Nonmitochondrial ATP/ADP Transporters Accept Phosphate as Third Substrate*§

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Chlamydiales and Rickettsiales as metabolically impaired, intracellular pathogenic bacteria essentially rely on “energy parasitism” by the help of nucleotide transporters (NTTs). Also in plant plastids NTT-type carriers catalyze ATP/ADP exchange to fuel metabolic processes. The uptake of ATP\(^{3-}\), followed by energy consumption and the release of ADP\(^{3-}\), would lead to a metabolically disadvantageous accumulation of negative charges in form of inorganic phosphate (P\(_i\)) in the bacterium or organelle if no interacting P\(_i\) export system exists. We identified that P\(_i\) is a third substrate of several NTT-type ATP/ADP transporters. During adenine nucleotide hetero-exchange, P\(_i\) is cotransported with ADP in a one-to-one stoichiometry. Additionally, P\(_i\) can be transported in exchange with solely P\(_i\). This P\(_i\) homo-exchange depends on the presence of ADP and provides a first indication for only one binding center involved in import and export. Furthermore, analyses of mutant proteins revealed that P\(_i\) interacts with the same amino acid residue as the γ-phosphate of ATP. Import of ATP in exchange with ADP plus P\(_i\) is obviously an efficient way to couple energy provision with the export of the two metabolic products (ADP plus P\(_i\)) and to maintain cellular phosphate homeostasis in intracellular living “energy parasites” and plant plastids. The additional P\(_i\) transport capacity of NTT-type ATP/ADP transporters makes the existence of an interacting P\(_i\) exporter dispensable and might explain why a corresponding protein so far has not been identified.

Most organisms possess the capacity to resynthesize the fundamental energy currency ATP by fusion of ADP and P\(_i\). Generally, in eukaryotes the major part of energy is produced in specialized organelles, the mitochondria. Mitochondrial ATP/ADP carriers (AACs)\(^2\) mediate the export of newly synthesized ATP in strict counter-exchange with cytosolic ADP and there-fore provide energy to the cellular metabolism (1). Plants additionally generate high amounts of ATP during photosynthesis in chloroplasts. However, under conditions of limiting or missing photosynthetic activity, plant plastids depend on external energy supply (2–4). Specific nucleotide transporters (NTTs) located in the inner plastid envelope membrane mediate the required energy import (5). These transporters structurally, functionally, and phylogenetically differ from mitochondrial AACs. They catalyze the import of cytosolic ATP in exchange with stromal ADP, are monomers consisting of 12 predicted transmembrane helices, and are related to the functionally heterogeneous group of bacterial NTTs (5).

Although most prokaryotic organisms are able to regenerate ATP and therefore are considered as energetically self-sustaining, the obligate intracellular living bacterial orders Chlamydiales and Rickettsiales are impaired in energy and nucleotide synthesis or even completely lost the corresponding pathways (6–8). Therefore, these bacteria, which comprise important human pathogens (9, 10), essentially rely on nucleotide and energy import. Bacterial NTTs catalyze the required import of a broad range of nucleotides and NAD or facilitate the counter-exchange of ATP and ADP (5, 11–15). The latter process has been termed “energy parasitism” and obviously is of high importance for the survival of rickettsial and chlamydial cells (5, 16–18).

Although import measurements on intact Escherichia coli cells expressing the corresponding proteins allowed characterization of many bacterial and plastidial NTT’s (12–15, 19–24), a very important physiological question is still not clarified. The uptake of ATP\(^{4-}\) in exchange with ADP\(^{3-}\) in absence of a concerted P\(_i\) export would result in a charge difference and a phosphate imbalance in the bacterial cell. In mitochondria, phosphate carriers metabolically cooperate with AACs because they provide P\(_i\) for ATP synthesis (25). Similarly, it was assumed that NTT-type ATP/ADP transporters cooperate with phosphate exporters to guarantee phosphate homeostasis in the bacterium or plastid. However, a P\(_i\) exporter interacting with ATP/ADP transporters is not known in “energy parasites” or plant plastids. Bacterial and plant phosphate transport systems rather facilitate P\(_i\) import or the counter-exchange of P\(_i\) and phosphorylated compounds and therefore do not allow net P\(_i\) export (26–29). Furthermore, the newly identified plastidial (proton-driven) phosphate transporters are not preferentially expressed under conditions or in tissues that require ATP provision to the plastid (30, 31).

Recently, we succeeded in the purification of the first recombinant NTT from Protochlamydia amoebophila (PamNTT1), a
parachlamydial endosymbiont of the protist Acantamoeba (32). The functional reconstitution of the highly pure PamNTT1 into artificial lipid vesicles for the first time allowed the biochemical characterization of a representative nonmitochondrial ATP/ADP transporter unaffected by the complex metabolic situation of the bacterial cell. We demonstrated that in contrast to mitochondrial AACs, PamNTT1 catalyzes a membrane potential independent, electroneutral adenine nucleotide hetero-exchange (32, 33). The latter could argue for a cotransport of a counterion compensating for the electrogenic ATP4−/ADP3− exchange.

Here, we investigated possible ions accompanying ATP or ADP transport. Interestingly, we uncovered that PamNTT1 and also rickettsial and plastidial ATP/ADP transporters accept an additional important substrate, which is Pi. We performed a comprehensive characterization of the Pi transport and gained new insights into the transport properties of ATP/ADP transporters.

EXPERIMENTAL PROCEDURES

Heterologous Synthesis of Selected NTTs and Mutant Proteins in E. coli—The heterologous synthesis of PamNTT1 and of two ATP/ADP transporters from the Rickettsia-related bacterium Caedibacter caryophilus (CcNTT) and Holospora obtusa (HoNTT) was performed on the basis of existing pET16b constructs (13, 22), which were transformed into BLR cells (Merck). Heterologous expression of the full-length cDNA encoding the plastidial ATP/ADP transporter from Arabidopsis thaliana (AtNTT1) led to very low amounts of recombinant transporters in BLR cells (data not shown). However, the truncation of the N-terminal extension (the putative leader peptide of 87 amino acid residues) and application of Rosetta2™ (DE3) pLysS expression cells resulted in an increased synthesis of AtNTT1. The truncated AtNTT1 sequence was amplified from the expression construct (34) by PCR using Pfu polymerase and the following oligonucleotides: AtNTT1shortNdeI sense, 5′-GGCCGCGGCTCATATGGAAGACGCAGCTG-3′, and the standard primer T7-term. The PCR construct contained one NdeI restriction site inserted by the sense primer and the XhoI restriction site of the plasmid. The amplificate was cloned into the correspondingly prepared original pET16b vector. The correct orientation of latter compounds further increased the stimulatory effects.

Nucleotide Exchange—The observation that PamNTT1 mediated adenine nucleotide exchange is electroneutral (32) was surprising. This carrier obviously transports a counterion that compensates for the charge difference of the hetero-exchange. The catalytic activity of bacterial and plastidial ATP/ADP transporters in contrast to bacterial proton-driven NTTs is not affected by the proton gradient across the membrane (12–15, 19–23). This argues against a cotransport of ATP and protons.

To detect possible cotransported ions, we analyzed the influence of diverse salt compositions on PamNTT1-mediated nucleotide transport in the liposomal system. We identified that the comparably low rates of ADP import into ATP loaded vesicles (ADP↓/ATP↑) were increased by 500 μM external Pi (3.7 times) and also by its structural analogue, arsenate (2.6 times) (supplemental Table S1). Higher concentrations (2 mM) of latter compounds further increased the stimulatory effects.

RESULTS

Pi Influences Nucleotide Exchange—The observation that PamNTT1 mediated adenine nucleotide exchange is electroneutral (32) was surprising. This carrier obviously transports a counterion that compensates for the charge difference of the hetero-exchange. The catalytic activity of bacterial and plastidial ATP/ADP transporters in contrast to bacterial proton-driven NTTs is not affected by the proton gradient across the membrane (12–15, 19–23). This argues against a cotransport of ATP and protons.

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Influence of the presence of Pi in the liposomal lumen on PamNTT1 mediated adenine nucleotide hetero-exchange. ATP$_{im}$/ADP$_{ex}$ transport and ADP$_{im}$/ATP$_{ex}$ transport was analyzed in absence (light gray bars, set to 100%) and in presence of internal Pi (dark gray bars, calculated according to the transport in absence of Pi). For this, PamNTT1 was reconstituted into liposomes loaded with 10 mM ADP or ATP or into vesicles containing 5 mM Pi plus 10 mM ADP or 5 mM Pi plus 10 mM ATP. External nucleotides and Pi were removed by gel filtration. Import of 50 μM α-32P-labeled ATP or ADP was allowed for 5 min and stopped by anion exchange chromatography. The data represent net values calculated by subtraction of uptake into vesicles lacking counter-exchange nucleotides and are the means of three independent experiments. Standard errors are displayed. For better comparison the presence of Pi and adenine nucleotides at the liposomal interior and exterior is graphically displayed.

The addition of other components had lower stimulatory or even inhibitory influences on the corresponding transport (supplemental Table S1).

In a previous study we demonstrated that only those reconstituted PamNTT1 proteins, which were inserted in the native orientation (right side out), displayed catalytic activity (32). To mimic the presence of Pi in the bacterium, we tested whether Pi application at the liposomal interior also influences adenine nucleotide hetero-exchange. When compared with the control (0 mM luminal Pi), the addition of internal Pi, stimulated ATP import in exchange with ADP (ATP$_{im}$/ADP$_{ex}$) ~4-fold, whereas ADP$_{im}$/ATP$_{ex}$ transport was not highly affected by internal Pi application (Fig. 1). Accordingly, not only external but also internal Pi supports ATP/ADP exchange. However, stimulation of the hetero-exchange exclusively occurred when ADP and Pi were present at the same side of the liposome (ADP plus Pi) (supplemental Table S1 and Fig. 1).

It had been suggested that Pi plays a regulatory role in ATP/ADP exchange across the rickettsial membrane (37). This based on the observation that high Pi concentrations marginally enhanced ATP but highly stimulated ADP import into isolated bacteria cells. Furthermore, only in the presence of Pi, ADP was able to compete with ATP for import, leading the authors to the assumption that Pi might increase (at least) the affinity of the rickettsial ATP/ADP transporter for ADP (37). To investigate the role of Pi in NTT-mediated adenylate exchange in more detail and separated from the complex cellular metabolism, we analyzed the effect of rising external Pi concentrations on adenine nucleotide import into PamNTT1-proteoliposomes loaded with internal Pi, plus the given counter-exchange nucleotides.

Changes in external Pi availability had low impact on ATP homo-exchange parameters and only slightly affected ADP homo-exchange (4-fold increased $V_{\text{max}}$ at 5 mM external Pi) (data not shown). In contrast to the homo-exchanges, the hetero-exchanges were substantially influenced by Pi addition (Fig. 2). As observed in the effector analysis (supplemental Table S1) ADP$_{im}$/ATP$_{ex}$ homo-exchange was significantly stimulated by external Pi. The highest rise in stimulation was obtained between 50 μM and 2 mM Pi (Fig. 2, dark gray bars). Increasing the external Pi concentration above 2 mM caused a further but comparably slight stimulation of the ADP import rates and suggests the beginning of a Pi saturation phase. Determination of the kinetic parameters revealed that the stimulatory influence of external Pi on ADP$_{im}$/ATP$_{ex}$ exchange can be traced back to an enhanced affinity (from 0 to 5 mM: 14-fold lower $K_{\text{m}}$) and an increased $V_{\text{max}}$ (from 0 to 5 mM: 3-fold higher $V_{\text{max}}$) of PamNTT1 for ADP.

Concentrations of more than 2 mM Pi in the transport medium led to a substantial reduction of the ADP$_{im}$/ATP$_{ex}$ transport rates. The $V_{\text{max}}$ of this exchange was high in the pres-
ence as well as in the complete absence of external P\textsubscript{i}, and thus the lowered import rates were mainly caused by a decreased affinity of Pam\textsubscript{NTT1} for ATP import (from 0 to 5 mM: 27-fold lower \(K_m\)) (Fig. 2).

It has to be mentioned that the transport rates of optimal ATP\textsubscript{in}/ADP\textsubscript{ex} exchange (0 mM external and 5 mM internal P\textsubscript{i}, \(\sim 1000\, \text{nmol mg protein}^{-1}\, \text{5 min}^{-1}\)) were much higher than that of P\textsubscript{i} stimulated ADP import into ATP plus P\textsubscript{i}-loaded vesicles (5 mM external and 5 mM internal P\textsubscript{i}, \(\sim 350\, \text{nmol mg protein}^{-1}\, \text{5 min}^{-1}\)) (Fig. 2). In this context it might be assumed that P\textsubscript{i} in the vesicle lumen hindered optimal ATP export. However, depletion of luminal P\textsubscript{i} did not result in a further relevant stimulation of ADP\textsubscript{in}/ATP\textsubscript{ex} exchange (data not shown; see also ADP\textsubscript{in}/ATP\textsubscript{ex} in Fig. 1). The opposed effects of external P\textsubscript{i} on ADP\textsubscript{in}/ATP\textsubscript{ex} versus ATP\textsubscript{in}/ADP\textsubscript{ex} exchange could be explained by (i) a cotransport of ADP and P\textsubscript{i}, and by (ii) a possible competition of P\textsubscript{i} with ATP during hetero-exchange.

**Pi Is Substrate of Pam\textsubscript{NTT1} but Only in Conjunction with ADP**—To analyze whether Pam\textsubscript{NTT1} is able to transport P\textsubscript{i}, we performed import studies using radioactively labeled P\textsubscript{i} (50 \(\mu\text{M}\) \(^{32}\text{P}\)). P\textsubscript{i} import was conducted with proteoliposomes loaded with interior P\textsubscript{i}, P\textsubscript{i} plus ADP, or P\textsubscript{i} plus ATP. In absence of exterior nucleotides no P\textsubscript{i} uptake into the vesicles was measurable when only P\textsubscript{i} or P\textsubscript{i} plus ATP were internally present (Fig. 3A, white circles and black diamonds). To our surprise P\textsubscript{i} was imported into liposomes loaded with P\textsubscript{i} plus ADP (Fig. 3A, gray squares). In a similar experiment we tested the effect of externally added ADP (Fig. 3B) or ATP (Fig. 3C) on P\textsubscript{i} transport. Application of external ADP did not markedly affect the rates of P\textsubscript{i} import into P\textsubscript{i} plus ADP loaded liposomes (Fig. 3, compare A and B, gray squares) but stimulated P\textsubscript{i} import into liposomes containing solely P\textsubscript{i} or P\textsubscript{i} plus ATP (Fig. 3, compare A and B, white circles and black diamonds). External ATP led to no or to comparably low P\textsubscript{i} import (compare the import rates of Fig. 3). The latter effect further argues for a possible competition of P\textsubscript{i} with ATP for import.

Our results show that P\textsubscript{i} indeed is a substrate of Pam\textsubscript{NTT1} but only when ADP is present at least at one side of the liposomal membrane. The observation that P\textsubscript{i} transport occurs also in the absence of adenine nucleotide counter-exchange (Fig. 3) suggests that Pam\textsubscript{NTT1} catalyzes, in addition to a possible P\textsubscript{i} plus ADP cotransport in exchange with ATP, an ADP-dependent P\textsubscript{i} homo-exchange. To provide further evidence for a P\textsubscript{i} homo-exchange, we analyzed P\textsubscript{i} import into ADP-loaded liposomes lacking luminal P\textsubscript{i}. The measured P\textsubscript{i} import rates were \(\sim 10\)-fold lower than the corresponding rates obtained with liposomes containing ADP plus P\textsubscript{i} (data not shown). This is a further argument supporting the postulated P\textsubscript{i} homo-exchange. To analyze whether P\textsubscript{i} homo-exchange is accompanied by a simultaneous unidirectional translocation of ADP, we additionally investigated \([\alpha-^{32}\text{P}]\text{ADP}\) import into proteoliposomes solely loaded with P\textsubscript{i}. Because in the absence of counter-exchange nucleotides P\textsubscript{i} but not ADP import occurred (data not shown), we concluded that ADP-dependent P\textsubscript{i} homo-exchange is not associated with a unidirectional ADP transport.

**Pam\textsubscript{NTT1} Catalyzes P\textsubscript{i} Homo-exchange and Cotransport with ADP**—In a further study we analyzed ADP and P\textsubscript{i} transport in more detail to address two important questions. First, is
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*PamNTT1* capable of transporting P\textsubscript{i} as cosubstrate of ADP during hetero-exchange, and second, what is the stoichiometrical ratio of the P\textsubscript{i} plus ADP cotransport.

ATP import in strict stoichiometrical exchange with one ADP plus one P\textsubscript{i} (H\textsubscript{2}PO\textsubscript{4}\textsuperscript{-}, with one negative charge) would be electroneutral. In this context it is important to mention that under the applied conditions an important part of P\textsubscript{i} carries one negative charge.

Unfortunately, determination of the ADP plus P\textsubscript{i} export parameters of the reconstituted PamNTT1 was impossible. We were not able to measure the slight decrease of the high internal label. Furthermore, traces of liposomal contaminations hampered the determination of exported radioactivity. However, we demonstrated above that exterior P\textsubscript{i} stimulates ADP\textsubscript{im}/ATP\textsubscript{ex} transport (supplemental Table S1 and Fig. 2, black bars) and that similarly interior P\textsubscript{i} stimulates ATP\textsubscript{im}/ADP\textsubscript{ex} transport (Fig. 1). Therefore, it seems justified to assume that P\textsubscript{i} acts as cosubstrate of ADP during hetero-exchange independent of the transport direction (ADP\textsubscript{im} + P\textsubscript{i}\textsubscript{im} / ATP\textsubscript{ex} or ATP\textsubscript{im} / ADP\textsubscript{ex} + P\textsubscript{i}\textsubscript{ex}). Accordingly, we investigated the coimport of P\textsubscript{i} and ADP.

For this, import measurements were performed with radioactively labeled ADP in the presence of nonlabeled P\textsubscript{i} as well as with labeled P\textsubscript{i} in the presence of nonlabeled ADP. One set of experiments was done with liposomes containing ATP plus P\textsubscript{i} and one was done with vesicles loaded with solely ATP (to disconnect the ADP-dependent P\textsubscript{i} homo-exchange from coimport with ADP). For determination of the ADP to P\textsubscript{i} coimport stoichiometry, we calculated P\textsubscript{i} import in relation to ADP import (ADP import was set as 1).

The presence of P\textsubscript{i} in the liposomal lumen led to a high P\textsubscript{i} to ADP import ratio (4.03 (± 0.45) P\textsubscript{i} to 1 ADP) (supplemental Fig. S1). Therefore, P\textsubscript{i} homo-exchange substantially exceeds coimport with ADP. However, depletion of luminal P\textsubscript{i} led to a significantly reduced P\textsubscript{i} homo-exchange and allowed the calculation of a nearly one-to-one stoichiometry for P\textsubscript{i} and ADP coimport (1.21 (± 0.16) P\textsubscript{i} to 1 ADP) in exchange with interior ATP (supplemental Fig. S1). It has to be mentioned that P\textsubscript{i} contaminations in the liposomal lumen or a marginal unidirectional P\textsubscript{i} transport might have caused a slight overestimation of the exact P\textsubscript{i}-to-ADP ratio.

**Determination of the Kinetic Properties and Identification of the Phosphate Binding Center of PamNTT1**—As demonstrated above, already low external ATP concentrations inhibited P\textsubscript{i} import (Fig. 3, compare B and C), whereas high exterior P\textsubscript{i} concentrations were required to substantially reduce ATP\textsubscript{im}/ADP\textsubscript{ex} transport (Fig. 2). These characteristics suggest that the two anions, ATP and P\textsubscript{i}, compete for uptake and that ATP is the preferred import substrate. On the other hand, P\textsubscript{i} exhibits positive effects on ADP transport (import and export) rather than competitive interference (Figs. 1 and 2). Accordingly, P\textsubscript{i} and ADP simultaneously fit into the binding center, whereas P\textsubscript{i} probably interacts with the NTT domain, which is otherwise occupied by the γ-phosphate of ATP.

In a previous study we showed that mutations of a lysine residue at position 527 in the plastidial ATP/ADP transporter from *A. thaliana* (*AtNTT1*) reduced ATP transport to a higher extent than ADP transport (34). Remarkably, this lysine residue is conserved in all plastidial and bacterial ATP/ADP transporters (supplemental Fig. S2). To test whether this amino acid residue is not only important for ATP but also for P\textsubscript{i} transport, we generated different PamNTT1 mutant proteins and analyzed their biochemical properties in the liposomal system. This was mandatory because *E. coli* possesses endogenous P\textsubscript{i} transporters that obscure NTT mediated P\textsubscript{i} translocation. We substituted the positively charged lysine by a positively charged arginine (*K446R*), by the neutral glutamine (*K446Q*), or by the negatively charged glutamate (*K446E*) to investigate a correlation between the charge of the amino acid residue 446 and the kinetic properties (*K\textsubscript{m} and *V\textsubscript{max} values) of PamNTT1.

The biochemical parameters of unmodified PamNTT1 for ATP and ADP transport were in the same range as previously described (32). Furthermore, the reconstituted wild type protein exhibited moderate affinities (*K\textsubscript{m} in the range of 140 μM) and high *V\textsubscript{max} values (9–16 μmol mg protein\textsuperscript{-1} h\textsuperscript{-1}) for ADP-dependent P\textsubscript{i} transport (Table 1). Application of internal ATP slightly and of external ATP highly reduced the affinity of PamNTT1 for P\textsubscript{i} uptake.

The conserved exchange of lysine 446 (*K446R*) led to a remarkable reduction of the *V\textsubscript{max} in particular of the ATP homo-exchange (Table 1). Furthermore, the affinity for ATP import in exchange with interior ADP plus P\textsubscript{i} was reduced, and that for ADP import into ATP plus P\textsubscript{i}-loaded vesicles was enhanced. Nevertheless, the basic properties of the mutant protein *PamNTT1-K446R*, such as the affinities for (ADP-dependent) P\textsubscript{i} import or ATP and ADP import during homo-exchange, as well as the preference of the ATP\textsubscript{im}/ADP\textsubscript{ex} transport over ADP\textsubscript{im}/ATP\textsubscript{ex} exchange, still resemble that of the unmodified *PamNTT1*.

In comparison with the wild type protein, the mutant protein with the neutral substitution (*PamNTT1-K446Q*) exhibited reduced affinities (and *V\textsubscript{max} values) for ATP import, whereas the affinities for ADP import, in particular in exchange with interior ATP, were significantly enhanced (Table 1). These changed biochemical characteristics led to a general adjust-

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**TABLE 1**

Comparison of the kinetic constants of reconstituted PamNTT1 and K446 mutant proteins

| Exchanges | PamNTT1 | K446R | K446Q | K446E |
|-----------|---------|-------|-------|-------|
| ATP/ATP + P\textsubscript{i} | 95 (27.6) | 123 (0.5) | 187 (6.5) | 620 (2.3) |
| ATP/ADP + P\textsubscript{i} | 8.6 (31.3) | 66 (1.0) | 274 (7.2) | 552 (2.4) |
| ADP/ATP + P\textsubscript{i} | 1401 (5.5) | 179 (0.3) | 68 (4.5) | 29 (1.6) |
| ADP/ADP + P\textsubscript{i} | 120 (7.1) | 96 (0.7) | 44 (4.0) | 159 (15.4) |
| P\textsubscript{i}/ADP + P\textsubscript{i} | 125 (13.6) | 271 (2.1) | |
| P\textsubscript{i} + ADP/ADP + P\textsubscript{i} | 154 (10.0) | 152 (0.5) | |
| P\textsubscript{i} + ADP/ATP + P\textsubscript{i} | 139 (15.9) | 220 (1.9) | 544 (0.4) |
| P\textsubscript{i} + ATP/ATP + P\textsubscript{i} | 295 (9.0) | 275 (0.7) | 550 (0.4) |
| P\textsubscript{i} + ATP/ADP + P\textsubscript{i} | 506 (13.3) | 598 (2.0) | |

Kinetic parameters of nucleotide or P\textsubscript{i} exchanges (exterior substrates/interior substrates) mediated by reconstituted PamNTT1 and the three mutant proteins (*PamNTT1-K446R, PamNTT1-K446Q, and PamNTT1-K446E*) were determined by application of rising concentrations of the respective labeled import substrates (5–1,500 μM). The used protein liposomes contained 5 μmol internal P\textsubscript{i} and the given nucleotides (10 μmol). Kinetic parameters of P\textsubscript{i} import were analyzed in the absence or presence of 50 μmol nonlabeled external nucleotides (added external nucleotides in squared brackets). *K\textsubscript{m} values are given in μM, and *V\textsubscript{max} values (in parentheses) are in μmol mg protein\textsuperscript{-1} h\textsuperscript{-1}, respectively. Transport was allowed for time spans in the linear phase of the corresponding import at 50 μM. The data are the means of three independent experiments. Standard errors of the low affinity and low velocity imports were below 20% and for the remaining imports below 14%.
ment of the import rates, the ATP homo-exchange equals ATP_{im}/ADP_{ex} transport, and also the high difference between ADP homo-exchange and ADP_{im}/ATP_{ex} transport of the wild type protein was reduced in PamNTT1-K446Q (Table 1 and supplemental Fig. S3). Because P_i transport of PamNTT1-K446Q was nearly completely diminished (supplemental Fig. S3), determination of the corresponding kinetic parameters was hampered. Solely in the presence of external ADP and internal P_i plus adenine nucleotides, very low affinities and V_{max} values for P_i import were calculable (Table 1).

Interestingly, the replacement of the positively charged lysine by the negatively charged glutamate completely blocked ADP-dependent P_i uptake (supplemental Fig. S3 and Table 1). In comparison with the unmodified PamNTT1 and the mutant PamNTT1-K446Q, the mutation in PamNTT1-K446E led to a further reduction of the K_m and V_{max} values of the ATP import (Table 1). Furthermore, the affinity for ADP import into ATP-loaded proteoliposomes was enhanced, whereas the V_{max} of this exchange was reduced. The moderate affinity for ADP import into ADP plus P_i-loaded vesicles, however, was accompanied by the highest V_{max}. Generally, the observed differences in the biochemical characteristics between the wild type protein PamNTT1 and the mutant PamNTT1-K446Q are more pronounced in the mutant PamNTT1-K446E. Our analyses of the mutant proteins revealed that a positively charged amino acid residue at position 446 is required for proper P_i import, for the preference of ATP_{im}/ADP_{ex} transport, and for the discrimination of ADP_{im}/ATP_{ex} exchange.

P_i Is Substrate of Several Nonmitochondrial ATP/ADP Transporters—The fact that PamNTT1 accepts P_i as third substrate might resolve inconsistencies in the phosphate metabolism of P. amoebophila. To analyze whether also phylogenetically more distantly related ATP/ADP transporters from other organisms possess the capacity to transport P_i, we investigated two carriers from the rickettsial species C. caryophilus (CcNTT) and H. obtusa (HoNTT) and one plastidial transporter from the higher plant A. thaliana (AtNTT1). These representative rickettsial and plastidial NTTs were heterologously expressed in E. coli, purified (supplemental Fig. S4), and reconstituted into liposomes. All recombinant carriers mediated ATP and ADP transport and therefore were functional in the liposomal system (supplemental Fig. S4). Generally, HoNTT exhibited highest net uptake rates that suggest a high activity of this reconstituted protein (Fig. 4 and supplemental Fig. S4). Import studies with radioactive P_i showed that AtNTT1 and also the two selected rickettsial NTTs were able to import P_i. In Fig. 4 the time-dependent P_i uptake in presence of exterior ADP is presented. P_i import into vesicles loaded with ADP plus P_i exceeded P_i import into proteoliposomes loaded with ATP plus P_i or with solely P_i. The rates of AtNTT1- and CcNTT-mediated P_i homo-exchange were lower than the rates of P_i (plus ADP) import into vesicles loaded with ATP plus P_i, whereas HoNTT exhibited higher rates for P_i homo-exchange than for P_i import in presence of exterior ADP and interior ATP plus P_i. Apart from slight differences in the substrate preference pattern, our results clearly demonstrate that ATP/ADP exchanging NTTs from distantly related organisms are capable for an

FIGURE 4. P_i import mediated by phylogenetically distantly related NTT-type ATP/ADP transporters. Heterologously expressed and purified ATP/ADP transporters from A. thaliana (AtNTT1), C. caryophilus (CcNTT), and H. obtusa (HoNTT) were reconstituted into liposomes loaded with 5 mM P_i or with 5 mM P_i plus the given nucleotides (10 mM). Time-dependent import of 50 μM radioactive P_i in presence of 50 μM nonlabeled ADP mediated by AtNTT1 (A), CcNTT (B), and HoNTT (C). Import into proteoliposomes loaded with P_i (open circle), P_i plus ADP (gray square), or P_i plus ATP (black diamond). The given values are net values (calculated by subtraction of P_i import in absence of external and internal nucleotides). The data are the means of at least three independent experiments, and the standard errors are displayed. The insets summarize the applied substrate conditions at the liposomal interior and exterior. Radioactive P_i is marked with an asterisk.
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ADP-dependent P_i transport, which resembles that of *PamNTT1* (Figs. 3 and 4).

**DISCUSSION**

Metabolically impaired energy parasites depend on ATP import from the host cell, and also plant plastids rely on energy supply from the surrounding cytosol when photosynthetic activity is reduced or missing. ATP/ADP exchange would lead to substantial P_i accumulation in the organelle or cell if no interacting export mechanism for phosphate exists. In this study we identified that P_i transport is a previously not identified intrinsic feature of nonmitochondrial ATP/ADP transporters (Figs. 3 and 4). P_i is transported simultaneously with ADP, and this cotransport is facilitated in a one P_i to one ADP stoichiometrical exchange with ATP (supplemental Fig. S1). The cotransport of one H_2PO_4^- (with one negative charge) would be in complete agreement with the recently documented electroneutrality of *PamNTT1* mediated ATP/ADP exchange (32). During hetero-exchange, P_i could act as a counterion compensating for the generation of a charge difference across the membrane.

A stimulatory influence of high P_i concentrations (in the millimolar range) on ADP import into isolated *Rickettsia prowazekii* cells had been observed more than three decades ago (38). Although a regulatory effect of P_i on the transport characteristics was discussed, the exact role of P_i in ATP/ADP exchange was not known (37). Interestingly, the simplest explanation, a possible cotransport of P_i and ADP, was never suggested for NTTs. Detailed analyses of *PamNTT1* (Figs. 2 and 3 and supplemental Fig. S1) and first studies with two carriers from *Rickettsiales* (Fig. 4, B and C) suggest that the regulatory principle of P_i is tightly associated to its function as a cosubstrate of ADP. The addition of P_i indeed increased the V_{max} and the affinity of *PamNTT1* for ADP transport into ATP loaded proteoliposomes. It is imaginable that a simultaneous entry of ADP and P_i might mimic the presence of a triphosphorylated adenine nucleotide (ATP), which is the preferred import substrate.

The capacity of the phylogenetically different NTTs to transport ADP plus P_i in exchange with ATP (i) is in line with the electroneutrality of nucleotide exchange (32), (ii) prevents harmful P_i accumulation in intracellular living bacteria and plant plastids, and (iii) implies that the phosphate exporter that was proposed for a long time in these bacteria and in plastids is represented by the “bifunctional” ATP/ADP plus P_i transporter.

Very recent analyses demonstrated that energy parasitism is not only restricted to *Chlamydiales* and *Rickettsiales* but also occurs in further important mammalian pathogens (23, 24). NTT-type carriers reside in the plasma membrane and in the mitochondrial relict (the so called mitosome) of the intracellular protist *Encephalitozoon cuniculi* (24). By catalyzing a highly specific ATP/ADP exchange, these NTTs provide energy to the cell or to the mitosome that lacks ATP synthesis. It is tempting to speculate that also these eukaryotic NTTs catalyze a P_i coexport with ADP.

To our surprise, NTT-type ATP/ADP transporters mediate a nucleotide exchange-independent but ADP-induced homo-exchange of P_i in addition to the P_i cotransport with ADP (Figs. 3 and 4, white circles). The P_i homo-exchange was induced by external as well as by internal ADP (Fig. 3). In the following we propose a scenario that could explain the induction of P_i homo-exchange independent of the side of ADP application.

ADP and P_i enter the binding center, for example at the proteoliposomal lumen, and are transported to the opposed side (Fig. 5). In absence of exchange nucleotides, ADP stays at the binding center, whereas the nonlabeled P_i is displaced by radioactive P_i. A subsequent reversely directed translocation of the two bound substrates and replacement of radioactive P_i by nonlabeled P_i at the interior causes the observed P_i homo-exchange (Fig. 5). This hypothesized mechanism presupposes that only one single binding center exists for import and export that opens from one side of the membrane to the other (Fig. 5, white circles).

By the help of mutant proteins we identified a correlation between the charge of the amino acid residue at position 446 and the transport properties of *PamNTT1*. A positive amino acid residue at position 446 allowed the insertion of ATP or P_i (plus ADP), a neutral amino acid residue or even more a negative amino acid residue stimulates ATP but suppresses P_i and ADP entry (Table 1). Substitution of Lys^{446} by arginine (K446R) entailed substantially lowered import rates for all substrates. We assume that structural differences between lysine and arginine rather than an unfavorable charge might have caused the decreased transport velocity (Table 1). The biochemical properties of the mutant proteins suggest that lysine 446 is an essen-
tial component of the ATP and Pi binding center or translocation pathway. We conclude that the positively charged lysine 446 interacts with the negative charge introduced either by the ϕ-phosphate of ATP or alternatively by Pi.

The fact that ATP import was highly reduced, whereas ATP export still occurred (ADP_{in}/ATP_{ex} transport) in the mutant protein PamNTT1-K446E might argue against a single binding center for ATP import and export. However, the \( V_{\text{max}} \) of ADP_{in}/ATP_{ex} exchange was remarkably low when compared with that of ATP homo-exchange (Table 1). This characteristic could be explained by an impaired ATP export capacity leading to a return transport of previously imported ADP. This would be in agreement with the characteristics of the mutated plastidial ATP/ADP transporter, which exhibited a highly reduced capacity for both ATP import and ATP export (34).

It becomes clear that additional studies are required to get more insights into structure/function relationships of NTTs, and it will be interesting to focus on this topic in the future. Furthermore, we wish to analyze transport parameters under conditions mimicking the energy state of intracellular living bacteria in their host cells. However, for this it is a pre-condition to determine \( P_i \) ATP, and ADP concentrations in the bacterium and the infected cell.

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