The MglA Component of the Binding Protein-dependent Galactose Transport System of Salmonella typhimurium Is a Galactose-stimulated ATPase*

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Binding protein-dependent transport systems mediate the accumulation of several ions, sugars, amino acids, and peptides in Gram-negative bacteria by using the energy of ATP hydrolysis and belong to a superfamily of membrane proteins which extends to eukaryotic cells and includes the multidrug resistance P-glycoprotein and the cystic fibrosis transmembrane conductance regulator. The binding protein-dependent galactose transport system of Salmonella typhimurium comprises four proteins which have been characterized previously by molecular cloning experiments (61,000-dalton MglA protein, with a stable proteolytic product of 38,000 daltons, 33,000-dalton MglB protein, 29,000-dalton MglC protein, 21,000-dalton MglE protein). By using a MglA hyperproducing strain, we have purified a galactose-stimulated ATPase which shows a single band in polyacrylamide gels under nondenaturing conditions and shows three bands at 51,000, 38,000, and 15,000 daltons on sodium dodecyl sulfate-polyacrylamide gels (our results suggest that the bands at 38,000 and 15,000 daltons represent proteolytic products of the 51,000-dalton protein). The ATPase activity coincides with the purified protein during the last two chromatographic steps of the purification procedure, and it cannot be isolated from a strain which does not contain the mglA gene. The MglA ATPase is stimulated 3-fold by galactose and hydrolyzes ATP to ADP and P_i (K_m ATP = 60 μM, K_m galactose = 0.3 mM, V_max = 140 nmol/min/mg of protein). The γ-phosphate of ATP is transferred neither to galactose nor to the protein itself. Vanadate, N-ethylmaleimide and 5-methoxyindole-2-carboxylic acid, a specific inhibitor of binding protein-dependent transport systems, inhibit the MglA ATPase.

The binding protein-dependent transport systems of Gram-negative bacteria form a class of at least thirty permeases implicated in substrate translocation and energy coupling. The binding protein-dependent galactose transport system of Salmonella typhimurium (methylgalactose permease) consists of a periplasmic galactose-binding protein (the 33,000-dalton MglB protein) and three inner membrane-associated proteins (MglA protein which exists under two forms of 51,000 and 38,000 daltons, 29,000-dalton MglC protein, 21,000-dalton MglE protein (5)). Former results based on a correlation between low ATP levels and reduced activity of the binding protein-dependent transport systems in vivo (6) led to the suggestion that these transport systems are energized by ATP hydrolysis. However other studies implicated the proton motive force (7), acetylphosphate (8), lipoic acid (9), or succinate (10) in binding protein-dependent transport systems. A central role for ATP has been recently supported by the discovery of a consensus ATP binding site in the sequence of the inner membrane components OppD, HisP, and MalK of several binding protein-dependent transport systems (11), by an ATP requirement for these transport systems in membrane vesicles (12, 13) and proteoliposomes (14–16) and by a demonstration that ATP hydrolysis occurs in vivo concomitantly with transport (17). However, whereas the periplasmic substrate-binding proteins have been thoroughly studied, the inner membrane components of these transport systems have not been well characterized. In the present study we report the purification of a novel ATPase from a bacterial strain which hyperproduces the mglA gene product of S. typhimurium. The purified protein shows a single band when analyzed by electrophoresis in nondenaturing polyacrylamide gels, and the ATPase activity coincides with the protein peak during the last steps of the purification procedure. The purified protein shows three bands which migrate on sodium dodecyl sulfate-polyacrylamide gels as 51,000-, 38,000-, and 15,000-dalton polypeptides; the 38,000- and 15,000-dalton polypeptides are probably degradation products of the 51,000-dalton protein (the 51,000- and 38,000-dalton bands have been recognized previously as MglA proteins in molecular cloning experiments) (5). The purified ATPase has a specific activity of 140 nmol/min/mg of protein; it is stimulated 3-fold by galactose and is inhibited by vanadate, N-ethylmaleimide, and 5-methoxyindole-2-carboxylic acid.

Experimental Procedures

Bacteria—Strain of Escherichia coli K12 LA 5709 (F mgl-lacY galE ptsF arg recA1 srl) carrying plasmid pHG15 or pHG16, two pBR322 derivatives containing, respectively, the mglA and mglB or the mglC and mglE genes of S. typhimurium were from the laboratory of Prof. W. Boos (Fachbereich Biologie, Konstanz University, Germany) (6). They were grown in LB-rich medium supplemented with 0.4% glucose (in order to repress the mgl genes) to an absorbance of 0.5 at 600 nm and then transferred for 2 h in the same medium but without glucose to obtain expression of the mgl genes.

Purification of the MglA Protein—The purification procedure started with 2 g of bacteria. The bacteria were washed with 30 ml of
100 mM Tris hydrochloride (pH 7.4) and suspended at 20 °C in 3 ml of 50 mM potassium phosphate (pH 6.8), 2 mM dithiothreitol, 0.2 mM ATP, 10 mM galactose, 20% sucrose; 1 ml of 10 mg/ml lysozyme (freshly prepared) was added at once, followed by EDTA (added slowly to a concentration of 5 mM). Triton X-100 (at a final concentration of 2%), 0.1 g of sonicated phospholipids (Asolectin from Associated Concentrates), 0.1 g of sonicated precipitate from Nase 1000, and 0.5 mM phenylmethylsulfonyl fluoride were then added. The whole was incubated for 1 h at 0 °C, centrifuged for 10 min at 40,000 \( \times g \), and the supernatant was used for purification. The supernatant was diluted five times in buffer A (20 mM Tris hydrochloride (pH 8.0), 0.5 mM EDTA, 5 mM MgCl\(_2\), 1 mM dithiothreitol, 15% glycerol, 0.2% Triton X-100, 0.2 mg/ml asolectin, and 0.5 mM phenylmethylsulfonyl fluoride); it was loaded on a blue-Sepharose column (blue-Sepharose CL-6B, from Pharmacia Fine Chemicals, 5 ml bed volume) at 20 °C equilibrated in the same buffer, and eluted with a linear gradient (2 x 20 ml) of 0–1 M NaCl in the same buffer. The protein-containing fractions were adjusted to 1% CHAPS; dialyzed overnight against buffer A containing 0.5% CHAPS, 0.1 mg/ml asolectin, and 0.5 mM phenylmethylsulfonyl fluoride; the dialyzed fractions were loaded on an ATP-agarose column (ATP-agarose attached through C8 spacer, from Sigma, 1 ml bed volume) at 20 °C equilibrated in the same buffer, and eluted with a linear gradient of 0–20 ml of 0–0.25 M sodium phosphate in the same buffer; the active fractions were pooled, dialyzed against buffer A containing 0.1% CHAPS and 0.05 mg/ml asolectin and concentrated by ultrafiltration on a Centricon 30 microconcentrator (Amicon, Grace Laboratories). ATP and GTPase Assays—The ATPase activity was measured at 20 °C by adding 1 ml of protein (diluted to a CHAPS concentration of 0.01%) to 3 ml of 300 mM \( [\text{H}] \)ATP (1.5 Ci/mmol), 1 mM MgCl\(_2\), and galactose when indicated. ATP and ADP were separated by chromatography on polyethyleneimine cellulose as described in Ref. 18. GTPase activity was measured in the same manner in the presence of \( [\text{H}] \)GTP instead of ATP. The appearance of product as a function of time was linear during the time course of the assays.

**Gel Electrophoresis**—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Laemmli (19) by electrophoresis in polyacrylamide gel under non-reducing conditions.

**Preparation of Anti-MglA Antibodies and Immunoblotting**—A rabbit was immunized with 40 pg of protein (pool from hydroxylapatite protein in incomplete adjuvant) was administered after 3 weeks. Bleedings were performed 10 days after the injection. Crude extracts from the E. coli strains LA 5709 pHG15 (containing the mglA and mglB genes) and LA 5709 pHG16 which does not hyperproduce MglA (not shown)) coincides with the peak of MglA protein (Fig. 2). This suggests that the ATPase described here is the MglA gene product. The ATPase activity was measured at 20 °C by adding 1 ml of protein (diluted to a CHAPS concentration of 0.01%) to 3 ml of 300 mM \( [\text{H}] \)ATP (1.5 Ci/mmol), 1 mM MgCl\(_2\), and galactose when indicated. ATP and ADP were separated by chromatography on polyethyleneimine cellulose as described in Ref. 18. GTPase activity was measured in the same manner in the presence of \( [\text{H}] \)GTP instead of ATP. The appearance of product as a function of time was linear during the time course of the assays.

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**RESULTS**

**Purification of the MglA ATPase**—The MglA protein was produced in a mgl mutant of E. coli transformed with a pBR322 plasmid containing the mglA and mglB genes of S. typhimurium. The purification (Table I) started with a 40,000 × g supernatant of a bacterial lysate which had been lysed by lysozyme-EDTA in the presence of Triton X-100 as described under “Experimental Procedures.” The purification consists of three chromatographic steps on blue-Sepharose, ATP-agarose, and hydroxyapatite columns in the presence of detergents. The purified protein shows a single band when analyzed by electrophoresis in polyacrylamide gel under non-denaturing conditions (Fig. 1A), and it shows three bands at 51,000, 38,000, and 15,000 daltons on sodium dodecyl sulfate polyacrylamide gels (Fig. 1B). These molecular masses are similar to those of the two forms (51,000 and 38,000 daltons) of the MglA protein determined from molecular cloning experiments (5), and the 15,000-dalton band is probably the second proteolytic product of the 51,000-dalton protein (these polypeptides and ATPase activity were not observed when a similar purification procedure was made from strain LA 5709 pHG16 which does not hyperproduce MglA (not shown)). The peak of ATPase activity eluted from the hydroxyapatite column (and from a subsequent gel permeation column (see below)) coincides with the peak of MglA protein (Fig. 2). This suggests that the ATPase described here is the MglA gene product. Furthermore, the immunoblot shown in Fig. 1C is consistent with the 51,000-, 38,000-, and 15,000-dalton proteins being the MglA protein and its proteolytic products. Antibodies raised against the purified MglA protein specifically recognize the three different polypeptides (molecular masses of 51,000, 38,000, and 15,000 daltons) in crude extracts from a MglA producing strain (lane 1); these polypeptides are not apparent in control extracts from a strain which does not

1 The abbreviation used is: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.
TABLE 1

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume</th>
<th>Total protein</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>40,000 × g supernatant</td>
<td>5 ml</td>
<td>50 mg</td>
<td>140 nmol/min/mg of protein</td>
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<tr>
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<td>30 ml</td>
<td>10 mg</td>
<td></td>
</tr>
<tr>
<td>Pool from ATP-agarose</td>
<td>6 ml</td>
<td>0.3 mg</td>
<td></td>
</tr>
<tr>
<td>Pool from hydroxylapatite</td>
<td>6 ml</td>
<td>0.3 mg</td>
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Fig. 1. A, electrophoresis of the MglA protein in nondenaturing polyacrylamide gel. Migration was from top to bottom. 0.1 μg of the purified protein eluted from the hydroxylapatite column was loaded onto the gel. The gel was stained with silver nitrate. B, sodium dodecyl sulfate-polyacrylamide gel of the MglA protein. Migration was from top to bottom; the concentration of acrylamide was 15%. The gel was stained with silver nitrate. kd, kilodaltons. 0.1 μg of the purified protein eluted from the hydroxylapatite column was loaded onto the gel. C, immunoblots of crude extracts from E. coli with antibodies raised against the MglA ATPase. Lane 1, strain LA 5709 pHG15 (containing the mglA and mglB genes). Lane 2, strain LA 5709 pHG16 (containing the mglC and mglE genes).

Fig. 2. Chromatography of the MglA protein on hydroxylapatite. A MglA protein preparation which had been purified by chromatography on blue-Sepharose and ATP-Agarose was applied to the hydroxylapatite column. Fractions were collected and analyzed for total protein by the Bradford method (C) and for ATPase activity (∝). The active fractions were analyzed on a 15% sodium dodecyl sulfate-polyacrylamide gel.

express MglA (lane 2). Two other bands (molecular masses, 45,000 and 70,000 daltons) are apparent in both lanes and were not obtained with the preimmune serum (not shown). They could be due to cross reacting material or to a strongly immunogenic minor contaminant in the protein used for immunization.

Kinetic Constants— Whereas the presence of detergent is necessary during the purification procedure, CHAPS inhibits considerably the MglA ATPase (not shown); for the kinetic experiments described below, the protein was dialyzed against 0.1% CHAPS and diluted so that the final CHAPS concentration was 0.005% in the assay mixture. In these conditions, the ATPase activity of the purified protein is stimulated 3-fold by galactose (Fig. 3A). ADP appears linearly as a function of time, both in the presence and in the absence of galactose. This suggests that the galactose stimulation of MglA is not due to an increased stabilization of the ATPase by galactose. Half-maximal stimulation of MglA occurs at 0.3 mM galactose. Glucose, arabinose, lactose, or maltose (5 mM) do not stimulate the ATPase (Fig. 3B). The ATPase does not produce galactose phosphate in the presence of galactose and ATP (not shown), eliminating the possibility that we had purified a galactokinase. The velocity of the ATPase shows a hyperbolic dependence on ATP concentration with an apparent Michaelis constant similar to that of the ATPase of E. coli with antibodies raised against the MglA ATPase. Lane 1, strain LA 5709 pHG15 (containing the mglA and mglB genes). Lane 2, strain LA 5709 pHG16 (containing the mglC and mglE genes).

Native Molecular Mass— When the protein was run on a gel permeation column (Bio-Gel P-200), the ATPase activity eluted as a single peak at a position corresponding to a native molecular mass of around 50,000 daltons. The ATPase activity and unique protein peak coincided and showed three bands at 51,000, 38,000, and 15,000 daltons (not shown); this suggests that the 51,000-dalton polypeptide is monomeric and that the proteolyzed form (38,000 and 15,000 daltons) migrates as a complex at the same position. The constant ratios of intensity of the protein bands at 51,000, 38,000, and 15,000
ATPase-10 pmol of the purified MglA protein were incubated with \([y^{32}P]ATP\) as described under "Experimental Procedures." No incorporation of \([y^{32}P]ATP\) in a trichloroacetic acid-precipitable product could be detected over a 2-h incubation, suggesting that the protein does not form a phosphorylated intermediate (not shown). This is in contrast to the phosphorylated intermediates of the P-type ATPases such as the Na,K-ATPase of eukaryotic cells (28) or the KdpA protein-dependent transport systems (30).

Absence of Autophosphorylation of the Galactose-dependent ATPase—10 pmol of the purified MglA protein were incubated with \([\gamma^{32}P]ATP\) as described under "Experimental Procedures." No incorporation of \([\gamma^{32}P]ATP\) in a trichloroacetic acid-precipitable product could be detected over a 2-h incubation, suggesting that the protein does not form a phosphorylated intermediate (not shown). This is in contrast to the phosphorylated intermediates of the P-type ATPases such as the Na,K-ATPase of eukaryotic cells (28) or the KdpA ATPase of E. coli (29). This result is in accordance with the failure to detect any phosphorylated protein in binding protein-dependent transport systems (30).

Inhibitors—Vanadate inhibits the MglA ATPase, with a half-maximal inhibitory concentration close to 40 \(\mu M\) (Table II). This inhibition is reminiscent of the vanadate inhibition of the high affinity histidine transport systems (31) and of several related ATPases such as the P-glycoprotein involved in the multidrug resistance of cancer cells (27). Arsenate, which is a strong inhibitor of binding protein-dependent transport systems in vivo (32) produces little inhibition of the MglA ATPase. This is in accordance with an indirect action of arsenate which acts in vivo by depletion of the intracellular ATP pool (33). The sulphydryl reagent N-ethylmaleimide inhibits the ATPase activity by about 70%, and this inhibition may be related to the possible involvement of thiol groups in the function of binding protein-dependent transport systems (9, 34). 5-Methoxyindole-2-carboxylic acid, a specific inhibitor of several binding protein-dependent transport systems (34), inhibits MglA with a \(K_i\) of 2 \(\mu M\). This suggests that the inhibitor acts directly on the ATP-hydrolyzing component of these transport systems. Azide, dicyclohexylcarbodiimide, arsenite, and bafilomycin A1 (a specific inhibitor of V-type ATPases (24)) do not inhibit MglA.

**DISCUSSION**

This report describes the purification and characterization of a novel galactose-stimulated ATPase. Several lines of evidence suggest that the ATPase described in this study is the mglA gene product of the binding protein-dependent galactose transport system. i) The purified ATPase (which shows a single band on polyacrylamide gels under nondenaturing conditions) shows three bands at 51,000, 38,000, and 15,000 daltons on sodium dodecyl sulfate-polyacrylamide gels; the 51,000- and 15,000-dalton polypeptides are not observed when a similar purification cloning experiments (5), and the 15,000-dalton band is likely to be the second degradation product of the 51,000-dalton protein. ii) Antibodies against the purified ATPase specifically recognize three polypeptides of 51,000, 38,000, and 15,000 daltons in a crude extract from a MglA producing strain. iii) The peak of ATPase activity eluted from the hydroxylapatite column and from the gel permeation column coincides with the peak of MglA protein; furthermore, the ATPase activity and the corresponding polypeptides are not observed when a similar purification is made from a strain which does not contain the mglA gene. iv) The MglA ATPase is specifically stimulated by galactose, while being unaffected by other compounds tested.

The discovery of the present ATPase supports the implication of ATP hydrolysis in binding protein-dependent transport systems (6, 11–16). The \(K_{m,ATP}\) of 60 \(\mu M\) is almost 20 times lower than the ATP concentration in bacterial cells (31); this may explain some conflicting results concerning the effects of ATP depletion (generally to concentrations in a 20–200 \(\mu M\) range) on the activity of binding protein-dependent transport systems (reviewed in Ref. 1). The specific activity of the ATPase (140 nmol/min/mg of protein) is lower than...
the specific activity of the arsenate-efflux ATPase encoded by some E. coli plasmids (26) or of the eukaryotic multidrug resistance P-glycoprotein (27). It is lower than the specific activity of the membrane complex of other binding protein-dependent transport systems (35, 36); however, it is similar to the specific activity of the purified MalK protein (37).

The ATPase activity of the MglA protein is specifically stimulated by galactose. This result suggests that MglA should possess a galactose binding site. This binding site would be capable of transport in the absence of the periplasmic binding protein or a coupling between substrate translocation on the MglA ATPase and ATP hydrolysis. Consequently, the stimulation of the ATPase activity of binding protein-dependent transport systems reconstituted in proteoliposomes by the liganded binding protein (16, 35) could be a due to the subsequent transfer of the ligand to the ATPase component of the transport system.

Whereas detergents are necessary for a successful purification of the MglA protein, CHAPS at low concentrations (as low as 0.01%) inhibits the ATPase and abolishes the stimulation by galactose. Strong inhibition of the multidrug resistance P-glycoprotein by extremely low detergent concentrations has also been reported (40).

The MglA ATPase does not appear to form a phosphorylated protein intermediate in contrast to the KdpA ATPase (high affinity K+ transport system of E. coli) (29), the Na+K+ ATPase (23), or the Ca2+ATPase of eukaryotic cells (41). This is in accordance with the failure to detect binding protein-dependent transport components as phosphorylated proteins in E. coli (30). The P-glycoprotein of eukaryotic cells which shows a high degree of homology with several binding protein-dependent transport components is not phosphorylated during transport either (27), although it could be regulated by phosphorylation (42).

The inhibition of the MglA ATPase by N-ethylmaleimide suggests the existence of an important thiol in the protein. This could be related to the possible implication of lipoic acid and 2-oxoacid dehydrogenases in the function of binding protein-dependent transport systems (9, 34). Whereas ATP appears to play a major role in these transport systems, lipoic acid and 2-oxoacid dehydrogenases could be implicated in their redox regulation (9, 34). The inhibition of MglA by 5-methoxindole-2-carboxylic acid is reminiscent of the specific inhibition of binding protein-dependent transport systems by this compound (34). Vanadate inhibits the MglA ATPase and inhibits also several binding protein-dependent transport systems (16, 31); this compound, which is a specific inhibitor of the P-type transport ATPases (i.e. containing a phosphorylated protein intermediate), inhibits also several other ATPases such as the P glycoprotein (27), myosin (43) or dynein (44).

The characterization of the present ATPase should help to understand the mechanism of other transport ATPases and of related membrane proteins such as the multidrug resistance P-glycoprotein (27) or the cyclic fibrosis transmembrane conductance regulator (4).

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REFERENCES

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