Expression of a Plastidic ATP/ADP Transporter Gene in \textit{Escherichia coli} Leads to a Functional Adenine Nucleotide Transport System in the Bacterial Cytoplasmic Membrane* 

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Recently, a second type of eucaryotic adenine nucleotide transporter located in the inner envelope membrane of higher plants has been identified at the molecular level (Neuhaus, H. E., Thom, E., Möhlmann, T., Steup, M., and Kampfenkel, K. (1997) \textit{Plant J.} 11, 73–82). Here we have analyzed the biochemical properties of this ATP/ADP transporter from \textit{Arabidopsis thaliana} (\textit{AATP1}, \textit{At}). This analysis was carried out by expressing a \textit{cDNA} encoding this carrier as a histidine-tagged chimeric protein heterologously in \textit{Escherichia coli}. Isopropyl-\textit{D}-\textit{thio-\textit{D}-galactopyranoside} (IPTG)-induced \textit{E. coli} cells were able to import radioactively labeled \([\alpha-^{32}\text{P}]\)ATP. Uninduced \textit{E. coli} cells did not import \([\alpha-^{32}\text{P}]\)ATP. Further control experiments revealed that IPTG induction did not promote import of other phosphorylated or unphosphorylated metabolites into the bacterial cell indicating the specificity of \([\alpha-^{32}\text{P}]\)ATP transport. \([\alpha-^{32}\text{P}]\)ATP uptake into induced \textit{E. coli} cells was linear with time for several minutes allowing for determination of kinetic constants. The apparent \textit{K}_m for ATP was 17 \textmu M which is close to values reported on the authentic protein in isolated plastids. ADP was a strong competitive inhibitor of \([\alpha-^{32}\text{P}]\)ATP uptake (\textit{K}_i, ADP 3.6 \textmu M). Other metabolites like AMP, ADP glucose, UTP, UDP, NAD, and NADP did not influence \([\alpha-^{32}\text{P}]\)ATP uptake. IPTG-induced \textit{E. coli} cells preloaded with \([\alpha-^{32}\text{P}]\)ATP exported radioactively labeled adenylates after exogenous addition of unlabeled ATP or ADP indicating a counter-exchange mechanism of transport. The biochemical properties of the heterologously expressed \textit{AATP1} gene product demonstrated that the protein is functionally integrated in the cytoplasmic membrane of \textit{E. coli}. This is the first report of the functional expression of a plastidic protein in \textit{E. coli} leading to new transport properties across the cytoplasmic membrane. The functional integration of a plastidic protein in the cytoplasmic membrane of \textit{E. coli} offers new possibilities for future studies of the structural and mechanistic properties of this transporter. Since IPTG induction allowed synthesis of a 67-kDa protein in \textit{E. coli}, which was subsequently specifically enriched by metal-chelate chromatography, this procaryotic heterologous expression system might provide a suitable system for overproduction of membrane proteins of eucaryotic origin in the near future.

Transport of metabolic intermediates across cellular membranes is necessary for maintenance of a coordinated metabolism. In most cases this transport is mediated by membrane proteins which function either as carriers or channels. In all plant cells plastids are crucially involved in fundamental anabolic or catabolic reactions (1). Chloroplasts, typically present in green tissues harbor the autotrophic apparatus allowing assimilation of carbon, nitrogen, and sulfur. Heterotrophic plant tissues contain various types of plastids which also fulfill unique functions since they are the sole location for synthesis of starch and fatty acids (1). Furthermore, heterotrophic plastids contain a highly active oxidative pentose phosphate pathway allowing oxidation of imported glucose 6-phosphate and the production of \textit{C\textsuperscript{5}}-sugar phosphates and reduced pyridine dinucleotides (2, 3).

The import of ATP from the cytoplasm is essential for many of the metabolic functions carried out within plastids: in isolated heterotrophic plastids, which lack the photosynthetic machinery for ATP production, synthesis of various plant storage products strictly depend on the supply with ATP from the cytoplasm (see Refs. 4–6). In addition, there is evidence that even autotrophic plastids import ATP from the cytosol under specific conditions. For example, work with isolated chloroplasts from pea leaves has shown that photosynthesis is strongly stimulated when adenine nucleotides are included in the incubation medium (7). Moreover, nocturnal degradation of transitory starch in chloroplasts is substantially stimulated by the uptake of ATP (8).

ATP import into all types of plastids is mediated by a specific ATP/ADP transporter (9, 5) which has been recently identified at a molecular level (10, 11). This carrier represents a second type of eucaryotic adenylate transporter that is not related to the adenylate translocator previously identified in mitochondria (for review, see Ref. 12). The preprotein of the plastidic ATP/ADP transporter (named \textit{AATP1}; \textit{ATP/ADP} transport protein 1) comprises 589 amino acids, possess a calculated molecular mass of 65 kDa, and exhibits 12 putative transmembrane domains (10). Heterologous expression of \textit{AATP1} in \textit{Escherichia coli} or bakers' yeast and subsequent reconstitution of proteins in proteoliposomes demonstrated that \textit{AATP1} catalyzes ATP transport (11).

The biochemical properties of this important type of eucaryotic ATP/ADP transporter are largely not determined. For example, the topological arrangement of \textit{AATP1} in a cellular...
membrane is unknown. In addition, in the near future the identification of consensus sequences of homologues to AATP1 will allow experimental attempts to analyze structure-function relationships in this type of transporter. The analysis of both, topological arrangements as well as structure-function relationships would be greatly facilitated if the expression of the AATP1 gene product in *E. coli* leads to a functional ATP/ADP transporter in the bacterial cytoplasmic membrane.

However, expression of highly hydrophobic proteins in *E. coli* is limited to a comparably few examples as most membrane proteins of heterologous origin are toxic for *E. coli*. Recently, a new strain of *E. coli* was identified that exhibits improved capacity for the overproduction of eucaryotic membrane proteins (13). Using this strain (C43, a derivative of the *E. coli* strain BL21) we attempted to express the first plant membrane protein in *E. coli* with the aim of functional integration of this transporter in the cytoplasmic membrane. The functional expression of an ATP/ADP transporter from the Gram-negative, obligate endoparasitic bacterium *Rickettsia prowazekii* into the cytoplasmic membrane of *E. coli* has already been described (14). As *A. Arabidopsis thaliana* and the rickettsial ATP/ADP transporter share more than 66% similarity in the amino acid sequence (10, 15) we analyzed whether the plastidic ATP/ADP transporter behaved in a similar manner to the bacterial protein when expressed in *E. coli*.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs for Heterologous Expression of AATP1 in *E. coli***—For expression in *E. coli* strain C43 plasmid pET6, encoding the entire recombinant AATP1 protein with an additional N-terminal tag of 10 histidine residues under the control of the T7 promoter, was constructed as follows: plasmid pC23 (10) was cleaved with *NdeI* at codon 77 of *AATP1* and *XhoI* within the multiple cloning site. The resulting 1.8-kilobase 3′-fragment of the *AATP1* cDNA was inserted into pET16b via *NdeI* and *XhoI* in a plasmid (pET4). A *NdeI* restriction site was introduced at the start codon of *AATP1* (plasmid pC23) by amplification with Taq polymerase via the polymerase chain reaction using gene-specific oligonucleotides: 5′-GAGTGGAAATAGAATATGAGCTTGAATCCACG-3′ (the lowercase letter indicates the introduced base exchange to create a *NdeI* site), and 5′-TATCCGACCACCAATAGCAGGACAAATGCGTCC-3′ (complementary primer to pET16b, base pair positions 1100 to 1136).

The polymerase chain reaction product (1.8 kilobases) was gel purified and cleaved with *NdeI* within the start codon and codon 77 of the *AATP1* cDNA. The resulting 228-base pair *NdeI* fragment was purified and then inserted into pET4 cuts with *NdeI*. The correct orientation of the 5′-*NdeI* fragment was subsequently verified by restriction analysis. The first 77 codons of *AATP1* of the resulting plasmid pET6 were sequenced with the dyeoxy chain termination method (16) using the T7-promoter specific primer 5′-AAATAGCCTCATAAG-3′ and found to be identical to the previously published cDNA sequence (10).

**Heterologous Expression of AATP1 in *E. coli***—The cDNA encoding the AATP1 protein (pET6) under control of the T7 promoter was transcribed after IPTG1 induction of the T7 RNA polymerase (17). *E. coli* (strain C43, kindly provided by Prof. John E. Walker, University of Cambridge, UK) cells transformed with plasmid pET6 or plasmid pET7 (see Ref. 11) were grown in YTAmp medium until an optical density (OD600nm) of 0.6 was reached. Induction of T7 RNA polymerase was initiated by addition of IPTG (final concentration 0.012%). Cells were further grown to an OD of 1.5 and subsequently collected by centrifugation for 10 min at 5,000 × *g* (4 °C, Sorvall RC5B centrifuge, rotor type GSA, Sorvall-Du Pont, Dreieich, Germany). The sediments were resuspended to an OD of 1.0 using incubation medium consisting of 50 mM potassium phosphate (pH 7.2) (14) and stored on ice until use.

**Radio labeling of AATP1 Protein Synthesized in *E. coli***—Strain C43 and Enrichment of the Histidine-tagged Chimeric Protein—Two ml of *E. coli* cells strain C43 harboring plasmid pET6 grown on the logarithmic phase were collected by centrifugation at an absorbance O600 of 0.4 to 0.5 and subsequently suspended in 0.8 ml of a methionine assay medium containing 0.6% NaHPO4, 0.3% KH2PO4, 0.1% NH4Cl, 0.05% NaCl, 1 mM MgSO4, 0.1 mM CaCl2, 0.4% glucose, and 0.1 mg/ml thiamine (Difco Laboratories, Heidelberg, Germany). T7 RNA polymerase was induced by adding 1 mM IPTG. After shaking the culture for 40 min at 37 °C, 20 μg/ml rifampicin (stock 10 mg/ml dissolved in methanol) was added to inhibit the *E. coli* RNA polymerase. *E. coli* cells were shaken for a 30 additional min after which 170 Kd of [35S]methionine were added to label newly synthesized proteins for 15 min at room temperature. Cells were sedimented by centrifugation and transferred to liquid nitrogen to destroy cell intactness.

Histidine-tagged proteins can be enriched by metal affinity chromatography we used the chelating Sepharose system by Novagen (Heidelberg, Germany). The cell sediment was resuspended in 1 ml of binding buffer medium consisting of 5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl (pH 7.9), and 1% Triton X-100 and incubated on ice for 30 min. Chromatography and elution of bound proteins was carried as recommended by the supplier (Novagen). Proteins dissolved in the fractions were precipitated by adding trichloroacetic acid (20% final concentration) and incubation on ice for 30 to 45 min. Proteins were sedimented by centrifugation and washed with 500 μl of ice-cold acetone prior to SDS-PAGE to remove remaining trichloroacetic acid. For SDS-PAGE, protein sediments were resuspended in 40 μl of SDS-PAGE sample buffer medium and incubated on ice for 30–45 min. Subsequently, the preparation was applied to a polyacrylamide gel (5% stacking gel, 15% resolving gel) for electrophoresis (gel thickness 0.1% SDS. After drying of the gels were autoradiographed for 2 to 4 days.

**Uptake of Radioactively Labeled ATP by Intact *E. coli* Cells and Synthesis of [α-32P]ATP—100 μl of IPTG induced *E. coli* cells harboring plasmid pET6 (or the given controls) were added to 100 μl of incubation medium containing radioactively labeled ATP or ADP and effectors at double concentration. α-[32P] Labeled adenine nucleotides were present at specific activities between 80 and 200 Ci/mmol. α-[32P] ATP was synthesized from [α-32P]ATP (NEN, Bad Homburg, Germany). For this 2 μl of [α-32P]ATP was added into a Eppendorf reaction vessel containing 20 μl of 50 mM Hepes-KOH (pH 7.2), 5 mM MgCl2, 1 mM glucose, 1 unit of hexokinase, and 10 μM unlabeled ATP. The incubation was conducted for 60 min and hexokinase was denatured by heat inactivation (2 min at 95 °C). Under the conditions indicated, [α-32P]ATP was totally converted to [α-32P]ADP (see below). Radioactively labeled ATP was used without further purification since the radioactivity was diluted about 150-fold leading to minute contaminations of the incubation medium with glucose and glucose 6-phosphate. We determined in control experiments that these contaminations did not interfere with transport of nucleotides (data not shown).

Uptake of nucleotides was carried out at 30 °C in a tempered Eppendorf reaction vessel incubator and terminated after the indicated time periods by transfer of the cells onto a 0.45-μm filter (25 mm diameter, Schleicher und Schull, Hannover, Germany) previously prewetted with incubation medium and under vacuum (for details see: Ref. 18). Cells were further washed to remove unimported radioactivity by addition of 2× incubation medium. The cells was subsequently transferred into a 20-ml scintillation vessel containing 5 ml of water. Radioactivity in these samples was quantified in a Canberra-Packard Tricarb 2500 scintillation counter (Canberra-Packard, Frankfurt, Germany).

For back-exchange experiments, IPTG induced *E. coli* cells were preincubated at room temperature in incubation medium containing 1 μM [α-32P]ATP (specific activity of 1 M Ci/mmol; for details, see Ref. 14). After 5 min, samples were diluted 1/100 with incubation medium containing AMP, ADP, or ATP (if indicated) at a concentration of 0.1 mM.

**Thin-layer Chromatography of Radioactively Labeled Adenine Nucleotides**—To analyze the degree of enzymatic conversion of [α-32P]ATP to [α-32P]ADP and to identify the type of adenine nucleotide exported from [α-32P]ATP preloaded *E. coli* cells, we employed a thin-layer chromatography system according to the method of Mangold (19). Radioactively labeled samples were loaded onto a 0.5-mm polyethylene amine-cellulose thin layer chromatography plate and dried with a fan. Separation was carried out for 0.5 min using 0.5 μM sodium formate (pH 3.4), 2 min with 2 μM sodium formate (pH 3.4), and the front was allowed to run for 15 cm with 4 μM sodium formate (pH 3.4). RF values of radioactively labeled adenine nucleotides were determined after autoradiography and correspond to RF values of unlabeled nucleotides visualized under UV light (19).

**Protein Quantitation**—The protein content of *E. coli* samples was quantified using Coomasie Brilliant Blue according to the method given in Bradford (20). Bovine serum albumin was used as the protein standard.

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1 The abbreviations used are: IPTG, isopropyl-1-thio-galactopyranoside; AATP1, ATP/ADP transport protein 1; ADP-Glc, ADP-glucose; Glc, glucose; Glc-6-P, glucose 6-phosphate; PAGE, polyacrylamide gel electrophoresis.
TABLE I

Uptake of various radioactively labeled compounds into E. coli

Radioactively labeled compounds were present at a final concentration of 50 μM. Incubation was carried out for 2 min as given under "Experimental Procedures." Transformed E. coli cells (strain C43) harboring pET6 were incubated with 50 μM [α-32P]ATP for the indicated time periods. Termination of uptake was carried out by rapid filtration. Data are the mean of three independent experiments. S.E. less than 7% of the mean values.

| Substrate       | Rate of transport | E. coli control | E. coli pET6 uninduced | E. coli pET6 induced |
|-----------------|-------------------|-----------------|------------------------|----------------------|
| [α-32P]ATP      | 0.001             | 0.002           | 0.66                   |
| [14C]Glc6P      | 1.0               | 0.8             | 1.1                    |
| [14C]Glc        | 5.0               | 5.1             | 4.8                    |

RESULTS

Recently, we constructed the plasmid pET7 encoding the entire AATP1 preprotein (possessing the N-terminal target peptide), expressed the cDNA in E. coli (strain WM 1576) by temperature induction, and measured adenylate uptake after reconstitution of the whole E. coli protein in proteoliposomes (11). The intact E. coli cells of strain WM 1576 did not show ATP uptake after induction2 which is in contrast to the heterologous expression of the rickettsial ATP/ADP transporter in E. coli (14). This discrepancy caused us to analyze whether other E. coli strains might be more useful for functional expression of AATP1 in the intact bacterial cytoplasmic membrane. For the expression of AATP1 cDNA, we used the E. coli strain C43 (kindly provided by Prof. John Walker, University of Cambridge, UK). In this strain the bacteriophage T7 polymerase promoter is induced by IPTG and thus allows protein synthesis.

Expression of AATP1 cloned in plasmid pET7 leads to a slow ATP uptake rate into the intact cell of 0.04 nmol/mg of protein and 2 min, whereas uninduced cells did not show import of radioactivity. However, to use E. coli as a putative source for recombinant AATP1 protein we included an additional N-terminal histidine tag to allow subsequent purification of the protein. Interestingly, expression of this gene leads to a rate of uptake into the intact E. coli cells of 0.66 nmol/mg of protein and 2 min (Table I), which is about 17 times faster than in E. coli cells harboring pET7 (see above). Expression of AATP1 cloned in plasmid pET6 allows significant higher rates of ATP import into E. coli after induction with IPTG.

Several control experiments were performed to gain insight into the specificity of adenylate transport across the bacterial cytoplasmic membrane. As shown in Table I, uninduced E. coli cells harboring pET6 did not import ATP at substantial rates. This observation is consistent with the inability of E. coli control cells that lack plasmid pET6 to import [α-32P]ATP after incubation with IPTG (Table I). In contrast to stimulation of [α-32P]ATP uptake, IPTG induction of E. coli harboring pET6 does not increase rates of glucose (Glc)- or glucose 6-phosphate (Glc-6-P) import when compared with corresponding control cells (Table I).

The time dependence of ATP uptake is shown in Fig. 1. The uptake of radioactively labeled ATP by IPTG induced E. coli cells harboring pET6 increased with time and the rate was linear for the first few minutes. Thereafter, ATP uptake declined progressively until after about 30 min of incubation when no substantial [α-32P]ATP uptake was detectable (Fig. 1). In contrast, uninduced cells did not import radioactively labeled ATP at significant rates (Fig. 1).

To our knowledge this is the first example of a plant membrane protein that is functionally present in the E. coli cytoplasmic membrane. We therefore analyzed the biochemical properties of the transporter in this heterologous system to check whether the properties of the native protein were retained. As visualized in Fig. 2A, rising concentrations of exogenous [α-32P]ATP led to increased rates of uptake reaching an apparent saturation at a concentration of about 100 μM (Fig. 2A). The estimated Lineweaver-Burk analysis (Fig. 2B) allows the estimation of an apparent Km for ATP of 17 μM and a Vmax of 29 nmol/mg of protein and hour.

To determine the kinetic characteristics of AATP1 for [α-32P]ADP we first had to synthesize radioactively labeled ADP from [α-32P]ATP (see “Experimental Procedures”). The purity of the final product was analyzed by thin-layer chromatography (Fig. 3A). It is clear from this chromatography that the conversion of ATP to ADP was essentially complete (Fig. 3A, lane 2) and dependent upon the presence of hexokinase (Fig. 3A, lane 3). The R2 values of both, ATP and ADP, respectively, are identical to those of unlabeled standards visualized under UV light (for details, see “Experimental Procedures”). With [α-32P]ADP as a substrate uptake was apparently saturated at an external concentration of about 50 μM (Fig. 2C). The deduced Lineweaver-Burk analysis yields an apparent Km of 12 μM and a Vmax of 80 nmol/mg of protein and hour (Fig. 2C).

The authentic ATP/ADP transporter analyzed in both, intact autotrophic or heterotrophic plastids, is relatively specific for ADP and ATP (9, 21, 22). The substrate specificity of the heterologously expressed AATP1 present in the bacterial cytoplasmic membrane was investigated by measuring the effect of various metabolic intermediates on ATP uptake (Table II). ADP exhibited the strongest inhibition on ATP import and reduced the transport rate below 6% of the control (Table II). Other metabolic intermediates like AMP, uridine nucleoside phosphates, NAD, or NADP did not substantially influence ATP uptake (Table I). As ADP is a substrate for the plastidic ATP/ADP transporter (Fig. 2, A and C) and as expected a strong inhibitor of ATP uptake (Table II) we determined the Ki of ADP on ATP uptake. From the data presented in Fig. 2B an apparent Ki of ADP on ATP uptake of 3.6 μM was calculated (according to Ref. 23).

Recently, it was shown that isolated maize endosperm amyloplasts import ADP-Glc and use the glucose moiety for starch biosynthesis (22). To verify whether the plastidic ATP/ADP transporter might be involved in nucleotide sugar uptake we analyzed a potential inhibitory effect of ADP-Glc on ATP import. Even a 2.5-fold excess of ADP-Glc does not substantially

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2 H. E. Neuhaus, E. Thom, and T. Möhlmann, unpublished data.
inhibit ATP uptake into the intact bacterial cell (Table II).

The plastidic ATP/ADP transporter is known to catalyze ATP movement in counter exchange to ADP (9, 21). Therefore, export of adenylates by E. coli catalyzed by AATP1 would be dependent on the presence of the appropriate externally applied substrate if a counter exchange mechanism was maintained. To reveal whether such a counter exchange mode of transport is present we preloaded IPTG induced E. coli cells harboring pET6 with radioactively labeled ATP for 5 min at room temperature, diluted the cells subsequently 1/100 in medium containing the indicated nucleotides, and quantified the remaining radioactivity in the bacterial cells. The presence of either ADP or ATP in the dilution medium induce a rapid export of \([\alpha-32P]\)ATP previously present in the E. coli cells (Fig. 4). This export occurred linear with time for the first minutes and progressively slowed down with incubation. Six minutes after dilution about 60% of the initial radioactivity present in the cells was exported (Fig. 4).

E. coli cells preloaded with \([\alpha-32P]\)ATP and diluted in medium either lacking nucleotides or supplemented with AMP did not show high rates of export (Fig. 4).

From the data reported in Fig. 4 it is clear that external ADP or ATP promote release of radioactivity from the intact bacterial cell. The presence of either ADP or ATP in the dilution medium induce a rapid export of \([\alpha-32P]\)ATP previously present in the E. coli cells (Fig. 4). This export occurred linear with time for the first minutes and progressively slowed down with incubation. Six minutes after dilution about 60% of the initial radioactivity present in the cells was exported (Fig. 4). E. coli cells preloaded with \([\alpha-32P]\)ATP and diluted in medium either lacking nucleotides or supplemented with AMP did not show high rates of export (Fig. 4).

From the data reported in Fig. 4 it is clear that external ADP or ATP promote release of radioactivity from the intact E. coli cells. However, the observation that radioactivity is exportable from the intact bacterial cell cannot be taken as proof that the compound released was indeed an adenine nucleotide. To analyze the chemical nature of the radioactivity released we separated the radioactively labeled compounds by thin-layer chromatography. For this purpose we preloaded the cells as above with \([\alpha-32P]\)ATP and removed unincorporated radioactivity by two washes with ice-cold incubation medium. Subsequently, the cells were resuspended in incubation medium containing unlabeled ATP or ADP and incubated for 6 min at room temperature. The cells were then sedimented for 2 min in a precooled (4 °C) Eppendorf centrifuge, the supernatant was then transferred to new reaction vessels and heated to 95 °C for 1 min. The resulting radioactively labeled products were analyzed by thin-layer chromatography and compared with control samples (Fig. 3).

**TABLE II**

| Effector  | Rate of transport | % of control |
|-----------|-------------------|--------------|
| None      | 20.7              | 100.0        |
| ADP       | 1.2               | 5.8          |
| AMP       | 17.6              | 84.3         |
| ADP-Glc   | 17.4              | 85.3         |
| UTP       | 18.8              | 91.1         |
| UDP       | 22.8              | 110.5        |
| UMP       | 20.7              | 100.1        |
| NAD       | 18.5              | 89.5         |
| NADP      | 20.5              | 99.3         |

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| NAD      | 18.5              | 89.5         |
| NADP     | 20.5              | 99.3         |
min to prevent any further reaction. An aliquot of this solution was used for separation by thin-layer chromatography. The radioactive components exported after addition of ATP (Fig. 3B, lane 1) or ADP (Fig. 3B, lane 2), respectively, exhibit the same radioactive pattern. The \( R_f \) values of the two spots in each lane are identical to unlabeled ATP and ADP. A small amount of radioactivity appears as a third compound of unknown nature that is distinct from ATP or ADP. We verified that this third compound is not AMP (data not shown) (Fig. 3B). However, from the data presented it can be concluded that at least 80 to 90% of the radioactivity exported appears as ADP or ATP in the incubation medium.

As the data above clearly show that IPTG application to \( E. coli \) harboring plasmid pET6 leads to the presence of a functional ATP/ADP transporter in the cytoplasmic bacterial membrane it was tempting to analyze IPTG-induced protein synthesis in more detail. Therefore, we applied IPTG in the presence of \(^{35}S\)methionine allowing detection of newly synthesized proteins by autoradiography. The autoradiograph presented in Fig. 5 shows that application of IPTG to \( E. coli \) harboring plasmid pET6 leads to synthesis of several proteins with varying molecular masses. A prominent protein at a molecular mass of about 67 kDa is only present in total protein from IPTG-induced \( E. coli \) cells (Fig. 5, lanes 2 and 1). This protein is specifically eluted from the metal-chelate affinity resin using an imidazole concentration of 200 mM (Fig. 5, lane 4), whereas no radioactively labeled protein was detectable in the corresponding fraction from the uninduced \( E. coli \) cells (Fig. 5, lane 3). A small amount of the 67-kDa protein is also detectable in the fraction eluted with 400 mM imidazole (Fig. 5, lane 6).

**DISCUSSION**

ATP uptake into chloroplasts and all other types of plastids is required for energization of a variety of anabolic reactions (1). Recently, we identified the cDNA encoding the transport protein responsible for movement of adenylates across the plastid envelope (10, 11). The data presented in this paper demonstrate that transformation of \( E. coli \) cells (strain C43) with plasmid pET6 leads, after IPTG induction, to adenylate transport across the bacterial cytoplasmic membrane (Table I, Figs. 1 and 24). We propose that this ATP import is mediated by the plastidic ATP/ADP transporter now functionally present in the bacterial cytoplasmic membrane. This conclusion is supported by several observations: (i) \( E. coli \) cells lacking the plasmid pET6 or uninduced cells harboring plasmid pET6 do not show any significant rates of ATP uptake (Table I); (ii) other metabolic intermediates, like Glc or Glc-6-P, do not show increased rates of uptake after IPTG induction (Table I); (iii) adenylate uptake occurs with low concentrations of substrate, exhibiting apparent substrate saturation at concentrations between 50 and 100 \( \mu \)M (Fig. 2A); (iv) ADP is a strong competitive inhibitor of ATP uptake (Fig. 2C); and (v) adenylates are moved across the cytoplasmic membrane in a counter exchange mode of transport (Figs. 4 and 3B).

Heterologous expression of membrane proteins is mostly carried out in eucaryotic expression systems like baker’s yeast, fission yeast, or \( Xenopus \) oocytes (24). The superiority of these expression systems is due to the comparably high tolerance of the corresponding cells to the heterologously expressed membrane proteins which behave often toxic to bacteria like \( E. coli \). Typically, overproduction of eucaryotic membrane proteins in \( E. coli \), when successful, leads to synthesis of insoluble inclusion bodies. These proteins exhibited activity only (if any) after reconstitution in proteoliposomes (25, 26). Recently, Miroux and Walker (13) identified \( E. coli \) strain C43 which was shown to be suitable for expression of both, eucaryotic membrane and soluble proteins. This strain has already been shown to allow overproduction of five highly hydrophobic membrane proteins from animal origin (13). The data presented in this paper clearly demonstrate that a plant membrane protein is also functionally synthesized in \( E. coli \) C43 (Table I, Figs. 1 and 2).

Recently, we have expressed AATP1 in \( E. coli \) strain WM 1576 (11). However, the use of that bacterial strain did not allow adenylate uptake into the intact cell to be measured and it was necessary to reconstitute the complete bacterial protein in proteoliposomes before activity could be detected (11). It was not possible from that study to conclude whether the lack of ATP/ADP exchange in the intact cell was due to the incorporation of the transporter into inclusion bodies (for example, see Refs. 25 and 26) or that the transporter was inactively present in the cytoplasmic membrane. The data reported in this article clearly show that AATP1 is actively present in the cytoplasmic bacterial membrane of strain C43.

AATP1 it is not the first example of the heterologous expression of an ATP uptake system in \( E. coli \). The heterologous expression of the rickettsial ATP/ADP transporter in \( E. coli \)
leads also to the functional presence of this translocase in the cytoplasmic membrane (14). The plastidic ATP/ADP transporter AATP1 shares more than 66% similarity (in the structural part) to the ATP/ADP transporter from the Gram-negative bacterium R. prowazekii (10, 15). Hence, it is not surprising that the plastidic adenylate transporter behaves similar to the rickettsial counterpart after heterologous expression. To analyze the synthesis of AATP1 protein in more detail, we induced protein synthesis in E. coli C43 harboring plasmid pET6 in the presence of radioactively labeled methionine (Fig. 5). This detection system allowed the demonstration that a 67-kDa protein is synthesized in E. coli after IPTG induction and that this protein binds, due to the N-terminal histidine tag, specifically to the metal chelating affinity resin (Fig. 5, lanes 4 and 6). The apparent molecular mass of about 67 kDa (Fig. 5, lane 4) fits with a previous report about the apparent molecular mass of the AATP1 preprotein as deduced after in vitro translation, SDS-PAGE, and autoradiography (11). Up to now the amount of AATP1 transporter present in IPTG-induced bacterial cells does not suffice for a Coomassie staining of the newly synthesized protein (data not shown). This observation concurs with data about the low degree of heterologous expression of the rickettsial ATP/ADP translocase in E. coli. In that system, the newly synthesized ATP/ADP transporter was only detectable using a highly sensitive peptide specific polyclonal antiserum but not by silver staining (27).

The function of AATP1 in the bacterial cytoplasmic membrane requires a correct integration of this highly hydrophobic protein in the lipid phase of the membrane. Bearing in mind that plastids are derived, according to the endosymbiotic theory, from prokaryotes, namely blue green algae (28), it is tempting to assume that similar protein translocation mechanisms are involved in the targeting of both, plastid inner envelope proteins and proteins located in the prokaryotic cytoplasmic membrane. The exact mechanism of how inner envelope proteins integrate into that membrane is still unclear. But, there is experimental evidence that chloroplasts initially import proteins from the cytosol and subsequently re-export the protein into the inner envelope (29, 30). If such protein targeting mechanism holds true for all types of proteins residing in plastid inner envelopes the protein translocation would occur, once they are in the stroma, via a similar route as known for proteins located in the bacterial cytoplasmic membrane (31).

We have demonstrated that AATP1 fused with a N-terminal histidine tag exhibits about 17-fold higher transport activity than without this extension (Table I, see above). The molecular basis for this difference is unclear but a favorable topological arrangement within the membrane due to the presence of the highly charged histidine tag is possible. Most inner membrane proteins of E. coli exhibit high positive charges on the cytoplasmically orientated surface (positive-inside rule) (32). As we have extended the N terminus of AATP1 by 10 histidine residues, a strong positive charge is present at this end of the protein which might facilitate an N-terminal cytoplasmic orientation of this transporter. Indeed, it has already been demonstrated that positively charged amino acids can be used to influence the topological arrangement of membrane proteins in the cytoplasmic membrane of E. coli (33, 34). An N-terminal cytoplasmic orientation of AATP1 in the intact E. coli cell would interestingly concur with the orientation of the rickettsial ATP/ADP transporter (27) known to catalyze ATP/ADP exchange in R. prowazekii (18) or E. coli when expressed heterologously (14).

The plastidic transporter AATP1 cloned in pET6 exhibits a significantly higher molecular mass than the rickettsial counterpart due to the presence of a leader sequence (about 100 amino acids; Ref. 10) and a histidine tag comprising 10 amino acid residues. The following observations indicate that these N-terminal amino acid extensions do not modify the transport properties of AATP1. (i) The apparent \( K_m \) values for ATP and ADP exhibited by the heterologously expressed AATP1 (Fig. 2, B and C) are close to values reported on isolated plastids from various tissues (5, 21). (ii) As shown for the authentic transporter in intact plastids (21), the heterologously expressed AATP1 protein exhibits a counter exchange mode of transport (Fig. 3). It is noteworthy to mention that the apparent \( K_m \) for ADP (12 \( \mu M \), Fig. 2C) and the apparent \( K_f \) for ADP on ATP uptake (3.6 \( \mu M \), Fig. 2B) differ significantly. Since a \( K_f \) value of a competitive inhibitor is close to the \( K_m \) of the protein for that compound (35) we would expect that these values are closer together. However, a similar observation has been made for the authentic plastidic ATP/ADP transporter reconstituted in proteoliposomes. Schünemann et al. (21) have shown that the chloroplastic transporter exhibits an apparent \( K_m \) for ATP of 25 \( \mu M \), whereas the \( K_f \) for ATP on ADP uptake is 94 \( \mu M \). At this point we are not able to determine whether such discrepancy between \( K_m \) and \( K_f \) is an intrinsic feature of plastidic ATP/ADP transporters.

An important characteristic of the plastidic ATP/ADP transporter is the reciprocal exchange nature of transport (9, 21) which allow adenylate exchange without a change of the adenylate pool size in each compartment. This reciprocal exchange nature of transport is now shown for the heterologously expressed plastidic ATP/ADP transporter present in the bacterial cytoplasmic membrane (Fig. 4) which occurs with a similar time course as observed for the rickettsial ATP/ADP transporter when present in E. coli (14). The observation that not all of the radioactivity previously present in E. coli can be exported indicates the use of ATP for synthesis of acid-precipitable material in this bacterium (14).

We have demonstrated recently that the plastidic ATP/ADP transporter in isolated maize endosperm amyloplasts is not able to import ADP-Glc (22). Here we show that the same holds true for the heterologously expressed ATP/ADP transporter from A. thaliana (Table I). The low inhibitory effect of ADP-Glc on ATP uptake is most likely due to contamination of commercially available ADP-Glc with ADP (21) known to be an extremely potent competitive inhibitor (Fig. 2B). The data presented in Table I should finally prove that the plastidic ATP/ADP transporter is not responsible for ADP-Glc import as previously claimed by others (36).

In conclusion, taking the plastidic ATP/ADP transporter as an example we have shown that E. coli strain C43 provides a suitable system for further biochemical characterization of membrane proteins since the general biochemical properties of this transporter have not changed in the cytoplasmic bacterial membrane. Recently, we have identified a second homologue to AATP1 in A. thaliana (AATP2) and several homologues in maize, potato, and wheat. Therefore, the identification of consensus sequences and subsequent site-directed mutations will allow the detailed analysis of the structure-function relationships in this second type of eucaryotic adenylate transporter. This work and the construction of chimeric proteins fused with the reporter gene lacZ are currently in progress to further characterize the mechanism and orientation of the transporter in the membrane.

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Expression of a Plastidic ATP/ADP Transporter Gene in *Escherichia coli* Leads to a Functional Adenine Nucleotide Transport System in the Bacterial Cytoplasmic Membrane

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