The Maltose Transport System of Escherichia coli Displays Positive Cooperativity in ATP Hydrolysis*

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Maltose transport across the cytoplasmic membrane of Escherichia coli is catalyzed by a periplasmic binding protein-dependent transport system and energized by ATP. The maltose system, a member of the ATP-binding cassette or ABC transport family, contains two copies of an ATP-binding protein in a complex with two integral membrane proteins. ATP hydrolysis by the transport complex can be assayed following reconstitution into proteoliposomes in the presence of maltose binding protein and maltose. Mutations in the transport complex that permit binding protein-independent transport render ATP hydrolysis constitutive so that hydrolysis can also be assayed with the transport complex in detergent solution. We have used both of these systems to study the role of two ATP binding sites in ATP hydrolysis. We found that both the wild-type and the binding protein-independent systems hydrolyzed ATP with positive cooperativity, suggesting that the two ATP binding sites interact. Vanadate inhibited the ATPase activity of the transport complex with 50% inhibition occurring at 10 μM vanadate. In detergent solution, the degree of cooperativity in the binding protein-independent complex decreased with increasing pH. The loss of cooperativity was accompanied by a decrease in ATPase activity and a decrease in sensitivity to vanadate. Because reconstitution of the complex into a lipid bilayer prevented the loss of cooperativity, we expect that ATP hydrolysis is cooperative in vivo. The mutations leading to binding protein-independent transport do not significantly alter the affinity, cooperativity, vanadate sensitivity, or substrate specificity of the ATP binding sites during hydrolysis. These results justify the use of the binding protein-independent system to investigate the mechanism of transport and hydrolysis.

The maltose transport system of Escherichia coli is one of a class of periplasmic binding protein-dependent systems that function to transport nutrients across the cytoplasmic membrane against a concentration gradient. These systems are part of a larger family of ATP-dependent transporters termed ATP binding cassette or ABC proteins (1). Members of the family are involved in many diverse functions, including ion transport (2), drug transport (3, 4), and polysaccharide transport (5, 6). A prominent feature of this family is its multi-domain, or multi-subunit structure, which includes two hydrophobic subunits capable of spanning the membrane in α-helical conformation and two hydrophilic subunits capable of binding ATP. The maltose transport complex consists of MalF, MalG, and two copies of MalK, an ATP-binding protein (9, 10). Reconstitution of the purified transport complex (MalFGK₂) into proteoliposome vesicles demonstrated that these three proteins, the periplasmic maltose binding protein (MBP) (11), and ATP are necessary and sufficient for maltose transport (9).

In proteoliposomes, MalFGK₂ requires both maltose and MBP to hydrolyze ATP (11). However, mutations in malF and malG, isolated as a consequence of their ability to support maltose transport in the absence of the MBP (12), permitted ATP hydrolysis to occur in the absence of maltose and MBP (11). These experiments formed the basis for the current model for transport (Fig. 1A), in which the interaction of the maltose-MBP complex with the transport complex shifts the conformation of the transport complex from an inactive form (I), which cannot hydrolyze ATP, to an active form (II), which can hydrolyze ATP and therefore transport maltose (III). This conformational change coordinates the appearance of maltose in the periplasm with ATP hydrolysis. The mutations that render transport binding protein independent stabilize the same active form (II) (Fig. 1B). This model predicts that during ATP turnover, the wild-type and mutant complexes are functionally identical. One goal of the present work is to test this hypothesis by characterizing hydrolysis in both the wild-type and the mutant systems. If they are similar, then we expect that the mutations have stabilized a normal intermediate in the reaction pathway and that use of the binding protein-independent mutants will facilitate the elucidation of the mechanism of ATP-dependent translocation. Unlike the wild-type transport complex, the mutant complex will hydrolyze ATP while in detergent solution, simplifying many biochemical and biophysical measurements.

Little is known about the molecular mechanism of ATP hydrolysis and transport in this family. The conservation of two nucleotide binding domains in this family of proteins suggests that both will be essential for transport activity. A second goal of this work is to examine the kinetics of ATP hydrolysis to look for interactions between the two ATP binding sites in the maltose transport complex. We find that ATP is hydrolyzed with positive cooperativity.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—Strain HN741 (E. coli K-12 argH his rpsL1 malT° malB131 ΔatpBC ilv::Tn10/ F’ lacI9 Tn5) (11) containing chromosomal deletions of the genes encoding the maltose transport system

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§The abbreviations used are: MBP, maltose binding protein; MDR, multidrug resistance protein; octyl glucoside, octyl β-D-glucopyranoside; dodecyl maltoside, n-dodecyl β-D-maltoside; Mes, 2-(N-morpholino)ethanesulfonic acid; Mops, 3-(N-morpholino)propanesulfonic acid; Caps, 3-(cytchexylamino)-1-propanesulfonic acid.

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and the FoF1-ATPase was simultaneously transformed with plasmids pFG23, carrying the malF and malG genes under control of the trc promoter, and pM11, carrying the malK gene under trc control (13). In some experiments, pFG42, carrying the malF500 allele, was substituted for pFG23. The plasmid pMS421 carrying lad” (14) was also present.

Freshly transformed cells were grown and induced for overexpression as described (9). Cell envelope fractions were prepared as described (11) and stored at -70°C until use.

Solubilization of Membrane Proteins—Membrane vesicles were thawed, washed by dilution in 20 mm Tris-HCl, pH 8, 1 mm EDTA, and collected by centrifugation at 100,000 × g for 30 min. Vesicles were resuspended in a solution of 20 mm Tris-HCl, pH 8, 5 mm MgCl₂, 1 mm dithiothreitol, and 20% glycerol, and proteins were solubilized by the addition of the detergents octyl glucoside or dodecyl maltoside to final concentrations of 1%. After a 20-min incubation at 0°C, the particulate fraction was removed by centrifugation for 30 min at 100,000 × g, and the supernatant was saved for direct assay (dodecyl maltoside) or preparation of proteoliposomes (octyl glucoside).

Preparation of Proteoliposome Vesicles—Proteins solubilized from membrane vesicles using octyl glucoside were reconstituted into E. coli phospholipid vesicles by detergent dilution (13). To stimulate ATP hydrolysis by the wild-type complex, MBP and maltose were trapped inside the vesicles by including them prior to dilution (11).

Assay of ATP Hydrolysis—For kinetic analyses, proteoliposome vesicles were incubated at room temperature in buffers containing, unless otherwise indicated, 50 mm Tris-HCl, pH 8, 5 mm MgCl₂, 1 mm dithiothreitol, and [γ-³²P]ATP (specific activity, 250–2,000 cpm/mmol). Assays with proteins solubilized in dodecyl maltoside contained in addition 20% glycerol and 0.01% dodecyl maltoside. Reactions were terminated with perchloric acid, and [³²P]Pi was assayed as described by Lill et al. (15). The computer program KALEIDAGRAPH (Synergy Software, Reading, PA) was used to fit kinetic data by unweighted least squares regression to the Michaelis-Menten equation

\[
\frac{v}{V_{\text{max}}} = \frac{[S]}{K_m + [S]} 
\]

and the Hill equation

\[
\frac{v}{V_{\text{max}}} = \frac{[S]^n}{K_{\text{Hill}} + [S]^n} 
\]

where \( v \) = velocity, \([S] = [\text{ATP}], V_{\text{max}} = \text{maximum velocity}, K_{\text{Hill}} = \text{the concentration of } S \text{ where } v = \frac{1}{2} V_{\text{max}}, \) and \( n \) = the Hill coefficient, or the number of substrate binding sites per molecule of enzyme. Chi square analysis was used to determine which equation best fit the data (16).

The ability of the ATPase to hydrolyze other nucleotides was tested as described above using sodium acetate buffer at pH 5 for the detergent-soluble form of the enzyme and Bis-Tris at pH 6 for the reconstituted systems, except that unlabeled substrates were employed and Pi was determined using a microtiter plate method (17). Absorbance was read at 650 nm.

Other Materials or Methods—[γ-³²P]ATP was obtained from Amer sham Corp. (PB10168). Sodium orthovanadate was obtained from Aldrich. Protein concentrations were determined as described previously (9) using the method of Schaffner and Weissmann (18).

RESULTS

pH Dependence of ATP Hydrolysis—ATP hydrolysis was assayed as a function of pH using either the wild-type transport complex or a transport complex generated from the malF500 allele, which had the highest constitutive ATPase activity of the binding protein-independent mutants tested (11). When assayed in proteoliposome vesicles following reconstitution, the MalF500GK2 complex displayed a pH optimum near pH 8, which was identical to that of the wild-type complex when stimulated by maltose and MBP (Fig. 2). The specific activity of the MalF500GK2 system was substantially higher than that of the wild type. We suspect that the MBP trapped inside the proteoliposomes stimulates ATP hydrolysis by only a fraction of the wild-type transport complexes at any given time.

The ATPase activity of the MalF500GK2 complex was also assayed in detergent solution following solubilization with dodecyl maltoside. Under these conditions, the complex displayed a more acidic pH optimum, pH 5.5. The activity of the detergent-soluble form of the MalF500GK2 complex was lower than that of the reconstituted form, except at low pH where the activities were comparable. In experiments where the detergent-soluble and reconstituted forms of the complex were incubated at room temperature for 2 h at pH levels from 4 to 11, then neutralized and assayed at the optimum pH levels irreversible inactivation occurred only at pH 4.

Kinetics of ATP Hydrolysis by MalF500GK2 in Detergent Solution—The ATPase activity of the MalF500GK2 complex was assayed in detergent solution as a function of ATP concentration at both pH 5 and pH 8. At pH 5, where the complex exhibited maximal activity, the velocity curve was sigmoid, indicating that the enzyme does not conform to simple Michaelis-Menten kinetics (Fig. 3A). Least squares fit to the Hill equation, a simplified version of the velocity equation describing an enzyme with multiple interacting substrate binding
sites, yielded a value of $n_{\text{app}} = 1.9 \pm 0.1$. A Hill coefficient ($n$) of 2 is indicative of positive cooperativity between two binding sites in the MalF500GK2 complex. The ATPase activity was not a function of enzyme concentration in the assay.

The cooperativity in hydrolysis is less apparent at pH 8, with $n_{\text{app}} = 1.2 \pm 0.1$ (Fig. 3B). To test the validity of including an additional term ($n$) in the fitting equation, an $F$ test on $\chi^2$, a measure of goodness of fit was performed (16). At pH 5, the fit to the Hill equation was far superior to that of the Michaelis-Menten equation ($p < 0.001$). However, at pH 8 the Michaelis-Menten equation with just two variables, $V_{\text{max}}$ and $K_m$, was adequate to describe the data ($p > 0.25$). This difference cannot be explained by the presence of a contaminating activity since the detergent-soluble membrane fraction isolated from the host strain without plasmids had little detectable ATPase activity when assayed at 100 $\mu$M ATP in this pH range (data not shown).

Table I summarizes several more experiments of the type shown in Fig. 3 in which the MalF500GK2 complex was assayed in detergent-soluble form at different pH levels. The half-saturation constant, $K_{0.5}$, is approximately 20 $\mu$M and is not greatly affected by changes in pH. As the data in Fig. 3 indicated, $n_{\text{app}}$ decreases with increasing pH.

Kinetics of ATP Hydrolysis in Proteoliposomes—ATP hydrolysis as a function of ATP concentration was measured following reconstitution of the MalF500 and wild-type systems into proteoliposome vesicles. As shown in Fig. 4A and in Table II, the reconstituted MalF500GK2 complex also displayed positive cooperativity with an average Hill coefficient of 1.5 at pH 6. Unlike MalF500GK2 in detergent solution, the Hill coefficient was essentially unchanged at pH 8, suggesting that cooperativity is not a function of pH in the reconstituted system. The wild-type system (Fig. 4B and Table II) also hydrolyzed ATP with positive cooperativity following reconstitution in the presence of maltose and MBP (average $n_{\text{app}} = 1.3$). Chi-squared analysis showed that the data fit better to the Hill equation than to the Michaelis-Menten equation in each of the three experiments in Table II ($p < 0.05$). Cooperativity was consistently seen only in potassium $P_i$ buffer at pH 6. Estimation of the Hill coefficient at pH 8 was impossible due to the presence of a low affinity, MBP-independent, alkaline phosphatase activity in the proteoliposome preparations. This activity interfered with assays of the wild-type complex more than the binding protein-independent complex because of the lower activity of the wild type. The $K_{0.5}$ was similar in the wild-type and the MalF500 systems and slightly higher than that seen in the detergent-soluble form of the MalF500 complex (Tables I and II).
Vanadate also inhibited the ATPase activity of the maltose transport complex. Like MDR, the detergent-soluble form of the MalF500 complex at pH 5 was 50% inhibited by vanadate concentrations close to 10 μM (Fig. 5). However, no inhibition was seen at pH 8. As shown in Fig. 6, the pH dependence of vanadate inhibition closely resembles the pH dependence of cooperativity reported in Table I. The data in Fig. 6 were fit to the equation

\[ y = A_H \left( \frac{H^+}{H^+ + K_a} \right) + A_0 \left( \frac{K_a}{H^+ + K_a} \right) \]  

(Eq. 3)

where \( y \) = percentage inhibition or \( n \), the Hill coefficient, \( H^+ \) = the proton concentration, \( K_a \) = the ionization constant, and \( A_H \) and \( A_0 \) represent the upper and lower limits of \( y \) for the protonated and unprotonated forms, respectively. \( A_H \) and \( A_0 \) were determined by least squares analysis, except in the analysis of the % inhibition, where \( A_0 \) was fixed at 100%. The pKₐ values of the transitions were estimated to be 5.2 ± 0.1 for inhibition by vanadate and 5.6 ± 0.1 for cooperativity (Napp). In contrast, in the reconstituted system, where cooperativity was not a function of pH, vanadate inhibited strongly at all pHs; in assays with 1 mM ATP, 100 μM vanadate inhibited 80–90% of the ATPase activity. The wild-type transport complex, assayed at pH 6 in potassium Pi buffer, was also inhibited by vanadate (not shown).

**DISCUSSION**

The conservation of two nucleotide-binding domains in the ABC transport family suggests that both are central to function, yet little is known about the role of these two sites in ATP hydrolysis and transport. To address this question, we examined the dependence of ATP hydrolysis on ATP concentration in the maltose transport system, and we discovered that ATP was hydrolyzed with positive cooperativity. Cooperativity likely results from interactions between the two MalK subunits, with the binding of ATP to the first subunit enhancing the affinity of binding to the second subunit. An alternative explanation, that ATP-induced oligomerization resulted in cooperativity, is unlikely because, in detergent solution, activity was independent of protein concentration. A tight binding site for nucleotides would lead to positive cooperativity only when the concentration of enzyme was high relative to the substrate concentration, which was not true in these experiments. Finally, a contaminating ATPase activity could generate negative but not positive cooperativity.

- The maltose system is the first ABC transporter in which cooperativity has been detected. This is of particular interest because kinetic analyses have been carried out on the ABC protein MDR in several different laboratories, but no positive cooperativity has been detected (21, 27–29). It is not clear if cooperativity was lost in vitro in the MDR system or if the two systems use different mechanisms to hydrolyze ATP despite their structural similarities.

- What is the physiological significance of positive cooperativity in this system? It is possible that positive cooperativity may play a key role in the reaction mechanism. The conformational changes required to facilitate the binding of ATP to the second site may be critical in coupling ATP hydrolysis to the transport event. In other transport ATPases, two mechanisms have been proposed to explain how ATP might stabilize the conformational changes required for transport. These include phosphorylation of the protein, as in the P-type ATPases, or tight binding of nucleotide, as in the F-type ATPases (23). To date, no evidence for either of these mechanisms has been found in any ABC protein.

- Cooperativity in ATP hydrolysis was a function of pH in the detergent-soluble form of the MalF500GK₂ complex but not in...
the reconstituted system. Since prior exposure to buffers in the pH range from 11 to 4.5 did not result in any irreversible inhibition of ATPase activity, this transition must relate to changes in the protonation state of the soluble transport complex. A critical residue(s) in the detergent-soluble form of the complex must be protonated for positive cooperativity to occur. In the reconstituted system, where there is no effect of pH, it can be argued that protein-lipid interactions either shift the pK_a of the residue(s) to more alkaline pH or, alternatively, stabilize quaternary interactions in the absence of the proton(s).

The pH dependence of ATP hydrolysis (Fig. 2) supports the hypothesis that a critical change is taking place in the detergent-soluble form of the complex near pH 5.5. Following reconstitution of the MalF500GK_2 complex into proteoliposomes, the pH dependence curve has a typical bell shape with a maximum at pH 8. The detergent-soluble form of the complex has a more unusual pH profile with a maximum at pH 5.5. Activity of the soluble form is substantially lower than the membrane-associated form at high pH, but it begins to rise at pH 6, ultimately matching the activity of the reconstituted form at pH 5. We interpret these curves to mean that protonation of the soluble form of the complex results in an increase in the efficiency of ATP hydrolysis as cooperative interactions are restored. Hence, we suggest that the form of the enzyme present at pH 5 in detergent solution is more representative of the actually transporting species in vivo, despite the fact that the pH in the cytoplasm is near neutrality (24). As discussed above, the presence of the membrane appears to prevent the loss of cooperativity and activity at neutral pH.

The susceptibility of the detergent-soluble form of the complex to the inhibitor vanadate also changes dramatically as a function of pH. ATP hydrolysis is inhibited at pH 5 but not at pH 8. The ionic state of vanadate does not change between pH 5 and pH 8 (25). The correlation between vanadate sensitivity and cooperativity in the detergent-soluble form (Fig. 6) strengthens the argument that the detergent-soluble form is undergoing a significant transformation near pH 5.5. This correlation is also seen in the reconstituted system, where both inhibition and cooperativity were seen at all pHs tested, and in the isolated MalK protein, where ATP hydrolysis followed Michaelis-Menten kinetics and was insensitive to vanadate (20). Deprotonation of the detergent-soluble form of the transport complex appears to alter the conformation of the ATP binding site such that vanadate can no longer inhibit and the two sites can no longer interact. While this conformational change, detected as a function of pH while studying the binding-protein-independent system in detergent-soluble form, may not represent a physiologically significant transformation, we are investigating the possibility that a similar conformational change may play an important role in the reaction cycle of the wild-type transport system under more physiological conditions.

Vanadate inhibits MDR by trapping ADP in one of the two ATP binding sites (26). Vanadate presumably acts in concert with magnesium and ADP to form a transition state analogue that is tightly bound by the enzyme. While the correlation between vanadate sensitivity and cooperativity found in our work could be purely coincidental, with two separate protonation events mediating the two changes, a far more interesting possibility is that this relationship reflects an integral part of the molecular mechanism of hydrolysis itself. For example, the ability to bind the transition state analogue tightly may be an essential feature of the signaling event between the two MalK subunits that results in cooperativity. Further experiments will be required to investigate this possibility.

On the basis of the work in this paper, it appears that the use
of the binding protein-independent (malF500) mutation is not significantly altering the characteristics of the ATPase in the reconstituted system, including the half-saturation constant, positive cooperativity, susceptibility to vanadate, and substrate specificity. These data support our model (Fig. 1) in which we propose that the MalF500 mutation stabilizes the complex in an active conformation (II) similar to the wild-type complex when it is stimulated by MBP. Thus, prudent use of the MalF500 system as well as the wild-type system should provide valuable insight into the mechanism of action of the transport complex. Using the manifestation of cooperativity as a guide, the best system for studying ATP hydrolysis is the reconstituted system that displayed cooperativity at all pHs. Where indicated, use of the detergent-soluble form of the complex at acid pH should also yield physiologically significant results.

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