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Complete replacement of basic amino acid residues with cysteines in *Rickettsia prowazekii* ATP/ADP translocase ☆

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Abstract

The ATP/ADP translocase (Tlc) of *Rickettsia prowazekii* is a basic protein with isoelectric point (p*I*)=9.84. It is conceivable, therefore, that basic residues in this protein are involved in electrostatic interactions with negatively charged substrates. We tested this hypothesis by individually mutating all basic residues in Tlc to Cys. Unexpectedly, mutations of only 20 out of 51 basic residues resulted in greater than 80% inhibition of transport activity. Moreover, 12 of 51Cys-substitution mutants exhibited higher than wild-type (WT) activity. At least in one case this up-effect was additive and the double mutant Lys422Cys Lys427Cys transported ATP five-fold better than WT protein. Since in these two single mutants and in the corresponding double mutant K_m's were similar to that of WT protein, we conclude that Tlc may have evolved a mechanism that limits the transporter's exchange rate and that at least these two basic residues play a key role in that mechanism.

Based on the alignment of 16 Tlc homologs, the loss of activity in the mutants poorly correlates with charge conservation within the Tlc family. Also, despite the presence of three positively charged and one negatively charged intramembrane residues, we have failed to identify potential charge pairs (salt bridges) by either charge reversal or charge neutralization approaches.

Keywords: ATP/ADP translocase; Rickettsia; Cysteine-scanning mutagenesis; Multiple mutations; Basic residue

1. Introduction

The obligate intracellular bacterium *Rickettsia prowazekii*, the aetiologic agent of epidemic typhus, grows and multiplies only within the cytoplasm of a eucaryotic cell. As an adaptation to this unusual ecological niche, *R. prowazekii* evolved to possess transport systems that allow it to take advantage of the many preformed metabolites that are abundant in the host cell. The ATP/ADP translocase (Tlc) that performs an exchange of ADP, generated as a result of rickettsial metabolism, for the high-energy ATP of the host cell, is an example of such system [1]. Although ancestral rickettsiae are the favorite candidate for the endosymbiote that led to mitochondria [2], and mitochondria possess a functionally similar system (mitochondrial ADP/ATP carrier or AAC) that transports mitochondrial ATP in exchange for cytosolic ADP [3], the physiological direction of ATP transport in these two systems is opposite [4]. Recently, a homologous ATP/ADP transporter was described in another obligate intracellular bacterium *Chlamydia trachomatis* [5,6] and, in addition, several members of the Tlc family were found in plant plastids [7–9]. To date, more than 16 members of the Tlc family of membrane transporters are known. The members of the family with known functions are involved in the transport of various ribonucleotides.

The functional similarity notwithstanding, mitochondrial and non-mitochondrial ATP transporters are quite dissimilar biochemically and structurally. Tlc and AAC share no sig-

Abbreviations: Tlc, *Rickettsia prowazekii* ATP/ADP translocase; WT, wild type, unless otherwise noted here refers to double C37A, C85A Tlc mutant; TM, transmembrane domain; p*I*, isoelectric point; AAC, mitochondrial ADP/ATP carrier; IPTG, isopropyl-β-D-thiogalactopyranoside; MTSEA, 2-aminoethyl methanethiosulfonate hydrobromide; MTSET, [2-(trimethylammonium)ethyl] methanethiosulfonate bromide; MTSES, sodium(2-sulfonatoethyl)methanethiosulfonate

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nificant homology at the nucleotide or amino acid levels. Tlc is insensitive [1,10] to carboxyatractyloside and bongkrekic acid, which are specific inhibitors of AAC [11–13]. Mitochondrial transporters are believed to function as homodimers and mitochondrial ATP carriers have an internal sequence homology indicative of a gene triplication of a common ancestor coding for a protein of about 100 amino acids [14,15], features not observed in Tlc family of proteins. The topological model of Tlc generated by antibody accessibility [16] and the dual reporter approach [17,18] suggests that Tlc polypeptide crosses the cytoplasmic membrane 12 times, with its N- and C-termini facing the cytoplasm, whereas AAC monomer spans membrane six times [19].

Despite many differences, mitochondrial and non-mitochondrial ATP transporters share one common feature: they all are very basic proteins with isoelectric points (p*I*'s) between 9.24 and 9.98. This suggests that electrostatic forces might be important for Tlc–substrate interactions. Alternatively, positively charged amino acid residues could be involved in shielding of negative charges on substrates (ATP and ADP) during translocation through hydrophobic membrane environment. However, to date, only 12 out of 43 positively charged residues were mutated in AAC [19–21] and only two positively charged residues were mutated in ATP/ADP transporter from Arabidopsis [22]. Therefore, this study was undertaken as the first systematic attempt to mutate all positively charged residues in any ATP/ADP transporter.

2. Materials and methods

2.1. Site-directed mutagenesis

Site-directed mutagenesis was performed essentially as described earlier [23], except reactions were scaled-up two-fold and Vent® DNA polymerase was used instead of *Pwo*. In several instances, a modified restriction site PCR [24] and overlap-extension PCR [25] were used to introduce mutations. First, the two Cys residues found in wild-type (WT) *tlc* protein were replaced with Ala in the cassette version of the synthetic WT *tlc* gene [26] using primers 68-3′ TTCCTGTTTCAGGATGTC***GGC***CAGTTTAACG and 68-5′ TTCATGATGTTC***GCC***ATCCTGCTGAACTAC. The Cys-less protein had been shown to have WT activity [27]. The resulting Cys-less synthetic cassette *tlc* gene was eventually cloned in a pET11a derivative thus generating pMA619 and used as a template in all subsequent mutagenesis reactions. pMA619 (Fig. 1) represents a pET11a derivative with the synthetic C-less *tlc* gene cloned between *Nde*I and *Bam*HI sites. The pET11a backbone in pMA619 was modified to remove a *Pst*I site from the *bla* gene and to introduce a polylinker. The overall strategy included mutagenesis of short (350–550 bp) fragments of the gene with subsequent substitution of the WT fragment in pMA619 with the corresponding mutated one using appropriate restriction enzyme sites found in synthetic gene. Multiple mutations

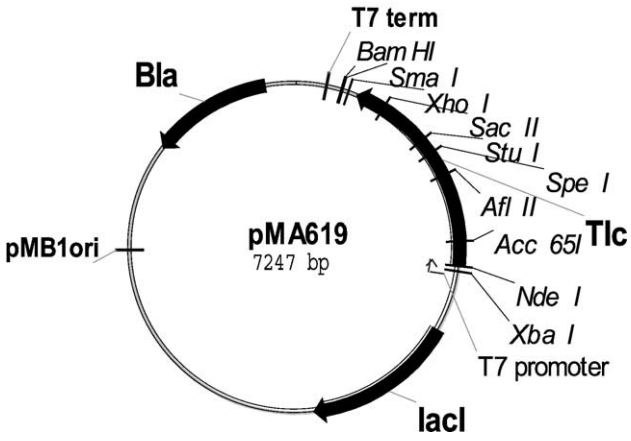

Fig. 1. The map of pMA619. Only relevant unique restriction sites are shown. *bla*, ampicillin-resistance gene; T7 term, T7 phage terminator; *lac*I, the gene for *lac* repressor, pMB1ori, origin of replication.

in Tlc were generated by combining single mutations in one molecule by subcloning corresponding fragments of single mutants, by doing sequential rounds of mutagenesis, or by designing primers with multiple mutations.

2.2. Transport activity assays

Activity of Tlc mutants was evaluated in *Escherichia coli* C41 cells [28] upon induction with isopropyl-β-D-thiogalactopyranoside (IPTG) essentially as described previously [26]. Protein content of the samples was determined using BC reagent kit (BioRad) according to the manufacturer's recommendations and ATP transport rates were expressed as picomoles ATP (milligram protein)$^{-1}$.

2.3. Reactions with MTS compounds

The effect of MTS compounds was determined after pretreatment of the cells with 0.78 mM 2-aminoethyl methanethiosulfonate hydrobromide (MTSEA), 2.5 mM [2-(trimethylammonium)ethyl] methanethiosulfonate bromide (MTSET) or 1 mM sodium(2-sulfonatoethyl)methanethiosulfonate (MTSES) for 5 min in KPi buffer before ATP uptake measurements. The compounds were prepared as 10×stocks in water immediately before use. Control samples were treated with water for the same period of time.

2.4. Cytoplasmic membrane preparations

E. coli cells were grown in 250-ml baffled-bottom flasks containing 50 ml of medium, induced as described above, and collected by centrifugation at 5000×*g* for 15 min at 4 °C. The cell pellet was resuspended in 1 ml KPi buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail and disrupted by sonication for 1 min. Unbroken cells and cell debris were removed by centrifugation at 10,000×*g* for 15 min at 4 °C. The membranes were then sedimented by centrifugation at 150,000×*g* for 45 min at 4 °C.

2.5. Western blotting

Purified cytoplasmic membranes were resuspended in water, adjusted to the same total protein concentration and solubilized in SDS-PAGE buffer. Electrophoretic separation of proteins was performed using Tris–Tricine buffer system (Amersham-Pharmacia Biotech). Proteins were transferred to PVDF membranes in a carbonate–bicarbonate buffer system [13], blocked in 5% non-fat dry milk or 3% BSA for 30 min and probed with antibodies developed against peptide corresponding to C-terminal portion of Tlc [16]. Western blots were developed with BCIP substrate (KPL) after incubation with goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma).

3. Results and discussion

3.1. Properties of single mutants

Every positively charged residue in *R. prowazekii* Tlc was individually mutated to Cys, thus generating a set of 51 single-Cys mutants. The transport activity in the resulting mutants varied between nearly 0 and more than 200% (Table 1). No major differences in Tlc expression levels could be detected by Western blotting (results not shown). Overall, mutation of only 20 out of 51 basic residues resulted in severe (>80%) inhibition of transport activity. According to the existing topological model [18], basic amino acid residues in Tlc are distributed asymmetrically (33 residues in cytoplasmic domains, 15 residues in periplasmic domains and 3 residues in transmembrane regions), which is consistent with "positive inside" rule [29]. However, asymmetry of distribution of the essential residues (>80% inhibition) between cytoplasmic and periplasmic compartments is less pronounced (11 residues in cytoplasmic domains, 7 residues in periplasmic domains and 2 residues in transmembrane regions). This might be a reflection of the symmetrical nature of transport conducted by Tlc.

Interestingly, in 12 cases (11 Lys residues and 1 Arg residue), replacements resulted in proteins that had higher transport activities as compared to WT (up-mutations). With two exceptions, those residues (up-residues) are located in cytoplasmic loops. Of the exceptions, one residue (Lys306) is located in periplasmic loop 8 and another (Lys483) in transmembrane helix 12, close to cytoplasmic face (Fig. 2). Up-residues are clustered in the two largest cytoplasmic loops 7 and 11 (four and three mutations, respectively).

Table 1
Properties of single mutants

Mutant	Location[a]	Conservation[b]	Percentage activity[c]	Mutant	Location[a]	Conservation[b]	Percentage activity[c]
K5C	Cyto1	25(25)	65±19 (6)	K257C	Cyto7	81(81)	120±38 (7)
R14C	Cyto1	50(56)	55±11 (6)	K259C	Cyto7	19(25)	170±8 (3)
K15C	Cyto1	13(19)	52±7 (5)	K260C	Cyto7	88(100)	142±61 (6)
K26C	Cyto1	63(63)	6±2 (6)	K262C	Cyto7	75(81)	145±34 (6)
K27C	Cyto1	88(88)	36±10 (5)	K264C	Cyto7	75(81)	89±10 (6)
R46C	Tm1	81(94)	7±1 (3)	K272C	Cyto7	50(50)	54±25 (5)
K49C	Peri2	94(100)	6±1 (3)	K278C	Cyto7	25(63)	19±13 (7)
K66C	Tm2	100(100)	4±1 (4)	K302C	Peri8	88(88)	3±1 (5)
K83C	Cyto3	81(88)	9±1 (4)	K304C	Peri8	63(63)	24±4 (3)
K89C	Cyto3	19(19)	49±6 (4)	K306C	Peri8	75(75)	142±31 (11)
H120	Peri4	88(88)	5±1 (2)	K312C	Peri8	13(25)	20±13 (6)
H123	Peri4	6(13)	51±1 (2)	R342C	Cyto9	81(88)	13±2 (3)
K124C	Peri4	6(6)	37±3 (3)	K343C	Cyto9	50(75)	48±16 (6)
K137C	Peri4	44(56)	12±2 (7)	K402C	Cyto11	81(94)	7±2 (6)
K141C	Peri4	13(19)	24±3 (4)	K405C	Cyto11	94(94)	5±1 (3)
K145C	Peri4	25(25)	58±22 (6)	K413C	Cyto11	88(94)	9±1 (4)
K176C	Cyto5	31(31)	215±52 (8)	K422C	Cyto11	13(13)	251±96 (10)
K181C	Cyto5	81(81)	19±7 (6)	R425C	Cyto11	19(100)	196±77 (9)
R182C	Cyto5	75(94)	14±4 (3)	K427C	Cyto11	81(88)	333±121 (9)
H207	Peri6	13(31)	24±9 (2)	R438C	Cyto11	44(69)	6±2 (6)
K209C	Peri6	19(19)	7±1 (3)	K441C	Cyto11	100(100)	9±5 (6)
H216	Peri6	25(25)	11±7 (2)	K483C	Tm12	44(63)	113±6 (3)
K218C	Peri6	13(13)	6±1 (3)	K487C	Cyto13	50(63)	95±39 (6)
R238C	Cyto7	31(38)	31±15 (8)	K495C	Cyto13	25(38)	242±97(6)
K242C	Cyto7	50(94)	48±18 (7)	K498C	Cyto13	19(19)	153±39 (7)
R249C	Cyto7	25(38)	51±13 (6)				

[a] Location of the mutated residue relative to the cytoplasmic membrane. Cyto, cytoplasmic; Tm, transmembrane; Peri, periplasmic. The numbers indicate the consecutive numbers of the corresponding domains.

[b] Conservation of corresponding residues based on alignment of 16 Tlc homologs. The first number indicates percentage identity, the number in parentheses indicates percentage similarity.

[c] Residual activity of corresponding single mutants expressed as mean±S.D. The number in parentheses indicates the number of independent experiments used to generate data.

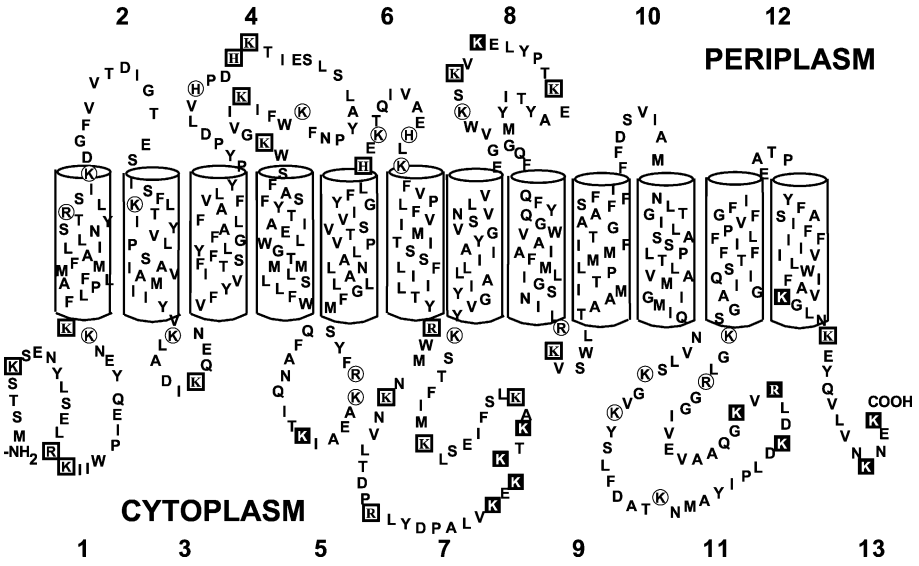

Fig. 2. Positioning of the mutated basic residues on the topological map of Tlc. Open circles, open squares and solid squares designate residues, whose replacement with Cys is inhibitory (<20% of WT activity), neutral (20–100% residual activity) and stimulatory (>100% activity).

Loops 7 and 11, which have 10 and 8 positively charged residues, seem to differ with respect to their tolerance of the replacements of positively charged residues with cysteines. Interestingly, in a previous study, we have found that insertions of short (25aa and 42aa) amino acid sequences are tolerated by loop 7, but not by loop 11 [17]. It appears that, with one exception, each of loop 7 mutants retains at least 30% of WT activity, suggesting that 9 of 10 positive charges in this loop are dispensable for function. On the other hand, residues in loop 11 are much less tolerant to replacements and, with exception of up-residues, mutants retain less than 10% of WT activity under similar conditions.

The residual activity of the mutants poorly correlates with charge conservation based on the alignment of 16 Tlc homologs (Table 1). One might expect high residual activity when conservation is poor and low activity when conservation is high. However, 33% (17 out of 51) of mutants demonstrate residual activity inconsistent with charge conservation at corresponding positions. For example, despite poor conservation of basic residues at positions 209 (19%) and 218 (13%) in periplasmic loop 6, the residual activity of corresponding mutants is relatively low (7% and 18% of WT, correspondingly). Conversely, Cys replacement mutants of better than 50% conserved positively charged residues at positions 14, 27, 242, 257, 260, 262, 264, 278, 304, 306, 343, 425, 427, 483 and 487 have fairly high residual activity (43%, 31%, 60%, 153%, 210%, 182%, 88%, 30%, 26%, 150%, 54%, 183%, 349%, 115% and 48% of WT, respectively). Interestingly, 7 of 12 up-residues (Lys257, 260, 262, 306, 427, 483 and Arg425) are located at positions with good charge conservation (81%, 100%, 81%, 75%, 88%, 63% and 100%, respectively). Also, a significant fraction (two of five) of Cys-replacement mutants at positions with 100% conserved positive charges retained more than 100% of WT activity, which is inconsistent with the initial hypothesis about significance of positive charges.

3.2. Effect of multiple replacements of positively charged residues

To get further insight into the role of Lys and Arg residues whose replacement with cysteins results in up-mutations, we constructed several additional mutations involving these residues (Lys422, Arg425 and Lys427) in loop 11 (Fig. 3). First, all possible double mutants of three up-residues in loop 11 were constructed (Fig. 3a). A double Lys422Cys Lys427Cys mutant in loop 11 exhibited transport rate that was almost equal to a sum of activities of corresponding single mutants and more than five-fold exceeded that of WT protein. The transport activities of other two double mutants were still higher than that found in WT control. An additive behavior of double Lys422Cys Lys427Cys mutant strongly suggests functional dispensability of Lys422 and Lys427 and suggests that these two mutations might affect different aspects of the transport process. Kinetic studies performed on mutants have shown that K_m in mutants Lys422Cys, Lys427Cys, and Lys422Cys Lys427Cys remained similar to those of WT protein (Fig. 4). This suggests that differences in transport rates are attributable primarily to changes in V_{max}. This also suggests that above residues are more involved in the translocation process than in the binding of negatively charged substrates. Therefore, it appears that Tlc has evolved a mechanism that limits the transporter's exchange rate and that Lys422 and Lys427 are playing a key role in that mechanism. Further, we reversed charges at the positions 422, 425 and 427 in Tlc by replacing positively charged residues with glutamate (Fig. 3b). The transport rate of K422E was higher than in WT and R425E did not change compared to WT, while the transport rate of K427E mutant

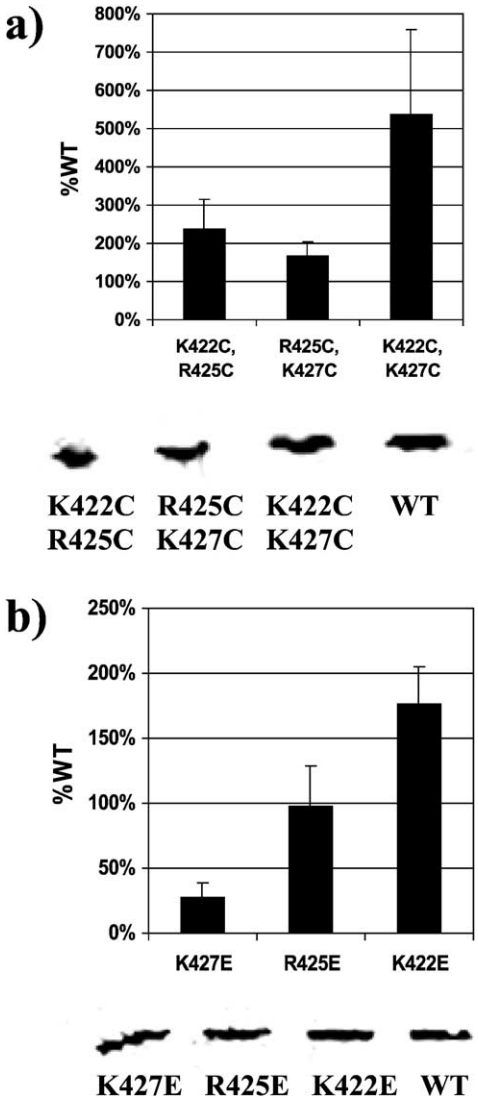

Fig. 3. Effect of replacements of up-residues in loop 11 on Tlc activity. (Panel a) Activities of double Cys-replacement mutants. (Panel b) Activities of the charge inversion mutants. Under each panel, a Western blot of membrane preparations from respective mutants is shown to demonstrate that steady-state Tlc levels were unchanged.

was inhibited (but 28% of WT activity remained). This again supports the idea of dispensability of positive charges at positions 422, 425 and 427.

Individual and multiple mutations of Arg and Lys residues in loop 11 suggested that at least some positively charged residues are non-essential for function. To strengthen this suggestion, we investigated how well multiple replacements are tolerated by Tlc. Thus, a mutant was constructed in which five clustered Lys residues in loop7 (Lys257, 259, 260, 262 and 264) were replaced with Cys. Interestingly, this quintuple mutant still retained about 20% of WT activity (Table 2). Therefore, this cluster mutation was combined with other mutations taking advantage of unique restriction endonuclease sites found in the synthetic *tlc* gene [26]. Introduction of Lys306Cys up-mutation into this quintuple mutant resulted in a protein with about three-fold increase in transport rate. Mutating two more residues in the resulting hextuple mutant (Lys15Cys and Lys176Cys or Lys422Cys and Lys427Cys mutations) reduced transport rates to about 30% of WT. A mutant with 10 positively charged residues replaced with Cys still retained 9% of WT activity, while replacement of 11 positively charged residues reduced activity to essentially background level (Table 2). This all strongly suggested dispensability of at least some positively charged residues in Tlc.

3.3. Potential involvement of Lys and Arg residues in formation of interhelical charge pairs

Charged residues are rarely found in transmembrane domains (TMs) of proteins because of the high free energy that such residues possess in hydrophobic environment. At least in some instances membrane proteins have been shown to minimize this energy through formation of interhelical charge pairs (salt bridges). Thus, two such bridges (between Asp237 and Lys358 and between Asp240 and Lys319) were identified in lactose permease (LacY) of *E. coli*. These bridges were initially identified through second site suppressor analysis [30,31] and later confirmed by biochemical techniques [32–34]. Remarkably, it was noticed that replacement of one member of a charged pair with a residue with a neutral side chain led to inactivation of the permease, whereas mutants in which neutral amino acids replaced both charged residues retained activity. Similarly, simultaneous charge reversal at both positions has been shown in some instances to produce an active protein, whereas the corresponding single mutants were inactive [34].

According to the current topological model [18], Tlc has three intramembrane positive charges, Arg46 (TM I), Lys66 (TM II) and Lys483 (TM XII), and only one intramembrane negative charge, Glu156 (TM IV). Of those three intramembrane positive charges, neutralization of only two (Arg46 and Lys66) led to loss of activity. It is currently impossible to identify charge pairs in Tlc by either second site suppressor analysis or by chemical cross-linking experiments because of the absence of a system that would allow for selection of Tlc function and because of the absence of sites to produce functional "split" translocases, respectively. Therefore, we constructed single Glu156Cys, Glu156Lys, Arg46Glu, and Lys66Glu as well as double Arg46Cys Glu156Cys, Lys66Cys Glu156Cys, Arg46Glu Glu156Lys, and Lys66Glu Glu156Lys mutants and tested if their behavior fits that expected of residues involved in the formation of charge pairs. All newly constructed single mutants were inactive, which would be expected of mutants of charge pair forming residues. However, the gain of function was not observed with any of the double mutants tested suggesting that Arg46, Lys66 and Glu156 may be involved in the formation of charge pairs with residues other than themselves (data not shown). Similar situation is observed in yeast AAC carrier, which has four positively charged residues in the transmem-

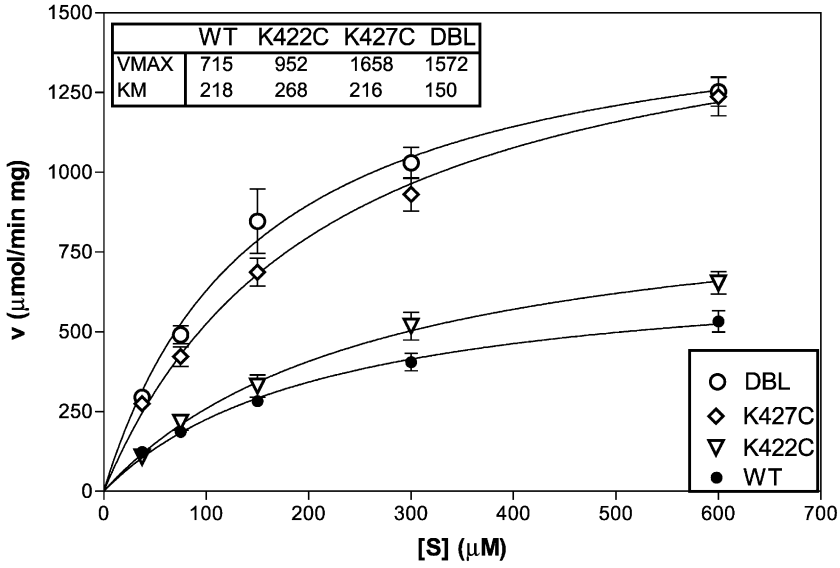

Fig. 4. Comparison of kinetics of ATP transport. The *E. coli* cells expressing the WT Tlc and mutants were grown, induced, washed and the kinetics determined in at least two independent experiments at 34 °C with triplicate points at 1 min for each substrate concentration. Non-linear regression analysis was based on the Michaelis–Menten equation and computed with the help of GraphPad Prism software.

brane helices. In that case, second-site suppressor analysis revealed that transmembrane positive charges are paired with extramembrane negative counterparts [35].

3.4. Effect of MTS compounds on the activity of single Cys replacement mutants

One of the advantages of Cys replacement mutagenesis is the availability of a battery of Cys-specific reagents that can be used for modification of the introduced Cys residues. This combination of Cys-scanning mutagenesis and subsequent modification with such reagents has been successfully used to elucidate membrane topology, spatial proximity between TMs and to identify intramembrane residues involved in formation of "water-filled channels" for substrate translocation (reviewed in Ref. [36]). We studied the effect of treatment with two membrane-impermeable sulfhydryl-specific reagents, MTSET and MTSES [37], on all 51 single Cys-replacement mutants. The modification of the cysteine's SH group with MTSET reintroduces the positive charge and modification with MTSES reverses the WT charge. With one exception, treatment with these MTS compounds had no effect on mutants. This is expected with Cys that are located in cytoplasmic loops and therefore inaccessible to modification. Treatment of only 1 of 15 mutants with predicted periplasmic localization affected activity. Although our experimental setup does not allow one to exclude the possibility that the other 14 periplasmic cysteins are inaccessible to modification, this seems unlikely. Modification of Lys306Cys with MTSET reduced the ATP transport to 28±5% of untreated control, whereas MTSES treatment reduced it to 11±1%. The more severe effect of MTSES treatment on Lys306Cys is consistent with charge reversal by site-directed mutagenesis (2.5% of WT activity in Lys306-Glu mutant vs. 285% in Lys306Arg mutant, data not shown). The inhibitory effect of MTSET as compared to the Lys306Arg mutation apparently results from the bulkiness of the formed adduct. Interestingly, ATP does not protect the Lys306Cys mutant from MTSES modification, which suggests that Lys306 does not directly interact with the substrate.

Table 2
Properties of multiple Cys-replacement Tlc mutants

Mutated residues	Number of residues mutated[a]	Residual activity of the mutant (number of experiments)[b]
257, 259, 260, 262, 264	5	20±5 (5)
257, 259, 260, 262, 264, 306	6	64±15 (6)
15, 176, 257, 259, 260, 262, 264, 306	8	28±9 (6)
257, 259, 260, 262, 264, 306, 422, 427	8	34±9 (4)
15, 176, 257, 259, 260, 262, 264, 306, 422, 427	10	9±3 (6)
15, 176, 257, 259, 260, 262, 264, 306, 422, 425, 427	11	3±1 (6)

[a] The total number of positively charged residues replaced in each multiple mutant.

[b] Percentage activity remaining in the multiple mutant as compared to WT. Numbers are mean±S.D. The number of independent experiments used to generate the data is given in parentheses.

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