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Large-scale purification, dissociation and functional reassembly of the maltose ATP-binding cassette transporter ($MalFGK_2$) of *Salmonella typhimurium*

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Abstract

The maltose ATP-binding cassette (ABC) transporter of *Salmonella typhimurium* is composed of a membrane-associated complex ($MalFGK_2$) and a periplasmic substrate binding protein. To further elucidate protein–protein interactions between the subunits, we have studied the dissociation and reassembly of the $MalFGK_2$ complex at the level of purified components in proteoliposomes. First, we optimized the yield in purified complex protein by taking advantage of a newly constructed expression plasmid that carries the *malK*, *malF* and *malG* genes in tandem orientation. Incorporated in proteoliposomes, the complex exhibited maltose binding protein/maltose-dependent ATPase activity with a V_{max} of 1.25 μmol P_i/min/mg and a K_m of 0.1 mM. ATPase activity was sensitive to vanadate and enzyme IIA^{Glc}, a component of the enterobacterial glucose transport system. The proteoliposomes displayed maltose transport activity with an initial rate of 61 nmol/min/mg. Treatment of proteoliposomes with 6.6 M urea resulted in the release of medium-exposed MalK subunits concomitant with the complete loss of ATPase activity. By adding increasing amounts of purified MalK to urea-treated proteoliposomes, about 50% of vanadate-sensitive ATPase activity relative to the control could be recovered. Furthermore, the phenotype of MalKQ140K that exhibits ATPase activity in solution but not when associated with MalFG was confirmed by reassembly with MalK-depleted proteoliposomes.

Keywords: ABC transporter; Maltose transport; Binding protein-dependent; Proteoliposomes; Assembly; *Salmonella typhimurium*

1. Introduction

The family of ATP-binding-cassette (ABC) transport systems comprises an extremely diverse class of membrane proteins that couple the energy of ATP hydrolysis to the translocation of solutes across biological membranes (for recent reviews, see Refs. [1,2]). Prominent members of the family include the P-glycoprotein (MDR) involved in multidrug resistance of certain cancer cells [3], the cystic fibrosis transmembrane regulator protein (CFTR) which is mutated in patients affected by cystic fibrosis [4], the TAP1–TAP2 peptide transporter, associated with antigen presentation [5], and the subfamily of binding protein-dependent ABC transport systems that mediate the uptake of a large variety of nutrients in prokaryotes [6].

A prototype ABC transporter is composed of four entities: two membrane-integral domains that presumably constitute a translocation pore, and two ATPase domains (also referred to as ABC subunits/domains), that provide the energy for the transport process. The ABC domains are characterized by a unique "signature sequence" (LSGGQ motif) of still unknown function [7]. The crystal structures of several prokaryotic ABC domains have recently been solved that agree largely on the overall folds. Accordingly, the structures can be subdivided in an F_1-type ATP-binding domain, encompassing both nucleotide binding motifs

Abbreviations: ABC, ATP-binding cassette; BSA, bovine serum albumin; DM, *n*-dodecyl-β-D-maltoside; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; MOPS, 4-morpholinepropanesulfonic acid; NTA, nitrilo triacetic acid; PMSF, phenylmethylsulfonyl fluoride; PTS, phosphoenolpyruvate phosphotransferase system; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis

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("Walker" A and B sites), a specific α-helical sub-domain, encompassing the LSGGQ motif and a specific antiparallel-β-subdomain [8–11].

In export systems, these modules are mostly fused to yield a single polypeptide chain, while prokaryotic ABC importers are built up from individual subunits [1,2]. The latter also require an additional extracellular (periplasmic) substrate-binding protein for activity [6].

The binding protein-dependent maltose/maltodextrin transporter of enterobacteria, such as *Escherichia coli* and *Salmonella typhimurium*, is a well-characterized model system for studying the mechanism of action of ABC transporters. Based on computational analysis, it belongs to a subclass of ABC importers designated CUT1 (carbohydrate uptake transporter) [12] or OSP (oligosaccharides and polyols) [13], respectively. Members of this subclass transport a variety of di- and oligosaccharides, glycerol phosphate and polyols and are recognized by their common subunit composition (two individual membrane-spanning subunits and two copies of a single ABC protein) and by an extension of approximately hundred amino acid residues at the C-terminus of the ABC protein [14].

The maltose transporter of *E. coli*/*S. typhimurium* is composed of the periplasmic maltose binding protein, MalE, and of the membrane-associated complex, $MalFGK_2$, consisting of one copy each of the hydrophobic subunits MalF and MalG and two copies of the nucleotide-binding subunit MalK [15,16]. Crystals of *Salmonella* MalK are available [17] but their structure could not be solved yet. However, the tertiary structure of a MalK homolog, isolated from the hyperthermophilic archaeon *Thermococcus litoralis*, was recently determined [9]. Two molecules are present per asymmetric unit that contact each other through the ATPase domains with the C-terminal domains attached at opposite poles. According to current transport models, the presence of substrate in the medium is thought to be signaled by liganded MalE via interaction with externally exposed peptide loops of MalF and MalG [18]. As a consequence, conformational changes of the latter are transmitted to the MalK subunits which, in turn, become activated. Hydrolysis of ATP would then trigger subsequent conformational changes that eventually lead to the translocation of the substrate molecule. Recent findings suggested that these steps occur rather simultaneously [19].

Assembly of the $MalFGK_2$ complex in vivo requires the initial formation of a MalK dimer that subsequently interacts with membrane-associated MalFG [20]. The latter spontaneously incorporate in the membrane alone, although the conformation of MalF is different when produced together with MalG [21]. When studied in membrane vesicles containing MalFG, binding of purified MalK was favoured in the presence of ATP [22]. Interaction of MalK with the hydrophobic subunits involves contact of residues in the helical subdomain connecting the Walker A and B motifs with conserved cytoplasmic loops ("EAA" motifs) in MalF and MalG [23–25].

Purified MalK displays a low spontaneous ATPase activity that is insensitive to vanadate [26–28], while in the $MalFGK_2$ complex, when incorporated into liposomes, ATP hydrolysis and ligand translocation are coupled, dependent on the binding protein, and vanadate-sensitive [16,28]. Moreover, ATP is hydrolyzed cooperatively [29] and two intact copies of the MalK subunit are required for activity [30].

Besides acting as an import system for maltose/maltodextrins, the $MalFGK_2$ complex is involved in the regulation of genes belonging to the maltose regulon by interaction of the MalK subunits with the positive transcriptional regulator, MalT [15,31]. Furthermore, the maltose transporter is subject to inhibition by enzyme IIA of the glucose transporter in a process called 'inducer exclusion' in the context of global carbon regulation in enteric bacteria [15,32]. Both activities are largely mediated by the C-terminal domain of the MalK subunits [33–35].

Obviously, specific interactions between the individual subunits as well as between the transporter and regulatory proteins are crucial for its role in intact cells. However, such interactions have, thus far, mostly been studied at the level of whole cells or membrane vesicles. Here, we describe for the first time the dissociation and reassembly of the purified $MalFGK_2$ transporter of *S. typhimurium* in proteoliposomes. The procedure will allow further studies on protein–protein interactions with purified components. Moreover, and as a prerequisite for these experiments, we report the construction of a new expression vector by which the yield in purified transport complex was substantially increased.

2. Materials and methods

2.1. Bacterial strains and plasmids

All cloning steps were carried out in *E. coli* strain JM109 (Stratagene). Plasmid pBB1 (p_{T5} *malKht*, ptrc, *malFmalG*, amp^r) was constructed by ligating a *Sal*I restriction fragment of pIG1 (p_{trc}, *malFmalG*, amp^r; derivative of pKK233-2, Göbling and Schneider, unpublished) with plasmid pGS91-1 (p_{T5} *malKht*, amp^r, Ref. [36]), previously digested with the same enzyme.

2.2. Purification of the $MalFGK_2$ complex

Cells of strain JM109(pBB1) were grown in tryptone-phosphate medium [37] at 37 °C to an $OD_{650} = 0.25$. Expression of *malK*, *malF*, *malG* was induced by the addition of 0.5 mM IPTG and growth continued to $OD_{650} = 4$. Cells were harvested by centrifugation for 10 min at 9000 × *g*, resuspended in 150 ml of buffer 1 [50 mM Tris–HCl, pH 8, 5 mM $MgCl_2$, 20% glycerol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF)] and disruptured by one passage through a French Press at 18,000 psi. Following a low speed spin for 15 min at 10,000 × *g*, membrane

vesicles were recovered by centrifugation for 1 h at 200,000 × *g*, resuspended in buffer 1 and stored at −80 °C until use. Solubilization of the transport complex was achieved by adding *n*-dodecyl-β-D-maltoside (DM, final concentration: 1.1%) (Biomol) [36,38] to membrane vesicles at 5 mg/ml in buffer 1. After incubation for 1 h on ice under constant stirring, solubilized proteins were separated from the remaining membranes by ultracentrifugation for 1 h at 200,000 × *g*. The supernatant was then mixed with Ni-nitrilo triacetic acid (NTA) agarose (Qiagen) (1 ml of slurry per 9 ml of supernatant), equilibrated with buffer 1 containing 0.01% DM (buffer 2) and incubated for 1 h on a shaking device in a cold room. Subsequently, the mixture was poured into a disposable column and the matrix was washed with 15 bed volumes of buffer 2, followed by 15 bed volumes of buffer 2 containing 20 mM imidazol. The transport complex was finally eluted with 15 bed volumes of buffer 2 supplemented with 50 mM imidazole. Peak fractions were combined, concentrated by ultrafiltration through Amicon filter YM30 and dialyzed against buffer 2 (500 × the sample volume). Finally, the protein was shock-frozen in liquid nitrogen and stored at −80 °C.

2.3. Purification of MalK variants

Wild-type MalK and variant Q140K were purified by a published procedure [37].

2.4. Purification of MalE

Maltose binding protein was purified from the osmotic shock fluid of the overproducing strain MM134(pES35) [28] as described in Ref. [39]. For some experiments, purified MalE was subjected to a denaturation/renaturation procedure [38] to remove tightly bound maltose.

2.5. Preparation of proteoliposomes

The $MalFGK_2$ complex was incorporated into liposomes by a detergent dilution procedure according to Ref. [38]. Typically, the reconstitution mixture (300 μl) contained liposomes (2.5 mg) preformed by ultrasonication from L-α-phosphatidylcholine Type IV-S (Sigma P-3644), 1% (w/v) octylglucoside, 50 μg purified $MalFGK_2$ complex, 120 μg maltose binding protein (MalE) and 60 mM maltose in 20 mM Tris–HCl, pH 8. After incubation for 30 min on ice under gentle stirring, 25 ml of 20 mM Tris–HCl, pH 8, 1 mM dithiothreitol (DTT) were added and the mixture was centrifuged for 1 h at 200,000 × *g*. Proteoliposomes formed by this procedure were resuspended in 100 μl of 20 mM Tris–HCl, pH 8, 1 mM $MgCl_2$, 10 μM maltose and analyzed for ATPase activity as described below.

Proteoliposomes for measuring maltose transport activity were essentially prepared as above with the following modifications: maltose binding protein and maltose were replaced by 5 mM ATP and after centrifugation, the proteoliposomes were resuspended in 100 μl of 20 mM Tris–HCl, pH 8, 3 mM $MgCl_2$.

2.6. Preparation of MalK-depleted proteoliposomes and reassembly with MalK variants

Proteoliposomes prepared as described above were resuspended in 50 mM Tris–HCl, pH 8, containing 100 mM NaCl and 6.6 M urea to a final volume of 400 μl. The mixture was gently stirred for 45 min on ice, diluted with 3.5 ml of 50 mM Tris–HCl, pH 8, 100 mM NaCl and MalK-depleted proteoliposomes were collected by centrifugation for 1 h at 300,000 × *g*. After resuspending the pellet in 100 μl of the same buffer, purified wild-type MalK or variant (10–200 μg) were added and the mixture was incubated for 1 h on ice, diluted with 1 ml buffer and centrifuged as above. Finally, the proteoliposomes were resuspended in 100 μl of 20 mM Tris–HCl, pH 8, 1 mM $MgCl_2$, 10 μM maltose and assayed for ATPase activity and protein content by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.7. Urea treatment of everted membrane vesicles

Everted membrane vesicles of strain JM109(pBB1) were prepared as described above and treated with varying concentrations of urea essentially as described in Ref. [40]. Briefly, membrane vesicles (10 mg/ml) were incubated in 50 mM 4-morpholinepropanesulfonic acid (MOPS)–KOH, pH 7, 100 mM NaCl, 1 mM DTT, 0.2 mM PMSF, containing the urea concentrations as indicated, for 1 h on ice. Subsequently, urea-soluble and urea-insoluble fractions were separated by ultracentrifugation for 30 min at 200,000 × *g* and subjected to SDS-PAGE. Detection of MalK and MalF was achieved by immunoblotting using polyclonal antibody raised against the respective proteins. Proteins were visualized by chemiluminescence (ECL, Amersham).

2.8. Analytical methods

Hydrolysis of ATP was assayed in microtiter plates essentially as described in Ref. [41].

The uptake of ^{14}C-maltose (10 μM; 0.86 μCi) in proteoliposomes was performed in 20 mM Tris–HCl, pH 8, 3 mM $MgCl_2$, in the presence of 1 μM MalE essentially as described in Ref. [42].

Protein was assayed by using the BCA kit from BioRad.

SDS-PAGE and immunoblot analyses were performed as described in Ref. [42]. For better separation of His_6-MalK, MalK, MalF and MalE from proteoliposomes, the pH of the separating gel was lowered to 8.4.

Quantitation of protein bands from proteoliposomes on SDS gels or immunoblots was carried out with the E.A.S.Y. Plus system (Herolab) using the software supplied by the manufacturer. Samples of purified MalK with known protein concentrations were run on the same gels and used as

standards. To correct for losses in proteoliposomes during the centrifugation step (see above), the calculated amounts of MalK protein were multiplied by a factor representing the ratio of the amounts of MalF protein present before and after centrifugation.

Competitive inhibition enzyme-linked immunosorbent assay (ELISA) was essentially carried out as described in Ref. [43].

3. Results

3.1. Large-scale purification and enzymatic properties of the $MalFGK_2$ complex

As a prerequisite for the intended study, we first set out to increase the yield in purified $MalFGK_2$ complex protein. Previously, two compatible plasmid vectors, bearing the *malK* gene (pGS91-1) and the *malFmalG* genes (pES62), respectively, were introduced into an *E. coli* host strain to achieve amplification of the transport proteins [36]. However, hardly controllable variations in plasmid copy numbers affected the stoichiometry of the gene products and resulted in inconsistent and rather low amounts of overproduced proteins. Thus, to eliminate these problems, we placed the *malFmalG* genes in a tandem orientation to *malK* on plasmid pGS91-1, yielding plasmid vector pBB1 (see Materials and Methods for details). Cells of *E. coli* strain JM109, transformed with pBB1 and grown to $OD_{650} = 4$ after induction of *mal* gene expression with IPTG typically yielded about 200 mg of membrane proteins per liter culture. From these, 4.5 mg of highly purified transport complex was routinely obtained (see also Fig. 1, insert). Compared to the two-plasmid expression system that, on average, allowed the purification of 0.6 mg of complex protein per liter culture, this corresponds to a 7–8-fold increase in protein yield.

The purified transporter displayed a low intrinsic ATPase activity in detergent solution (0.036 μmol P_i/min/mg protein) that could be stimulated 5-fold by the addition of maltose loaded MalE (Fig. 1). When incorporated into liposomes, the spontaneous rate of ATP hydrolysis was 0.03 μmol P_i/min/mg which could be substantially increased by loading the proteoliposomes with liganded MalE. Under these conditions, a V_{max} of 1.25 μmol/min/mg of protein and a K_m of 0.1 mM were obtained. The latter value is in excellent agreement with Michaelis constants reported for the purified MalK subunit (0.07–0.1 mM) [27,37].

In the absence of maltose, MalE was also capable to initiate ATP hydrolysis (0.39 μmol P_i/min/mg of protein).

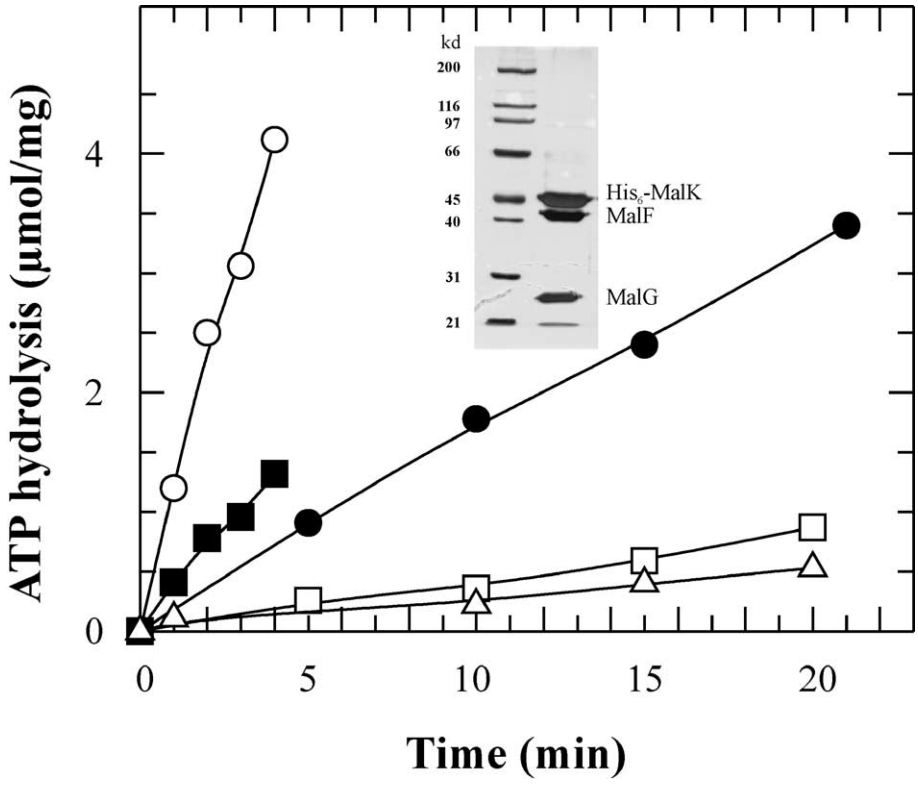

Fig. 1. ATPase activity of the purified $MalFGK_2$ complex. ATP hydrolysis catalyzed by the $MalFGK_2$ complex in proteoliposomes or in detergent solution was analyzed by monitoring the release of inorganic phosphate as described under Materials and Methods. Symbols: open circles, MalE/maltose-loaded $MalFGK_2$-containing proteoliposomes; closed squares, MalE-loaded $MalFGK_2$-containing proteoliposomes; closed circles, $MalFGK_2$ complex in detergent solution in the presence of MalE/maltose; open squares, $MalFGK_2$ complex in detergent solution; open triangles, $MalFGK_2$-containing proteoliposomes. *Insert*: Silver-stained SDS gel of the purified preparation (2 μg).

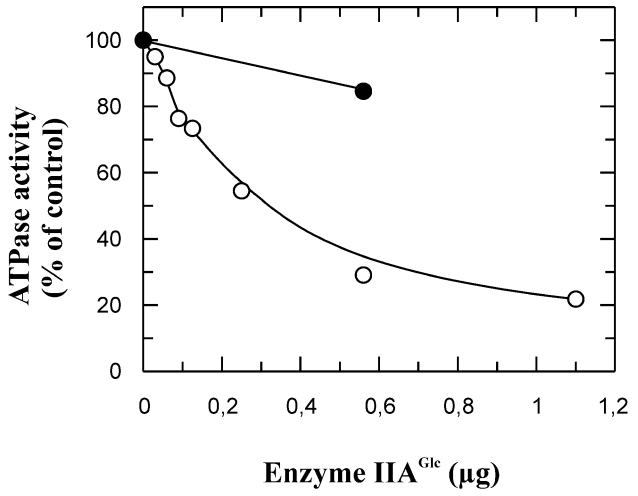

Fig. 2. Inhibition of MalE-maltose-stimulated ATPase activity by purified enzyme IIA^{Glc}. Proteoliposomes (corresponding to 1 μg of complex protein originally mixed with phospholipids) (open circles) or MalK (1 μg) (closed circles) were incubated with the indicated amounts of enzyme IIA^{Glc} at 37 °C for 5 min and subsequently assayed for ATPase activity under standard conditions. Control activities (100%) were 1.1 and 0.13 μmol P_i/min/mg for proteoliposomes and MalK, respectively.

This was not due to residual, tightly bound maltose as a MalE preparation that was subjected to a denaturation/renaturation procedure displayed essentially the same activity. Thus, and in agreement with other data [44], the unliganded binding protein interacts with the membrane-bound complex.

The V_{max} value given above is based on the assumption that all added complex protein was incorporated into liposomes. However, quantitation of MalK in SDS gels revealed that only 46% of the protein was actually recovered with the proteoliposomes (average of 10 independent experiments). Moreover, as determined by competitive inhibition ELISA, only 50% of the incorporated transport complexes were oriented with the MalK subunits facing the medium and thus contributed to the enzymatic activity (average of two independent experiments). Taking these numbers into account, the actual V_{max} value for ATP hydrolysis would be 5.4 μmol P_i/min/mg of protein.

The ATPase activity of the purified $MalFGK_2$ complex in proteoliposomes was sensitive to vanadate with half-maximal inhibition at 60 μM. Furthermore, purified enzyme IIA^{Glc} of *E. coli* (kindly provided by F. Titgemeyer) also strongly reduced the ATPase activity, reaching 50% inhibition at 0.3 μg (Fig. 2). In contrast, soluble MalK was hardly affected (18% inhibition in the presence of 0.6 μg enzyme IIA^{Glc} per microgram MalK). From these data, it was calculated that a 25-fold molar excess of enzyme IIA^{Glc} (molecular mass: 18.2 kDa) over $MalFGK_2$ (molecular mass: 171 kDa) would be required for full inhibition. This value is close to the ratio of 18 determined for whole cells [45].

Finally, we demonstrated that the observed MalE/maltose-dependent ATPase activity of the transport complex in proteoliposomes was coupled to the uptake of radiolabelled maltose. An initial rate of uptake of 61 nmol/min/mg of protein (average of three experiments) was measured. This rate is more than an order of magnitude higher than that reported for the *E. coli* transporter (1.2 nmol/min/mg of protein) [19,30]. As both preparations exhibit similar ATPase activities, we conclude that the preparation described here displays a better coupling between ATP hydrolysis and substrate translocation. Again, when taking into account the amount of transport proteins incorporated into liposomes and the fact that only 50% of the molecules are in an orientation that can contribute to the activity, the actual rate would be 266 nmol/min/mg.

Together, we conclude that the transport complex purified by the procedure described has all the earmarks of a functionally coupled system and thus, is suited for studying dissociation and reassembly processes.

3.2. Dissociation of the $MalFGK_2$ complex in proteoliposomes

To gain further insight into subunit–subunit interactions in the transport complex, we set out to develop a protocol

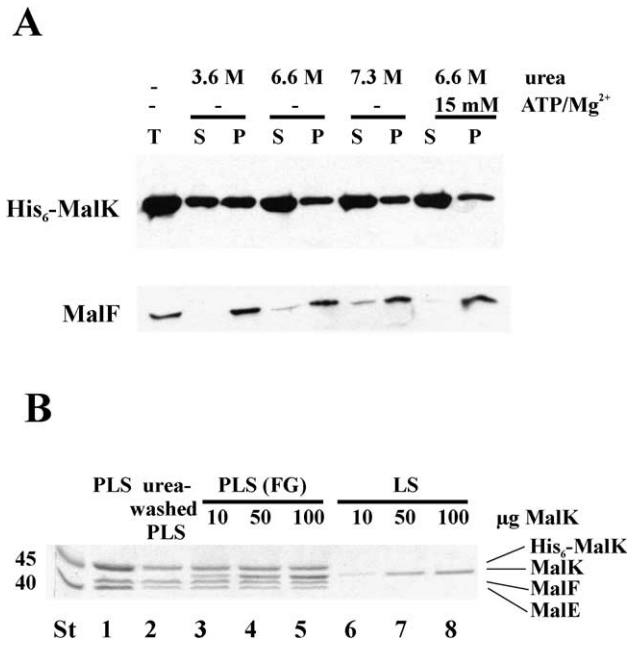

Fig. 3. Dissociation and reassembly of $MalFGK_2$ complex. (A) Removal of MalK from everted membrane vesicles of strain JM109(pBB1) by urea. See Materials and Methods for details. Immunoblots developed for detection of MalK (upper part) and MalF (lower part) are shown. T, total membranes (control); S, supernatant (urea-soluble fraction); P, pellet (urea-insoluble fraction). (B) Depletion of MalK from proteoliposomes and rebinding. An SDS-PAGE of a typical experiment is shown. Intact $MalFGK_2$-containing proteoliposomes (PLS) were treated with 6.6 M urea to remove exposed His_6-MalK and subsequently reassembled with increasing concentrations of purified (tagless) MalK as described under Materials and Methods (lanes 1–5). Control liposomes lacking the transport complex were incubated with MalK under the same conditions (lanes 6–8). Please note that under the conditions used to separate His_6-MalK, MalF and MalK, the MalG protein runs out of the gel. St, molecular weight markers ($\times 10^3$).

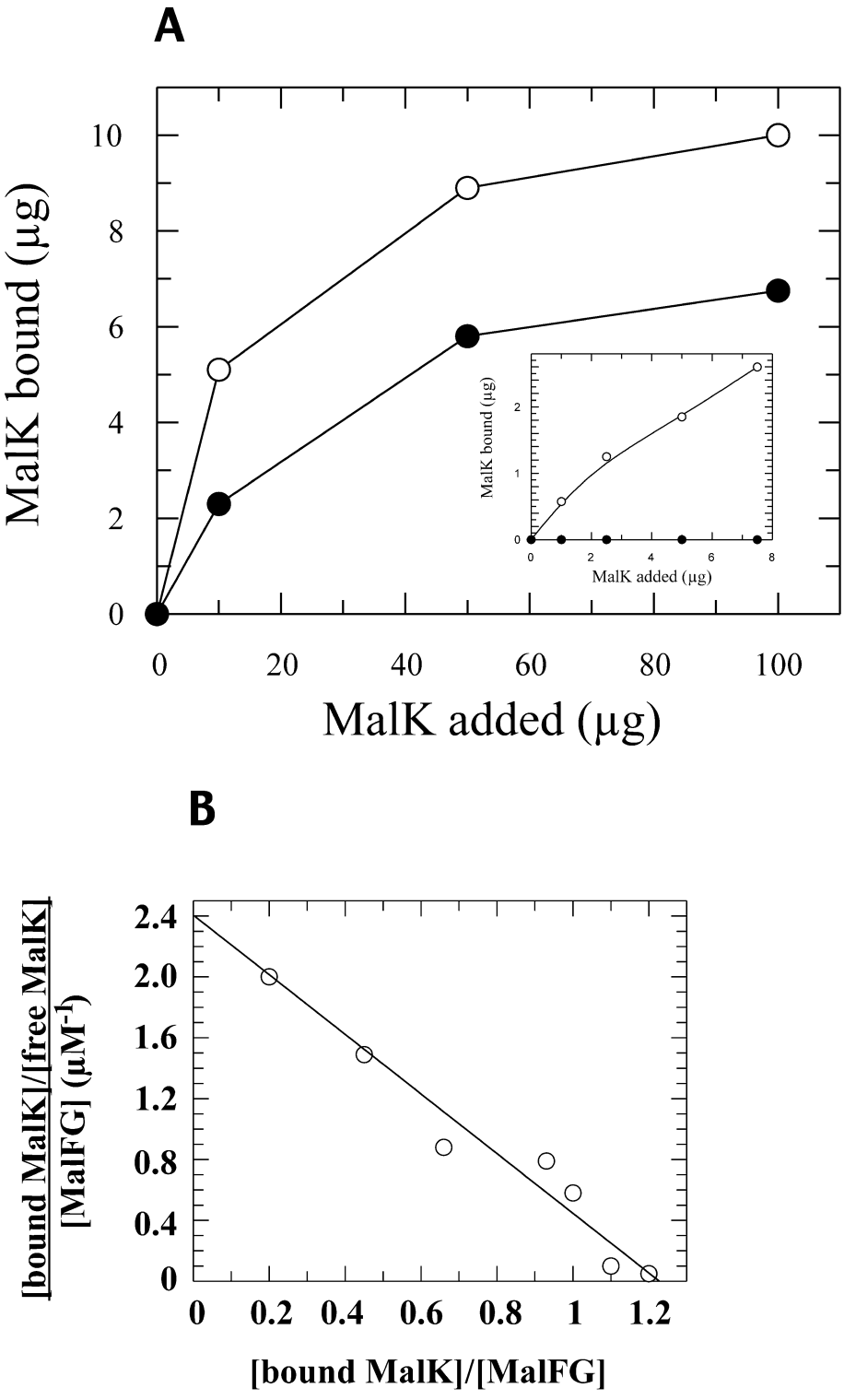

Fig. 4. Quantitative analysis of rebinding of MalK to MalFG-containing proteoliposomes. (A) Reassociation of increasing amounts of MalK with FG-containing proteoliposomes and control liposomes, respectively, was carried out as described under Materials and Methods. After centrifugation to remove unbound MalK, proteoliposomes and liposomes were subjected to SDS-PAGE and stained with Coomassie blue. Quantitation of bound MalK was determined by densitometric scanning, using samples of purified MalK of known protein concentrations as standards. Binding of low amounts of MalK (1–8 μg) was quantitated by analyzing immunoblots developed with the ECL chemoluminescence kit (Amersham). (B) Scatchard analysis of the data given in (A).

for the removal of MalK from proteoliposomes. Initial experiments using inside-out membrane vesicles of strain JM109(pBB1) had shown that up to 69% of MalK was released from MalFG by treatment with 6.6 M urea (Fig. 3A, upper part). The residual amount of membrane-associated MalK could neither be extracted by adding MgATP (15 mM each) nor by raising the urea concentration to 7.3 M. Possibly, the subpopulation of MalK that resists solubilization by urea is either aggregated and/or unspecifically associated with the membrane. Under the conditions used, most if not all MalF was recovered with the pellet fractions suggesting that treatment with urea did not result in significant desintegration of the membrane (Fig. 3A, lower part).

When similar experiments were performed with MalE/maltose loaded proteoliposomes containing the purified $MalFGK_2$ complex, incubation with 6.6 M urea resulted in the complete loss of ATPase activity. In contrast, proteoliposomes treated with lower concentrations of urea (3.6–5 M) exhibited residual ATPase activities (0.6–0.35 μmol Pi/min/mg), suggesting incomplete dissociation of MalK. Again, the presence of MgATP did not change these results. Quantitation by densitometric scanning of SDS gels of the amount of MalK present in the proteoliposomes before and after treatment with 6.6 M urea revealed that 46% of total MalK (average of four experiments) were removed (Fig. 3B, lanes 1 and 2). Since only half of the complex molecules contribute to the enzymatic activity (see above), we conclude that the proteoliposomes were fully depleted of medium-exposed MalK subunits.

3.3. Reassembly of a functional transport complex with purified MalK

MalK-depleted proteoliposomes were then incubated with increasing concentrations of purified MalK protein to study reassembly of a functional complex. To distinguish between residual and newly bound MalK proteins, a tag-less variant that migrates faster in SDS gels was used. As shown in Figs. 3B (lanes 3–5) and 4A, increasing amounts of MalK associated with the MalFG-containing proteoliposomes. Concomitantly, MalE/maltose-dependent and vanadate-sensitive ATPase activity was recovered that, at maximum, corresponded to 53% of the activity measured with intact proteoliposomes (Fig. 5). Recovery of enzymatic activity could not be increased by the presence of MgATP during reassembly. Control experiments revealed that MalK also associates with MalE/maltose loaded liposomes lacking MalFG, but only at higher concentrations and to a lesser extent (Fig. 3B, lanes 6–8, and Fig. 4A). However, and most importantly, these liposomes did not hydrolyze ATP (Fig. 5). Taking into account that purified MalK exhibits an intrinsic ATPase activity (0.2 μmol P_i/min/mg), lipid actually inhibited this activity. A similar result was reported for the homologous HisP subunit of the histidine transporter of *S. typhimurium* [41].

Scatchard plot analysis of data shown in Fig. 4A revealed that 1.2 mol of MalK reassociated with 1 mol of MalFG complex (Fig. 4B), rather than 2 mol as in the native complex [16]. This finding is consistent with the 53% of ATPase activity that was maximally recovered. Thus, about half of the MalK-depleted MalFG complexes retained the capability to functionally interact with MalK.

3.4. Reassembly with the MalKQ140K variant

With a protocol for dissociation of the $MalFGK_2$ complex at hand, we then studied the reassembly process with a MalK mutant protein. The MalKQ140K variant was recently shown to exhibit normal intrinsic ATPase activity in the soluble state but largely failed to hydrolyze ATP when assembled with MalFG [36]. Thus, the mutation might cause a defect in the activation of ATPase activity upon interaction of the complex with liganded MalE. Consequently, reassembly with MalFG should result only in a low recovery of MalE/maltose-dependent ATPase activity. As shown in Fig. 4, this was indeed the case.

4. Discussion

In this communication, we describe the dissociation and functional reassembly of the $MalFGK_2$ complex of *S. typhimurium*. To our knowledge, this is the first report on such a study for a multi-subunit ABC transporter at the level of purified components in proteoliposomes. Treatment with 6.6 M urea resulted in complete extraction of exposed MalK proteins as judged from the loss in MalE/maltose-dependent ATPase activity and from quantitation of residual MalK in SDS gels. These data basically confirmed the results obtained with membrane vesicles of strain JM109(pBB1) of which the majority of MalK protein was released under the same conditions. Urea was also the preferred chaotrope to dissociate the MalK-homolog HisP of the histidine transporter of *S. typhimurium* in inside-out membrane vesicles [40]. Here, only low amounts of HisP protein were recovered with the soluble fraction after treatment with 3.6 M urea and even 7.3 M urea did not release more than 40% of HisP. However, addition of MgATP to 6.6 M urea extracted essentially all HisP [40]. These and other data led the authors to conclude that HisP is rather tightly bound to its cognate membrane-integral components HisQM but becomes disengaged upon binding of MgATP [46]. Clearly, such a notion is not consistent with our results. Rather, in the case of the maltose transporter, the more efficient solubilization of MalK by the same concentration of urea both in membrane vesicles and in proteoliposomes (Fig. 3A and B) suggests a more solvent-exposed status of MalK within the complex. Moreover, this status is not drastically altered when ATP is bound as indicated by the failure of MgATP to increase the percentage of extractable MalK. Possibly, these differing results reflect specific structural

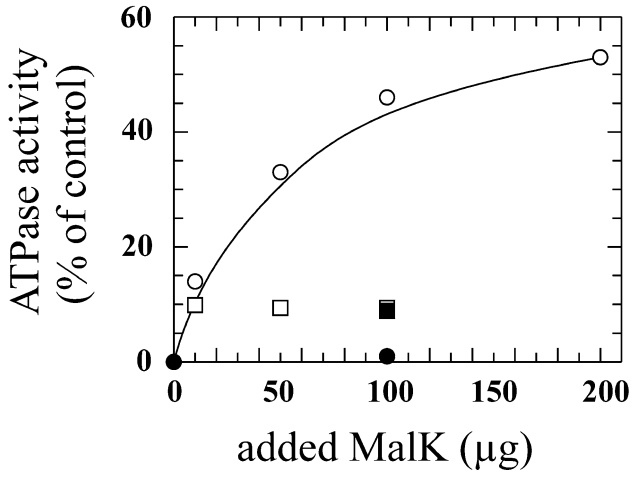

Fig. 5. ATPase activity of the reassembled complex. The ATPase activity of MalE/maltose-loaded MalFG-containing proteoliposomes that were previously depleted of endogenous His_6-MalK was monitored after incubation with increasing amounts of purified MalK as described in Materials and Methods. Symbols: open circles, complexes reassembled with wild type MalK; filled circles, MalK bound to control liposomes lacking MalFG; open squares, complexes reassembled with MalKQ140K; filled square, reassembled $MalFGK_2$ complex in the presence of vanadate (50 μM). The data represent the average of at least three independent experiments. Control activity (100%): 1.25 μmol P_i/min/mg.

requirements of MalK to perform its regulatory functions. These involve physical interactions with cytoplasmic proteins at its C-terminal domain which is lacking in HisP [9,15,47].

Reassembly of a functional complex from MalK-depleted proteoliposomes and purified MalK occurred linearly and specifically as demonstrated by our failure to detect association of MalK (at low concentrations) with pure lipid vesicles (Fig. 4A, insert). Moreover, recovery of ATPase activity was saturatable but with no indication of positive cooperativity. Rebinding of MalK was also independent of added MgATP which appears to be in contrast to findings by Mourez et al. [22] who observed a stimulatory effect of MgATP on reconstitution of MalK with MalFG-containing membrane vesicles. However, in their study, membranes were prepared from a strain lacking the *malK* gene, thus producing MalFG subcomplexes that, unlike in proteoliposomes, were never in contact with MalK before. It seems not unreasonable to assume that such 'naïve' MalFG proteins might preferentially recognize the ATP-bound form of MalK [22,48] in the initial assembly process.

Recent experiments by Kennedy and Traxler [20] suggested that assembly of the maltose transport complex in vivo requires the formation of a MalK dimer that is formed independently of MalFG. In contrast, in case of the histidine transporter, assembly is viewed to occur by recruiting HisP subunits independent of each other. This conclusion was drawn from the finding that the amount of HisP rebound to HisP-depleted HisQM-containing membranes increased linearly, while the ATPase activity of the restored transport complex increased sigmoidally with the amount of HisP added, indicating that the HisP subunits are dimerized (activated) only while in contact with HisQM [40]. By using a similar in vitro approach, our data are in support of the former view, since the observed increase in reconstituted ATPase activity upon rebinding of MalK to MalFG-containing proteoliposomes was clearly noncooperative (Fig. 5). These apparent discrepancies are most likely explained by assuming that, unlike HisP, MalK already exists in dimeric form at rather low protein concentrations. This notion is consistent with the finding by Kennedy and Traxler [20] who also observed formation of heterodimers of MalK and a variant in vitro by simply mixing crude cell extracts. Moreover, pre-existing dimers would also explain the linear dependency of the ATPase activity on increasing MalK concentrations in solution (unpublished results).

Taken together, we have succeeded in the dissociation and functional reassembly of the maltose transporter in proteoliposomes. The protocol provides a valuable tool for further studying the interactions between the subunits at the level of purified components, for example, by means of monoclonal antibodies, competing synthetic peptides or site-specific crosslinking.

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