{array}{ccc} E_{cyt} & = & E_{occl} \\ \| \text{-Mtl} & & \\ Mtl{:}E_{cyt} & = & Mtl{:}E_{occl} \end{array}$$

[a] (A) Induced fit. E_{equi} has been replaced with $^{*}E_{cyt}$. The binding site on both E and *E are accessible from the cytoplasmic volume. (B) Translocation. E_{equi} has been replaced by E_{per}. The binding site on the latter is only accessible from the periplasmic volume. (C) Occlusion. E_{equi} is replaced by E_{occl}. The binding site on the latter is not accessible from either side of the membrane, and states E_{occl} and $Mtl{:}E_{occl}$ do not interconvert.

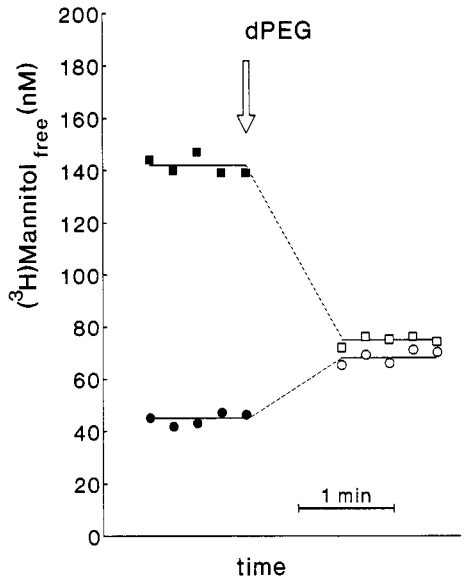


FIGURE 5: Equilibrium binding to RSO and ISO vesicles at 4 °C. Suspensions of RSO and ISO membrane vesicles (0.39 and 0.5 mg/mL, respectively) were made 200 nM in [^{3}H]mannitol. The response of the flow dialysis system was followed for 7 min. The mixtures were then removed from the cell and left for 90 min at 4 °C after which the samples were reloaded and the recording was continued. The plot shows the equilibrium concentration of free mannitol after 90 min for the RSO (■) and the ISO (●). Subsequently, 0.5% of the detergent decylPEG was added to the RSO (□) and ISO (○) membranes, and the free concentration was measured after equilibration of the system.

(Lolkema et al., 1990). The binding to the RSO vesicles that contained the same amount of enzyme II^{mtl} was much less at 4 °C, only 58 nM (Figure 5, ■). This level of binding does not change within 90 min after addition of the [^{3}H]mannitol to the membranes. Therefore, binding of mannitol to enzyme II^{mtl} embedded in the membrane of RSO or ISO vesicles does not result in the same situation at 4 °C, and no recruitment of sites from the cytoplasmic to the periplasmic side of the membrane could be detected. The implication of this result will be treated in the Discussion.

Discussion

Transitions of the Enzyme versus Passive Diffusion. Two transients in the kinetics of mannitol binding to ISO vesicles were attributed to transitions between states of enzyme II^{mtl}. The mannitol concentration-independent transient was attributed to the recruitment of binding sites to the cytoplasmic phase of the membrane ($E_{equi} \rightarrow E_{cyt}$, see Scheme I) and part of the mannitol concentration-dependent transient was attributed to a transition that followed upon the initial binding event ($Mtl{:}E_{cyt} \rightarrow Mtl{:}E_{equi}$). It is important to show that both transients really are related to the enzyme and not due to passive diffusion of mannitol across the membrane and subsequent binding to binding sites at the inner face of the ISO vesicle. In the latter case the passive diffusion would determine the rate of equilibration of the binding since binding to RSO vesicles showed kinetics that was fast relative to the response time of the system (Figure 1B). The best indication that passive diffusion of mannitol across the membrane at 4 °C is too slow to play a role comes from the experiment described in Figure 5. The level of binding to ISO and RSO vesicles was compared under conditions where the total concentrations of enzyme II^{mtl} in the two preparations were identical. The ISO membranes bound 155 nM mannitol, whereas the RSO vesicles bound only 58 nM mannitol. The latter level of binding was stable over a 90-min period. Therefore, even though there is a pool of binding sites of at least 155 nM, most of these are not accessible within 90 min in RSO vesicles, indicating no significant leaking of mannitol into the vesicles. Apparently, the cytoplasmic membrane is highly impermeable to mannitol at 4 °C.

Further evidence for the enzymatic transitions comes from the asymmetry in the kinetics and the size of the transients in the opposite directions. For instance, it could be argued that the slow increase in binding from 274 to 441 nM after addition of a high concentration of mannitol demonstrated in Figure 4 might be due to a slow leaking of mannitol into the vesicle ($t_{1/2}$ = 5 min) and subsequent binding to internal binding sites. However, if this were the case, addition of excess unlabeled mannitol after equilibration should result in the rapid release of 274 nM of mannitol followed by a release of the other 167 nM with a half-time of 5 min. In contrast, the whole population of bound mannitol is released with a half-time of about 3 min (Figure 3, insert). The same argument holds for the mannitol-dependent transient (compare Figures 2A and 3).

Analogy between States E_{equi} and $Mtl{:}E_{equi}$. Analysis of the mannitol concentration-dependent phase of the kinetics of binding of mannitol to enzyme II^{mtl} embedded in the membrane of ISO vesicles indicated that the binding to the cytoplasmic-facing binding site was followed by a transition to a state that we denoted as $Mtl{:}E_{equi}$ (see Scheme I). On the other hand, for a fraction of the binding sites, there was a transition preceding binding, $E_{equi} \rightarrow E_{cyt}$, giving rise to a mannitol concentration-independent component in the binding kinetics. It is likely that states E_{equi} and $Mtl{:}E_{equi}$ represent the same states with respect to the nature of the binding site on enzyme II^{mtl}, the only difference being that a molecule of mannitol is bound to $Mtl{:}E_{equi}$ and not to E_{equi}. We have

anticipated this by using the same subscript for the two states. Transitions $E_{equi} \Leftrightarrow E_{cyt}$ and $Mtl:E_{equi} \Leftrightarrow Mtl:E_{cyt}$ would represent the same change of the unloaded and loaded carrier, respectively. The very fact that analogous transitions exist for the unloaded and loaded carrier implies that the transitions represent conformational changes of the protein, rather than the physical movement of the substrate from one binding site to another. Evidence has also been presented earlier against the movement of substrate from one binding site to another. We showed that, after equilibration, the rate of exchange between [^{3}H]mannitol bound to ISO membranes and free [^{1}H]mannitol was independent of the concentration of the latter up to 1 mM, indicating that a vacant binding site on the $Mtl:E_{equi}$ complex is not very likely (Lolkema et al., 1990). Using the same kind of reasoning, the same conclusion was reached in the case of the lactose carrier of *E. coli* (Lolkema et al., 1991c).

Therefore, the states E_{cyt} and E_{equi} seem to represent different conformations of the protein with a single binding site.

Accessibility of the Binding Site on E_{equi} from the Cytoplasmic Side of the Membrane. It was stated in the results that the state $Mtl:E_{equi}$ was only accessible through the state $Mtl:E_{cyt}$. A possible explanation for the transition $Mtl:E_{cyt} \rightarrow Mtl:E_{equi}$ would be an "induced fit" type of mechanism; i.e., binding of mannitol to the enzyme would induce a conformational change in the protein leading to a stronger binding, but leaving the substrate in contact with the cytoplasmic volume. A kinetic scheme according to such a mechanism, including the two conformations for the unloaded carrier, is depicted in Scheme IIA (subscript "equi" has been substituted for "cyt"). Under the experimental conditions described in this paper, states $*E_{cyt}$ and $Mtl:*E_{cyt}$ would only communicate with the cytoplasmic volume through states E_{cyt} and $Mtl:E_{cyt}$, respectively. Therefore, for the scheme to properly describe our results, either the affinity of mannitol for state $*E_{cyt}$ would have to be lower than for state E_{cyt} or the rate constants for the corresponding association/dissociation equilibrium would have to be much smaller. Our data indicate that the latter is the case. The difference in free energy between states E_{cyt} and E_{equi} could be quite accurately estimated from the experiment shown in Figure 4 and turned out to be very small. On the other hand, the difference in free energy between states $Mtl:E_{cyt}$ and $Mtl:E_{equi}$ could only be evaluated qualitatively. The single exponential for the exchange, after equilibration of the binding, between mannitol bound to the ISO vesicles and excess unlabeled mannitol suggests that the free energy of state $Mtl:E_{equi}$ is significantly lower than the free energy of state $Mtl:E_{cyt}$. At least qualitatively, this is confirmed by the kinetic data. The mannitol concentration-dependent phase of the binding kinetics, which is determined by the association step itself and the transition to state $Mtl:E_{equi}$, was completed in about 2 min (e.g., see Figure 2A) whereas the same steps in the reverse direction as measured by the exchange between bound and free mannitol (Figure 3, O) resulted in a half-time of over 3 min.

In conclusion, the relative free energies of the states requires that the affinity of state $*E_{cyt}$ for mannitol is higher than the affinity of state E_{cyt} for mannitol, and therefore, for the explanation given in Scheme IIA to comply with the experimental data, the accessibility of the binding site on state $*E_{cyt}$ from the cytoplasmic volume ought to be kinetically restricted. Direct access of state $*E_{cyt}$ should still be possible by increasing the mannitol concentration in a binding experiment like the one described in Figure 4. We did not see any effect up to a concentration of 2.4 μM mannitol (Table I), and though this does not exclude that Scheme IIA might be the correct one, it does allow for the more general conclusion that the accessibility of the binding site in state E_{equi} from the cytoplasmic volume is very low.

The Physical Meaning of States E_{equi} and $Mtl:E_{equi}$. We tend to believe that the transitions that we have detected in the kinetics of binding of mannitol to ISO membranes are related to the function of enzyme II^{mtl} to transport mannitol across the membrane. For this purpose we will extrapolate the general conclusion stated above to its extreme: the binding site in states E_{equi} and $Mtl:E_{equi}$ is not accessible from the cytoplasmic volume at all. Physically this would mean that the protein itself would form a barrier between the binding site and the cytoplasmic water phase. Upon binding of mannitol to the cytoplasmic-facing binding site, the protein would fold around the substrate as a first step in the translocation of the substrate to the other side of the membrane. The closing of the protein at the cytoplasmic side of the membrane could leave the substrate or the empty binding site hidden in the protein. On the other hand, the closing at one side could be in concert with the opening of the binding site to the other side of the membrane (see Scheme IIB,C). In the former case (Scheme IIB) equilibrium binding to ISO and RSO membranes would be the same, a conclusion that we reached in a previous report based upon experiments performed at room temperature (Lolkema et al., 1990). However, the present results indicate clearly that equilibrium binding to RSO and ISO vesicles did *not* result in the same situation when the experiments were performed in the cold room (Figure 5). Therefore, Scheme IIC is more likely to be the correct one.

Mechanics of Mannitol Translocation. The discrimination between translocation and occlusion of the substrate is fully based upon a comparison of the characteristics of the binding of mannitol to enzyme II^{mtl} embedded in the membrane of ISO and RSO vesicles. These membrane preparations are prepared according to considerably different procedures (see Materials and Methods) which could cause enzyme II^{mtl} to behave slightly differently in the two types of membranes and, potentially, lead us to the wrong conclusion. Provided that this is not the case, the present results suggest the model depicted in Figure 6 for the mechanics involved in the translocation of mannitol by enzyme II^{mtl}. Translocation would involve two major conformational changes in the protein: (i) closing of the binding pocket to one side of the membrane and (ii) opening of the binding pocket to the other side of the membrane. In between these two changes are, for both the loaded and unloaded binding site, well-defined states in which the binding site is not accessible from either side of the membrane. The enzyme works like a sluice, thereby preventing leakage from one compartment to the other. Both the transitions from the states with the binding sites opened up to the cytoplasmic side of the membrane to the two occluded states could be demonstrated by lowering the temperature to 4 °C. At this temperature the binding pocket does not seem to open to the periplasmic side of the membrane.

The Binding Sites and Subunit Structure of Enzyme II^{mtl}. Binding of mannitol to ISO membranes behaves like binding to a single population of binding sites, and we have treated it accordingly. The binding to RSO vesicles seems to represent a physically distinct population of sites. It is not a surprise that two distinct sites seem to exist since enzyme II^{mtl} is believed to function as a dimer. Scatchard analysis of the binding of mannitol to purified enzyme II^{mtl} solubilized in detergent revealed two different binding sites per dimer, indicating some interaction between the binding sites on the monomeric sub-

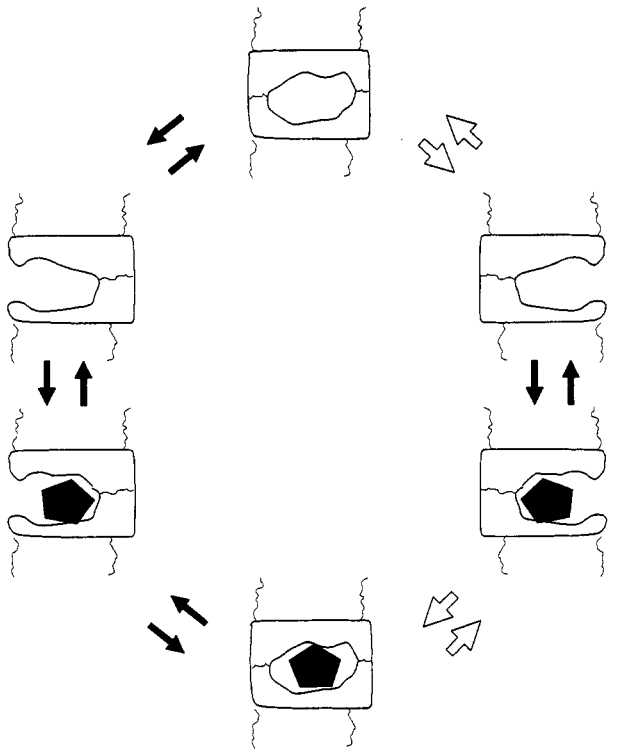


FIGURE 6: Model for the mechanics of mannitol translocation catalyzed by enzyme II^{mtl}. The mannitol "loaded" or "unloaded" binding site can be opened to either side of the membrane, but never at the same time. In between these two states is a well-defined state where the binding site is closed. This leads to occlusion of the mannitol molecule. With the cytoplasm and periplasm on the left- and right-hand side, respectively, the transitions indicated by solid arrows were demonstrated in this study. The pentangle symbolizes the mannitol molecule.

units (Pas et al., 1988). In combination with the present results this seems to suggest that the binding site on one subunit is oriented in one direction whereas the site on the other subunit is oriented in the opposite direction. At 4 °C binding of mannitol to ISO membranes would be to the binding site on just one of the subunits, leaving the site on the other subunit unoccupied because it would be oriented to the interior of the vesicle. With RSO vesicles this would just be the other way around. Binding to the subunit with the binding site oriented to the periplasm would equilibrate rapidly on the time scale of the present experiments (Figure 1B) whereas the conformational changes described in this paper would be in the subunit with the binding site oriented to the cytoplasm.

From this suggestion it is only a small step to propose a functional coupling between the orientation of the binding sites on the two subunits within the dimer during turnover of the enzyme. The cooperativity between the two subunits may be such that the catalytic cycle of the individual subunits is coupled at the level of the translocation step. The activation energy for the translocation of the binding site on one subunit would be much lower if at the same time the binding site on the other subunit would translocate in the opposite direction. At present we are trying to get more experimental evidence both for the opposite orientations of the binding sites on the dimer and for the conformational coupling between the sites during turnover.

References

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Colowick, S. P., & Womack, F. C. (1969) *J. Biol. Chem. 244*, 774–777.

Grisafi, R. L., Scholle, A., Sugayama, J., Briggs, L., Jacobson, G. R., & Lengeler, J. W. (1989) *J. Bacteriol. 171*, 2719–2727.

Kaback, H. R. (1971) *Methods Enzymol. 22*, 99–120.

Khandekar, S. S., & Jacobson, G. R. (1989) *J. Cell. Biochem. 39*, 207–216.

Lolkema, J. S., & Robillard, G. T. (1990) *Biochemistry 29*, 10120–10125.

Lolkema, J. S., & Walz, D. (1990) *Biochemistry 29*, 11180–11188.

Lolkema, J. S., Dijkstra, D. S., ten Hoeve-Duurkens, R. H. & Robillard, G. T. (1990) *Biochemistry 29*, 10659–10663.

Lolkema, J. S., ten Hoeve-Duurkens, R. H., Dijkstra, D. S., & Robillard, G. T. (1991a) *Biochemistry 30*, 6716–6721.

Lolkema, J. S., Dijkstra, D. S., ten Hoeve-Duurkens, R. H., & Robillard, G. T. (1991b) *Biochemistry 30*, 6721–6726.

Lolkema, J. S., Carrasco, N., & Kaback, H. R. (1991c) *Biochemistry 30*, 1284–1290.

Pas, H. H., Ellory, J. C., & Robillard, G. T. (1987) *Biochemistry 26*, 6689–6696.

Pas, H. H., ten Hoeve-Duurkens, R. H., & Robillard, G. T. (1988) *Biochemistry 27*, 5520–5525.

Reenstra, W. W., Patel, L., Rottenberg, H., & Kaback, H. R. (1980) *Biochemistry 19*, 1–9.

Robillard, G. T., & Blaauw, M. (1987) *Biochemistry 26*, 5796–5803.

Robillard, G. T., & Lolkema, J. S. (1988) *Biochim. Biophys. Acta 974*, 493–519.

Roossien, F. F., & Robillard, G. T. (1984) *Biochemistry 23*, 5683–5685.

Roossien, F. F., van Es-Spiekman, W., & Robillard, G. T. (1986) *FEBS Lett. 196*, 284–290.

Saier, M. H., & Reizer, J. (1991) *J. Bacteriol.* (in press).

Saier, M. H., Simoni, R. D., & Roseman, S. (1976) *J. Biol. Chem. 251*, 6584–6597.

Stephan, M. M., & Jacobson, G. R. (1986) *Biochemistry 25*, 4046–4051.

van Weeghel, R. P., Meyer, G. H., Keck, W. H., & Robillard, G. T. (1991a) *Biochemistry 30*, 1774–1779.

van Weeghel, R. P., Meyer, G. H., Pas, H. H., Keck, W. H., & Robillard, G. T. (1991b) *Biochemistry 30*, 9478–9485.

White, D. W., & Jacobson, G. R. (1990) *J. Bacteriol. 172*, 1509–1515.