Molecular and Biochemical Analysis of MalK, the ATP-hydrolyzing Subunit of the Trehalose/Maltose Transport System of the Hyperthermophilic Archaeon Thermococcus litoralis*

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We report the cloning, sequencing, and expression of malK encoding the ATP-hydrolyzing subunit of the maltose/trehalose transport system of the hyperthermophilic archaeon Thermococcus litoralis. According to the deduced amino acid sequence, MalK consists of 372 amino acids with a calculated molecular weight of 41,787. It shows 47% identity with the MalK protein of Escherichia coli and high sequence conservation in important regions. C-terminal His-tagged MalK was purified. The soluble protein appeared monomeric by molecular sieve chromatography and showed ATPase activity. Enzymatic activity was highest at 80 °C with a \( K_m \) of 150 \( \mu M \) and a \( V_{max} \) of 0.55 \( \mu mol \) of ATP hydrolyzed/min/mg of protein. ADP was not a substrate but a competitive inhibitor (\( K_i \) 230 \( \mu M \)). GTP and CTP were also hydrolyzed.

ATPase activity was inhibited by N-ethylmaleimide but not by vanadate. The strong homology found between the components of this archaeal transport system and the bacterial systems is evidence for the evolutionary conservation of the ABC transporters in these two phylogenetic branches.

High affinity binding protein-dependent ABC transporters were originally discovered in Gram-negative bacteria. They consist of a binding protein as their major substrate recognition site, located in the periplasm, two hydrophobic membrane proteins forming the translocation pore, and, peripherally associated with them at the inner face of the membrane, two additional subunits. By ATP hydrolysis the latter provide the energy for the accumulation of substrate (1). In the case of the Escherichia coli maltose/maltodextrin transport system, malE is the gene for the periplasmic-binding protein (MBP or MalE), the membrane components are encoded by genes malF and malG, and the two ATP-hydrolyzing subunits, by MalK. These genes form a cluster on the E. coli chromosome where malF, malG, and the two ATP-hydrolyzing subunits, by MalK. These genes form a cluster on the E. coli chromosome where malF, malG, and the two ATP-hydrolyzing subunits, by MalK. These genes form a cluster on the E. coli chromosome where malE, malF, malG constitute an operon that is oriented divergently to malK (2). Binding protein-dependent ABC transporters have also been found in thermophilic bacteria (3, 4). Recently, we described an ABC transporter for maltose/trehalose in the hyperthermophilic archaeon Thermococcus litoralis (5). This transport system has several unusual properties. It shows an extremely high affinity (\( K_m \) of about 20 \( \mu M \)) at 85 °C, the optimum growth temperature of this organism, and it recognizes with equal affinity its very different substrates, maltose and trehalose, and is not inhibited by maltodextrins. The soluble high affinity maltose/trehalose-binding protein (TMBP) is anchored to the membrane by a lipid anchor. malE, the gene encoding TMBP, is the first gene in an operon with malF and malG as distal genes (6). Here we report the molecular characterization of MalK and the biochemical properties of the encoded purified His-tagged protein.

MATERIALS AND METHODS

Cloning of T. litoralis malK—Chromosomal DNA was prepared from T. litoralis by the method of Owen and Borman (7). Because the trehalose/maltose system of Thermococcus litoralis is nearly identical to that of Pyrococcus furiosus, we searched the P. furiosus sequence data base at the Center of Marine Biotechnology, University of Maryland Biotechnology Institute, for homology to the E. coli malK gene. The open reading frame with the highest homology was chosen for the synthesis of primers for amplification of the corresponding malK gene from T. litoralis. The two primers (5’-GCCCCATGGCTGGTGTTAGGCTTGTA-3’ and 5’-CTGGATCCCAATATTGCTTTTCCTG-3’) contained the endonuclease restriction sites for NcoI and BamHI (underlined), respectively. After digestion with the corresponding restriction enzyme, the fragment was ligated into plasmid pCS19 (obtained from C. Spiess). This plasmid is a derivative of pQE60 (a his-tag vector from Qiagen) and contains the lacI* gene ligated into the XbaI site of pQE60. The resulting open reading frame (His6-MalK) carried a C-terminal extension of 10 amino acids: GSRSYHHHHH. The resulting plasmid was named pGG100.

Purification of His6-MalK—E. coli strain SF210 (8) was transformed with pG100 selecting for ampicillin resistance. The transformants were pooled and aliquoted, frozen in liquid nitrogen, and stored at −80 °C. These aliquots were used to inoculate overnight cultures subsequently used for large scale cultures. Cultivation was done in 6 liters of N2A medium (10 g NZ-amide A (Sheffield Products Inc.), 5 g of yeast extract, and 7.5 g of NaCl/liter) containing 200 mg of ampicillin/liter. After the culture reached an optical density at 578 nm (\( A_{578} \)) of 1.0, 150 mM isopropyl-\( \beta \)-D-thiogalactopyranoside was added. 12 h later the culture was harvested by centrifugation. The pellet was resuspended in 80 ml of buffer 1 (50 mM Tris, pH 7.5, containing 500 mM NaCl), ruptured in a French pressure cell at 16,000 pounds/square inch, and centrifuged for 15 min at 19,000 \( \times g \). The supernatant was heated to 80 °C for 10 min. After centrifugation of the precipitated proteins (30 min at 19,000 \( \times g \)), imidazol was added to the supernatant to a final concentration of 20 mM. The solution was loaded onto a Ni\(^{2+}\)-nitrilotriacetic-acid-agarose column (4-ml bed volume) from Qiagen equilibrated with the same buffer. After washing the column with 80 ml of buffer 1 (containing 20 mM imidazol), MalK was eluted with buffer 1 containing 100 mM imidazol. MalK-containing fractions (30 ml) were pooled and dialyzed against 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl. The enzyme was stored at 4 °C without loss of...
activity.

ATPase Assay and Analytical Techniques—ATPase activity was determined colorimetrically (9). 10 μg of MalK was added to 0.5 ml of ATPase buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl2). The solution was heated to 80 °C, and the assay was initiated by adding ATP at 1 mM final concentration. After 5 min, the reaction was stopped by freezing in liquid nitrogen. After thawing, the amount of liberated Pi was determined according to Chan et al. (9). ATPase activity is given in units defined as the amount of enzyme liberating 1 μmol of P_i/min under the assay conditions. Protein determination was according to Bradford (10) with reagents from Bio-Rad. SDS-polyacrylamide gel electrophoresis was according to Laemmli (11).

Nucleotide Sequence Accession Number—The sequence of malK from T. litoralis (strain DSM 5473, maintained in the laboratory of Dr. H. Santos, Instituto de Tecnologia Quı´mica e Biol-gica, Universidade Nova de Lisboa) as shown in Fig. 1 has GenBank™ accession number AF121946. The sequence of malK of T. litoralis strain DSM 5473 maintained in the laboratory of Jocelyne DiRuggiero (Center of Marine Biotechnology, University of Maryland, Baltimore, MD 21202) has the accession number AF126010.

Preparation of Antibodies and Western Blot Analysis—A chicken was immunized five times with 100 μg of purified MalK. 14 days after the last immunization, antibodies were prepared from 10 eggs (12). Western blot analysis was done as described previously (13, 14) using the primary antibody (17 mg/ml) in a dilution of 1 to 10,000.

RESULTS

Cloning and Sequencing of the T. litoralis malK Gene—The previous analysis of T. litoralis DNA harboring the malEFG cluster did not reveal the presence of malK. Upstream of malEFG we found an apparently unrelated gene (orf1) encoding a plant fructokinase homolog (GenBank™ accession number AF096373) and downstream, two genes (orf2 and orf3) with homology to a gene encoding a sugar phosphorylase (synthase) (15) and to cymJ of Klebsiella oxytoca (16), respectively.

Progress in the P. furiosus sequencing project revealed the presence of two similar but distinct genes that were homologous to E. coli malK. One of them was located close to the previously identified malG gene, directly adjacent and distal to cymJ. We realized that the mal gene cluster as well as the adjacent genes of T. litoralis were nearly identical to the corresponding cluster of P. furiosus. Primers designed from the P. furiosus malK sequence were used to clone the T. litoralis malK gene, using T. litoralis DNA provided by the laboratory of Dr. H. Santos (Instituto de Tecnologia Quı´mica e Biológica, Universidade Nova de Lisboa). Dr. Santos obtained the T. litoralis strain DSM 5473 from the Deutsche Sammlung von Mikroorganismen und Zellkultur GmbH (Braunschweig, Germany).

The polymerase chain reaction fragment was cloned into an expression vector that attached a 10-amino acid extension containing 6 histidine residues to the C terminus, resulting in plasmid pGG100. The recombinant malK gene was sequenced, and the nucleotide and deduced amino acid sequence are shown in Fig. 1. The malK sequence has 30 base pair changes in comparison to the corresponding sequence of the same T. litoralis strain DSM 5473, which is maintained in the laboratory of J. DiRuggiero. This resulted in a difference of four amino acids in the two MalK protein sequences. In contrast, comparison of the malK sequence from T. litoralis (maintained in the laboratory of J. DiRuggiero) to that of P. furiosus showed only an exchange of 7 base pairs resulting in two altered amino acids. When the sequence of T. litoralis MalK (maintained in the laboratory of H. Santos) was compared with the corresponding sequence of P. furiosus, 37 base pair changes were observed resulting in the exchange of 6 amino acids. The important conclusion from this comparison is that T. litoralis and P. furiosus do contain the nearly identical binding protein-dependent trehalose/maltose ABC transport system. Attempts to identify in T. litoralis a second and nonidentical mal gene cluster that is present in P. furiosus did not yield any result.

Fig. 2 shows an alignment of T. litoralis MalK protein with MalK proteins from several bacteria and a putative MalK protein from another archaeon (Pyrococcus horikoshii). As expected, the degree of sequence identity is high among the archaeal MalK proteins (80%), but surprisingly, it is also very high with MalK proteins from both Gram-positive and -negative bacteria (≤50%).

The conserved regions are clustered around the recognized functional domains of MalK, i.e. Walker A and B motif and the ABC motif LSGGQ (linker peptide) (17) as well as the recently recognized H motif overlapping the switch region (18). The sequence homology is still noticeable in the C-terminal half of the protein, which is lacking in some ABC proteins. In contrast, sequence comparison of the T. litoralis MalEFG subunits with the corresponding bacterial proteins revealed only 25–35% identity (6).

The T. litoralis MalK sequence (from the laboratory of H. Santos) has 50 positively and 52 negatively charged amino acids (11 and 9 more than the E. coli sequence, respectively) of a total of 372 amino acids (371 amino acids in E. coli MalK). Also, T. litoralis MalK contains 20 phenylalanines, which is 9 more than in E. coli, and 8 of these additional phenylalanines are in the less conserved C-terminal portion.

Purification of Recombinant T. litoralis MalK—Recombinant MalK protein was purified from strain SF120, which lacks several proteases (8). Strain SF120 was transformed with pGG100 and grown at 28 °C in rich medium (NZA) to an A660 of about 1 before the expression of malK was induced with isopropyl-1-thio-β-D-galactopyranoside. Under these conditions, MalK does not form inclusion bodies but remains as a soluble protein in the cytoplasm. It was purified from the soluble cellular extract by heat treatment (80 °C for 10 min), Ni2+−nitritotriacetic acid-agarose affinity chromatography, and dye ligand chromatography on Red agarose. SDS-polyacrylamide gel electrophoresis was used to follow the purification protocol (Fig. 3). The total yield of purified recombinant MalK from a 6-liter culture was routinely between 40 and 60 mg. Molecular sieve chromatography gave no hint for multimerization. MalK eluted as a symmetric peak with a calculated molecular weight of 40,000 (data not shown). Samples of MalK with and without ATP were investigated by dynamic light scattering (19) at different temperatures using a DynaPro mass spectrometry instrument. Values of mean particle radius and estimated molecular weight were essentially identical for both samples (data not shown).

After the last purification step (Fig. 3), the sample still contained small amounts of a contaminating protein of about 29,000 daltons that could not be removed. N-terminal amino acid sequencing of the contaminant revealed that it contained a part of the MalK sequence starting with methionine-178. Inspection of the upstream DNA sequence suggested that this protein might originate from a second translational start site. Apparently, restart from within an open reading frame of DNA from hyperthermophilic archaea heterologously expressed in E. coli is not uncommon (20).

Biochemical Characterization of MalK—The purified protein showed ATPase activity. Fig. 4 shows that the activity was highest at 80 °C but hardly measurable at room temperature. Incubating the protein in buffer at 80 °C resulted in loss of activity with a half-life time of 45 min. At 80 °C we determined a pH optimum of 7.0 (Fig. 5), a Km of 155 μM, and a Vmax of 0.55 μmol of ATP hydrolyzed/min/mg of protein (Fig. 6). ADP and ATPγS were not substrates but competitive inhibitors for ATP

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2 R. Weiss, manuscript in preparation.
hydrolysis ($K_i$ values of 230 $\mu$M). GTP and CTP were also hydrolyzed with $K_m$ values of 430 $\mu$M and 870 $\mu$M, and $V_{\text{max}}$ values of 0.45 and 0.32 mmol/min/mg of protein, respectively (Fig. 5). ATPase activity was inhibited by 10, 60, and 97% after incubating the enzyme with 10 $\mu$M, 100 $\mu$M, and 1 mM N-ethylmaleimide for 5 min. The additional presence of 1 mM ATP during incubation largely prevented inactivation by 1 mM NEM (75% remaining activity). In agreement with the property of MalK from Salmonella typhimurium, ATPase activity was not inhibited by 10 mM vanadate (21).

It had been reported that the intrinsic tryptophan fluorescence of MalK from S. typhimurium is quenched by ATP, indicating a conformational change (22). We saw the same magnitude of an apparent fluorescence quenching by ATP when using T. litoralis MalK. However, we also observed this quenching with serum albumin and conclude that ATP and other nucleotides generally interfere with fluorescence measurement by a filter effect, reducing both excitation and emission. Thus, we believe that the apparent reduction in fluorescence by ATP cannot be taken as evidence for a protein conformational change.

Even though the protein has ATPase activity and showed a surprisingly high sequence identity with E. coli MalK, the recombinant T. litoralis MalK was not able to complement a malK mutant of E. coli for growth on maltose at 37 °C when expressed from the isopropyl-1-thio-$\beta$-D-galactopyranoside-induced plasmid pGG100. It was also unable to affect mal gene expression as does E. coli MalK when overproduced (23). The malK gene from the T. litoralis strain maintained in the laboratory of J. DiRuggiero has also been cloned and overexpressed, and the protein was purified in the same manner as described above. This protein showed the same biochemical characteristics as the other MalK species. Thus, the difference in four amino acids does not seem to have an effect on the activity of the protein.

**Induction Pattern**—The purified His-tagged protein was used to obtain polyclonal antibodies in chicken. This antiserum was able to recognize MalK in extracts of T. litoralis. We tested
the amount of MalK in T. litoralis by Western blotting of extracts from cells grown in peptone with and without the addition of maltose, trehalose, and yeast extract. Fig. 7 shows that MalK was induced by maltose, trehalose, and yeast extract. The induction appeared less pronounced than the induction of the cognate TMBP and the corresponding transport activity in intact cells (Fig. 7) (6).

DISCUSSION

In this publication we describe the cloning of malK from the hyperthermophilic archaeon T. litoralis, its expression in E. coli, and the purification and biochemical characterization of the recombinant protein. With the exception of malK, the organization of the T. litoralis trehalose/maltose transport operon malEFG (Fig. 1) is very similar to that of E. coli and other bacterial binding protein-dependent ABC transport systems (1). At present it is unclear whether or not malK is expressed from a different promoter than malEFG. The Western blot analysis shown in Fig. 6 suggests that malK is coregulated with malEFG. The open reading frames orf3 and malK are separated by only five nucleotides, indicating translational coupling of the two genes that might form an operon. 32 nucleotides upstream of the translational start of orf3 the sequence TTTTAAA points to the presence of an element identical with the consensus sequence of archaeal promoters (Box A) (24).

The T. litoralis MalK protein exhibits an astonishingly high sequence identity of 50% with the E. coli protein (25–27). It is noteworthy that the T. litoralis protein has 23% more positively and 17% more negatively charged amino acids than the E. coli protein, which is in agreement with the notion that ionic interactions are important for high thermostability (28). Even the nonidentical part of T. litoralis MalK appears highly homologous to the E. coli protein. Walker A and B motifs are well conserved as is the intervening linker region LSGGQQ, the established ABC signature (29). Purified His-tagged MalK heterologously expressed in E. coli shows ATPase activity that is comparable in its kinetic constants with the well studied MalK protein from S. typhimurium (21).

E. coli MalK is known to be involved in the regulation of mal
gene expression. Several amino acids in its C-terminal part define a domain responsible for this repressor function (23, 30). Repression works via an interaction of MalK with the transcriptional activator MalT (31). None of the amino acids of *E. coli* MalK that supposedly participate in the MalK-MalT interaction are conserved in the *T. litoralis* MalK sequence.

At present it cannot be directly proven that the MalK protein, purified and characterized in this publication, is really part of the trehalose/maltose transport machinery. This can only be inferred from the position of the malK gene next to the malEFG cluster and from the induction pattern. The two genes orf2 and orf3 that separate malEFG from malK encode open reading frames with homology to a sugar phosphorylase (synthase) (15) and a CymJ protein. Whereas the role (if any) of orf2 in a (trehalose/maltose) transport gene cluster is unclear, the presence of cymJ has been observed previously in a gene cluster of *Klebsiella oxytoca* encoding a binding protein-dependent ABC transporter for cyclic dextrins (16). The function of CymJ remains unknown.

There are more differences between the two *T. litoralis* malK sequences than to the corresponding sequence from *P. furiosus*. This is probably due to mutations that accumulated over the multiple cell generations. Because the two *T. litoralis* strains have been maintained in different laboratories under different conditions, it is not surprising that the mutations appear.

We found that the sequences of the mal genes and adjacent
genes in *T. litoralis* are nearly identical to those in the *P. furiosus* genome. Interestingly, such a gene cluster has not been found in the genome of the closely related archaeon *P. horikoshii*. There, the homologous *malE malF malG orf2* and *orf3* cluster is present but lacks the downstream *malK* (32). Yet, this genome contains several genes encoding ABC proteins with high homology to MalK from *T. litoralis*, the one fitting best showing 80% sequence identity (cf. Fig. 2). This raises the possibility not only of a gene transfer between *P. furiosus* and *T. litoralis* but also between bacteria (likely Gram-positive bacteria (33)) and archaea (34). Analysis of the many complete genomes that have been published in the last few years has shown that in archaea most of the information-processing genes (*i.e.* for replication, repair, transcription, and translation) are more closely related to eukaryal genes, whereas their metabolic genes are more closely related to bacterial genes.

Nevertheless, binding protein-dependent transport systems must have appeared early in evolution. A gene cluster supposedly encoding a maltose transport machinery also exists in *T. maritima* which, like *T. litoralis* in the archaea, is one of the most deeply branched bacteria with a maximum growth temperature of 90 °C (35).

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2 Flanked by inverted sequence elements in *P. furiosus*, this gene cluster is evidence for a recent horizontal gene transfer between two organisms (J. DiRuggiero, G. Greller, R. Horlacher, and W. Boos, manuscript in preparation.)