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Accessibility of Cysteine Residues in a Cytoplasmic Loop of CitS of *Klebsiella pneumoniae* Is Controlled by the Catalytic State of the Transporter[†]

Iwona Sobczak and Juke S. Lolkema*

Molecular Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Haren, The Netherlands

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ABSTRACT: The citrate transporter CitS of *Klebsiella pneumoniae* is a secondary transporter that transports citrate in symport with two sodium ions and one proton. Treatment of CitS with the alkylating agent *N*-ethylmaleimide resulted in a complete loss of transport activity. Treatment of mutant proteins in which the five endogenous cysteine residues were mutated into serines in different combinations revealed that two cysteine residues located in the C-terminal cytoplasmic loop, Cys-398 and Cys-414, were responsible for the inactivation. Labeling with the membrane impermeable methanethiosulfonate derivatives MTSET and MTSES in right-side-out membrane vesicles showed that the cytoplasmic loop was accessible from the periplasmic side of the membrane. The membrane impermeable but more bulky maleimide AmdiS did not inactivate the transporter in right-side-out membrane vesicles. Inactivation by *N*-ethylmaleimide, MTSES, and MTSET was prevented by the presence of the co-ion Na⁺. Protection was obtained upon binding 2 Na⁺, which equals the transport stoichiometry. In the absence of Na⁺, the substrate citrate had no effect on the inactivation by permeable or impermeable thiol reagents. In contrast, when subsaturating concentrations of Na⁺ were present, citrate significantly reduced inactivation suggesting ordered binding of the substrate and co-ion; citrate is bound after Na⁺. In the presence of the proton motive force, the reactivity of the Cys residues was increased significantly for the membrane permeable *N*-ethylmaleimide, while no difference was observed for the membrane impermeable thiol reagents. The results are discussed in the context of a model for the opening and closing of the translocation pore during turnover of the transporter.

The sodium-ion dependent citrate transporter CitS of *Klebsiella pneumoniae* transports divalent citrate in symport with two sodium ions and one proton (1, 2). The transporter functions in the anaerobic citrate degradation pathway of *K. pneumoniae* and is encoded in an operon together with two other enzymes of the pathway, citrate lyase and the primary sodium ion pump, oxaloacetate decarboxylase (3). The *citS* gene encodes a highly hydrophobic protein of 446 amino acid residues and a predicted molecular mass of 47 557 Da (4). CitS belongs to the family of 2-hydroxycarboxylate transporters (2HCT)¹ that contains secondary transporters exclusively found in bacteria (5). Substrates of the transporters in the family share a 2-hydroxycarboxylate motif like in citrate, malate, and lactate. The family contains sodium ion symporters (CitS, MaeN of *Bacillus subtilis*) and proton symporters (CimH of *B. subtilis*, MaeP of *Streptococcus bovis*) but also precursor/product exchangers (CitP of *Leu-*

conostoc mesenteroides, MleP of *Lactococcus lactis*, CitW of *K. pneumoniae*) (6–11). The citrate/lactate exchanger CitP and the malate/lactate exchanger MleP found in lactic acid bacteria are involved in secondary proton motive force generation (10, 12, 13).

Studies on the membrane topology of CitS revealed that the protein contains 11 transmembrane segments (TMSs) that traverse the membrane in a zigzag fashion with the N- and C-terminus on the cytoplasmic and periplasmic face of the membrane, respectively (14–16). In the secondary structure model (Figure 1), the N-terminal half is separated from the C-terminal half by a long cytoplasmic loop that is observed in many transporters of this type. Three other loops are considerably longer than found usually in bacterial transporters, and it was speculated that they may have a role in structure and/or function of the transporter (16). The loop between TMS V and VI constitutes a hydrophobic segment termed Vb that is long enough to span the membrane but is translocated into the periplasm during insertion. The cytoplasmic loop between TMS VIII and IX forms an amphipathic helix (AH) that is believed to play a role in the co-insertion of the two adjacent TMSs (17). Finally, the cytoplasmic loop between TMS X and XI, here called region Xa, forms (together with segment Vb) one of the two most conserved regions in the family and is suspected to play an important role in substrate binding. Chimeric transporters in which the 46 C-terminal residues containing the Xa region of the CitP

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* Corresponding author. Phone: +31 50 3632155. Fax: +31 50 3632154. E-mail: j.s.lolkema@biol.rug.nl.

¹ Abbreviations: 2HCT, 2-hydroxycarboxylate transporter; TMS, transmembrane segment; RSO, right-side-out; PMS, phenazine methosulfate; NEM, *N*-ethylmaleimide; AmdiS, 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid; MTSEA, 2-aminoethyl methanethiosulfonate hydrobromide; MTSES, sodium (2-sulfonatoethyl)methanethiosulfonate; MTSET, [2-(trimethylammonium)ethyl] methanethiosulfonate bromide.

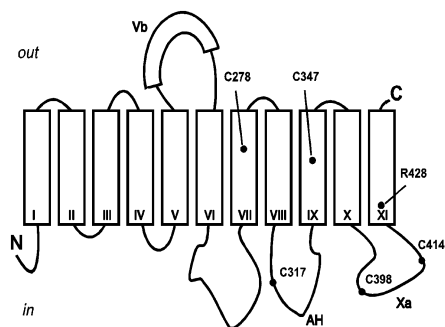


FIGURE 1: Membrane topology model of the CitS transporter protein. The CitS protein contains 11 TMS indicated by the boxes. The length of the loops more or less correlates with the number of residues in the loop. Segment Vb corresponds to a hydrophobic region that was predicted to be transmembrane. Loop Xa is one of the best conserved regions in the protein family. The positions of the five endogenous cysteine residues were indicated. Arg-428 in TMS XI is believed to be involved in substrate binding.

and MleP proteins were exchanged showed that this part constitutes part of the substrate binding pocket (18). Moreover, a conserved arginine residue at the cytoplasmic side of TMS XI was shown to be essential in CitS of *K. pneumoniae* (Arg-428; unpublished), in CitP of *L. mesenteroides* (Arg-425; (19)), and in CimH of *B. subtilis* (Arg-432; (20)). The broad specificity of the exchangers in the family allowed for the identification of Arg-425 of CitP as the residue that directly interacts with one of the carboxylate groups on the substrates (18, 21). The position of the conserved arginine residue in the model suggests that the binding site in this family of transporters is located closely at the cytoplasm/membrane interface.

Recently, we showed that conserved residues Arg-420 and Glu-428 in the Xa region of CimH of *B. subtilis* were accessible from the periplasmic side of the membrane by membrane impermeable reagents (20). It was suggested that a water-filled substrate translocation pathway would extend all the way to the cytoplasmic loop, or alternatively, region Xa would form a reentrant looplike structure similarly as reported for the glutamate transporters GltT of *Bacillus stearothermophilus* and GLT-1 in the central nervous system (22, 23). Here, we confirm and extend the observations made with CimH of *B. subtilis* by showing that two nonconserved Cys residues in region Xa of CitS of *K. pneumoniae* are accessible from the periplasmic side of the membrane. The importance of the region is demonstrated by showing that the accessibility of the residues is directly linked to the translocation event. The accessibility provides an assay for different conformational states of the protein during turnover.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Media, and Growth Conditions. *Escherichia coli* strains DH5 α and ECOMUT2 (24) were routinely grown in Luria Bertani Broth (LB) medium at 37 °C under continuous shaking at 150 rpm. When appropriate, antibiotics such as ampicillin and chloramphenicol (ECOMUT2 strain) were added at a final concentration of 50 and 30 μ g/mL, respectively.

All genetic manipulations were done in *E. coli* DH5 α while the CitS protein was expressed in *E. coli* ECOMUT2 harboring plasmid pBADCitS that contains the gene coding for CitS with an N-terminal His-tag. Expression of CitS was

induced for 1 h by adding 0.1% arabinose when the optical density of the culture measured at 660 nm (OD₆₆₀) was 0.6. Citrate metabolism was detected as blue colonies on Simmons citrate agar indicator plates (Difco). The host *E. coli* ECOMUT2 grew poorly without change of color (white colonies) on Simmons agar plates.

Construction of pBADCitS and the Cysteine Mutants of CitS. Plasmid pBADCitS was constructed by recloning the *citS* gene (*NcoI/XbaI*) from plasmid pHisCitS (15) into the pBAD24 vector (25) as follows. Plasmid pGltThisIII is pBAD24 containing the *gltT* gene coding for glutamate transporter of *B. stearothermophilus* (24). The *gltT* gene was cut out from the plasmid using the *NcoI* and *XbaI* restriction enzymes and replaced with the *citS* gene. The resulting plasmid codes for the CitS protein with an N-terminal His-tag. Expression of the gene is under control of the arabinose promoter.

Cysteine mutants of CitS were constructed by PCR using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Plasmid pHisCitS was used as the template. All mutants were sequenced (Baseclear, Leiden, The Netherlands) to confirm the presence of the desired mutations and recloned into the pBAD24 vector as described above.

Preparation of the Right-Side-Out Membrane Vesicles. Right-side-out (RSO) membrane vesicles were prepared by the osmotic lysis procedure as described previously (26). Membrane vesicles were resuspended in 50 mM potassium phosphate (KPi) pH 6.0 and rapidly frozen and stored in liquid nitrogen. Membrane protein concentration was determined by the DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA).

Transport Assay in RSO Membranes. Uptake was measured by the rapid filtration method. RSO membranes were energized using the K-ascorbate/phenazine methosulfate (PMS) electron donor system (27). Membranes were diluted to a final concentration of 0.5 mg/mL into 50 mM KPi pH 6.0 containing 25 mM Na⁺ or as indicated otherwise, in a total volume of 100 μ L at 30 °C. Under a constant flow of water-saturated air, and while stirring magnetically, 10 mM K-ascorbate and 100 μ M PMS (final concentration) were added, and the proton motive force was allowed to develop for 2 min. Then, [1,5-¹⁴C]-citrate (114 mCi/mmol Amersham Pharmacia, Roosendaal, The Netherlands) was added to a final concentration of 4.4 μ M. The uptake was stopped by the addition of 2 mL of ice-cold 0.1 M LiCl, followed by immediate filtration over cellulose nitrate filters (0.45 μ m pore size). The filters were washed once with 2 mL of the 0.1 M LiCl solution and assayed for radioactivity. The background was estimated by adding the radiolabeled substrate to the vesicle suspension after the addition of 2 mL of ice-cold LiCl, immediately followed by filtering. Specific activities of CitS wild type and mutants were compared by correcting the uptake rates for the level of expression of the proteins determined by Western blotting. When indicated, experiments were carried out in low sodium KPi buffer that contains at most 0.005% Na⁺, while standard KPi buffers may contain up to 0.3%.

Treatment of Membranes with Thiol Reagents. RSO membranes were treated with 1 mM NEM for 10 min and with 0.25 mM AmdS, 1 mM MTSEA, 2 mM MTSET, and 10 mM MTSES for 20 min in 50 mM KPi pH 7.0 at room temperature. The treatment was stopped by addition of an

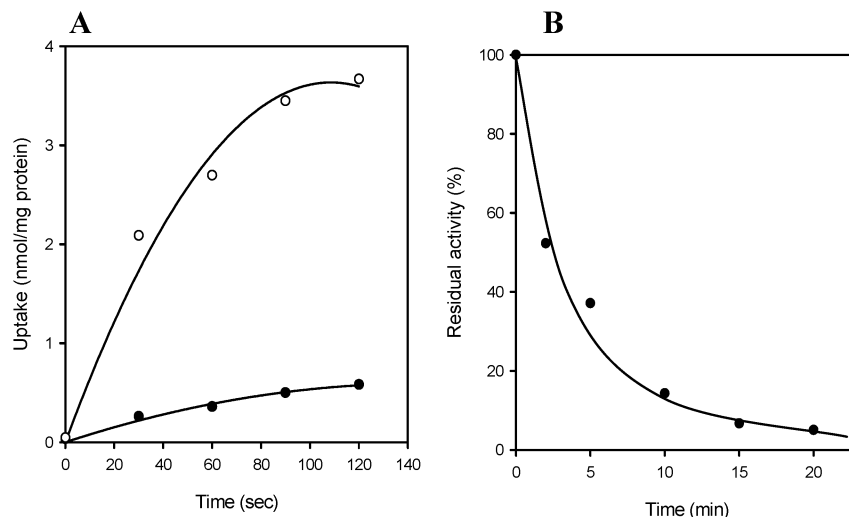


FIGURE 2: Citrate uptake activity of wild type CitS. (A) [1,5- ^{14}C]-citrate uptake in RSO membrane vesicles containing wild type CitS before (open circles) and after (solid circles) treatment with 1 mM NEM for 10 min. (B) Residual citrate uptake rate after treatment with 1 mM NEM for the indicated time. Initial rates were expressed as the percentage of the initial rate catalyzed by untreated CitS (3.5 nmol/min per mg of protein).

excess of 1 mM DTT in case of NEM and Amdis, and 1–10 mM L-cysteine in case of the MTS-reagents, after which the sample was diluted twice into 50 mM KPi pH 5.0. The resulting suspension was pH 6.0 and was immediately used to measure uptake activity.

The effect of sodium ions on the treatment was measured by adding a mixture of NaCl and KCl yielding a total concentration of 70 mM chloride. Upon dilution into the assay mixture, the Na^+ and K^+ concentrations were adjusted to 70 mM.

The effect of citrate was measured by adding the indicated citrate concentrations from a 0.5 M stock solution adjusted to pH 6.0. Following the treatment, the membranes were washed twice with 600 μL of 50 mM KPi pH 6.0 by centrifugation for 20 min in a Beckman OptimaTLX ultracentrifuge in a rotor TLA100.2 to remove the substrate. Membranes were resuspended in 50 mM KPi pH 6.0 containing 25 mM Na^+ and assayed for uptake activity. The washing procedure resulted in a loss of 10–15% of the uptake activity.

The effect of the pmf on the treatment was studied in RSO membrane vesicles diluted in 50 mM KPi pH 7.0 to a final concentration of 0.5 mg/mL and incubated for 2 min at 30 $^\circ\text{C}$ in the presence of Ascorbate/PMS as described above. After 2 min NEM, MTSET, or MTSES was added to a final concentration of 0.3, 1, and 3 mM, respectively, and incubated for another 3 min. The membranes were washed once as described above and resuspended in 50 mM KPi pH 6.0.

Immunoblot Analysis. Samples containing 20 μg of total membrane protein were loaded onto a 12.5% sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE). After electrophoresis, the proteins were transferred to poly(vinylidene difluoride) (PVDF) membranes (Roche Diagnostic GmbH, Mannheim, Germany) by semi-dry electroblotting. The blots were analyzed using monoclonal antibodies directed against the His-tag (Dianova, Hamburg, Germany). Antibodies were visualized using the Western-light chemiluminescence detection kit (Tropix, Bedford, MA). Expression levels were estimated by determining the chemiluminescence intensity

of each band using a Lumi-Imager F1 imager (Roche Diagnostic GmbH, Mannheim, Germany).

Materials. Sodium (2-sulfonatoethyl) methanethiosulfonate (MTSES), 2-(trimethylammonium)ethyl methanethiosulfonate bromide (MTSET), and 2-ethylammonium methanethiosulfonate hydrobromide (MTSEA) were purchased from Anatrace Inc. (Maumee, OH). NEM was purchased from Sigma-Aldrich BV (Zwijndrecht, The Netherlands), and Amdis was purchased from Molecular Probes Europe BV (Leiden, The Netherlands).

RESULTS

Inactivation of CitS with *N*-Ethylmaleimide. Transport activity of CitS using [1,5- ^{14}C]-citrate as the substrate was measured in right-side-out vesicles prepared from *E. coli* ECOMUT2 harboring plasmid pBADCitS, which encodes the wild type CitS protein. In the presence of a proton motive force (pmf) generated by the artificial electron donor system Asc/PMS, the membranes took up citrate at a initial rate of 3.5 nmol/min per mg of protein (Figure 2A, open circles). As shown before (4), no uptake activity could be detected in RSO membrane vesicles that were prepared from the host cells that do not express CitS (not shown). Prior treatment of the membranes containing CitS with 1 mM thiol reagent *N*-ethylmaleimide (NEM) for 10 min resulted in 80% inhibition of the citrate uptake rate (Figure 2A, solid circles). Treatment with NEM for increasing amounts of times revealed a half time of inactivation of 3 min, and eventually, full inactivation (Figure 2B).

Identification of the Reactive Cysteine Residues. The CitS protein contains five cysteine residues (CCCCC, see Figure 1). Two cysteine mutants were constructed, SSSCC and CCCSS, in which the first three (Cys-278, Cys-317, and Cys-347) and last two (Cys-398 and Cys-414) cysteine residues were mutated into serine residues, respectively. *E. coli* ECOMUT2 expressing the two mutants grew as dark blue colonies on Simmons agar indicator plates indicative of citrate uptake and metabolism. Uptake activity in RSO membranes indicated that both mutants showed significant citrate transport activity, while the expression levels as

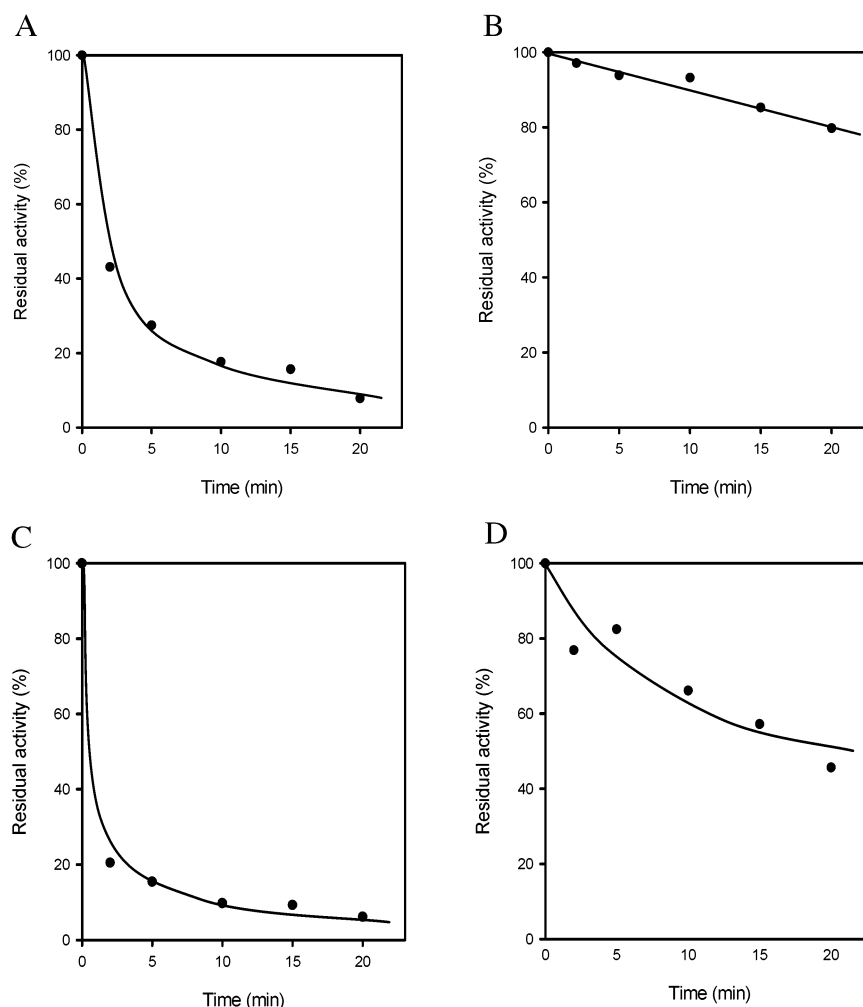


FIGURE 3: Effect of NEM on the citrate uptake activity of cysteine mutants. Residual activity of the [1,5- 14 C]-citrate uptake after treatment of RSO membrane vesicles containing CitS mutants SSSCC (A), CCCSS (B), SSSSC (C), and SSSCS (D) for the indicated time with 1 mM NEM. Residual activity was presented as the percentage of the initial rate catalyzed by untreated membranes.

determined by Western blotting were similar as observed for the wt protein. The specific activities of the CCCSS and SSSCC mutants were 56 and 79% relative to the wild type, respectively (not shown).

Treatment of mutant SSSCC with 1 mM NEM revealed an inactivation pattern with a half time of about 2.5 min, very similar to that observed for the wild type (Figure 3A). In contrast, mutant CCCSS was quite insensitive to NEM; the loss of activity was 20% after 20 min of incubation (Figure 3B). The results show that the inactivation of wild type CitS was mainly due to labeling of one or both of the two cysteine residues located in loop Xa.

The specific activity of the two single cysteine mutants SSSSC and SSSCS that were constructed next was 28 and 57% relative to wild type, respectively. Mutant SSSSC was much more sensitive to NEM than observed for the wild type and the SSSCC mutant. The half time of inactivation was less than 1 min (Figure 3C). Interestingly, the cysteine mutant SSSCS was inactivated by NEM much more slowly resulting in about 50% residual activity after 20 min of treatment (Figure 3D). Clearly, both cysteine residues are accessible to NEM, and labeling results in inactivation of the transporter. However, the reactivity in the two single cysteine mutants, SSSCS and SSSSC, is not the same as in the SSSCC mutant. The presence of Cys-398 reduces the reactivity of Cys-414.

Accessibility of the Cysteines in CitS. Amdis is a maleimide derivative containing a bulky, negatively charged group, and therefore, in contrast to NEM, membrane impermeable. Treatment of membranes containing CCCCC and SSSCC with Amdis did not result in a significant decrease of uptake activity (Figure 4).

The methanethiosulfonate (MTS) derivatives MTSEA, MTSET, and MTSES represent thiol reactive reagents that react with cysteine residues in proteins to form mixed disulfides. They differ in size, membrane permeability, and charge of the groups attached to the reactive MTS moiety. MTSEA and MTSET are positively charged, whereas MTSES is negative. The reactivity of the reagents decreases in the order MTSET > MTSEA > MTSES (28). Treatment of RSO membrane vesicles expressing CitS and cysteine mutants SSSCC and CCCSS for 20 min in the absence of sodium ions, with 1 mM MTSEA, 2 mM MTSET, and 10 mM MTSES, resulted in significantly reduced uptake activity for all the three reagents (Figure 4A). The same pattern of inactivation was observed with RSO membranes containing the SSSCC mutant, indicating that labeling of the two cysteine residues in loop Xa was responsible for the inactivation (Figure 4B). Membranes containing mutant CCCSS were largely insensitive to MTSET and MTSES, but the uptake activity was reduced down to 30% after treatment with MTSEA (Figure 4C). MTSEA is known to effect the

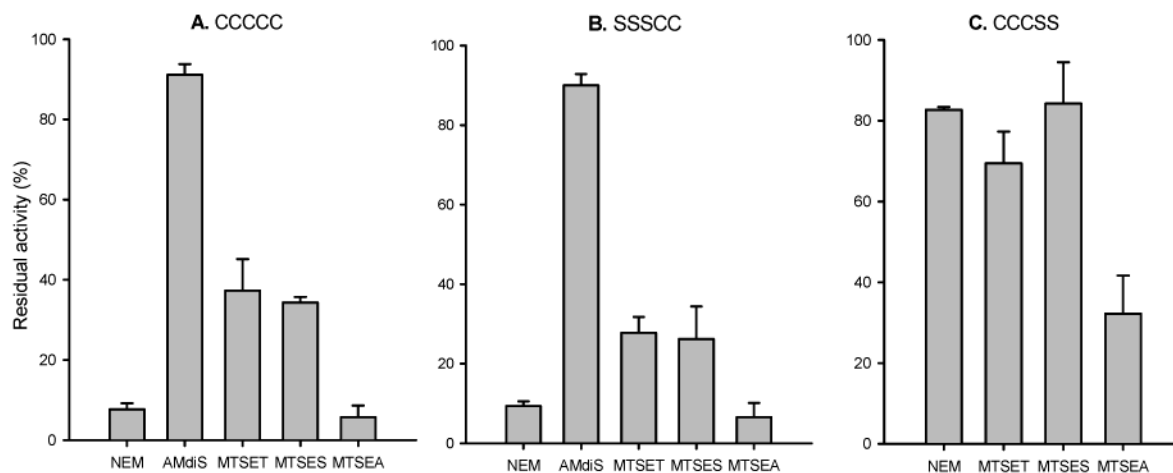


FIGURE 4: Inactivation of CitS by various thiol reagents. RSO membrane vesicles containing wild type CitS (A) and the mutants SSSCC (B) and CCCSS (C) were treated with the indicated thiol reagents after which the activity was measured and plotted as the percentage of the rate obtained with untreated membranes. The reagents were used at concentrations of 1 mM NEM, 0.25 mM AmdIS, 1 mM MTSEA, 2 mM MTSET, and 10 mM MTSES. The bars represent the average and standard deviation obtained from at least three independent measurements.

pmf generating system in RSO membranes (29), and it is concluded that the CCCSS mutant itself is not sensitive to treatment by MTS reagents. It follows, in addition, that the high decrease of uptake activity by MTSEA observed in the membranes containing the wild type and the SSSCC mutant, in part, was due to the reduced capacity of the membranes to generate proton motive force.

In conclusion, the cysteine residues in cytoplasmic loop Xa are accessible to the small membrane impermeable MTS reagents MTSET and MTSES but not to the bulky and impermeable maleimide AmdIS, added at the periplasmic side of the membrane.

Effect of Na^+ on Uptake and Inactivation by Thiol Reagents. Transport activity of the CitS protein is strictly coupled to translocation of two sodium ions (1). The rate of uptake of citrate in whole cells of *E. coli* expressing CitS increased sigmoidally with increasing Na^+ concentration. Half of the maximal rate was obtained at a concentration of 3.5 mM Na^+ at pH 6.0. In RSO membrane vesicles containing CitS, a similar sigmoidal dependence was observed (Figure 5, closed circles). The rate increased more than proportional with Na^+ concentrations up to 1 mM (Figure 5B). Half of the maximal rate of citrate uptake was obtained at a slightly lower Na^+ concentration than observed in whole cells (2 vs 3.5 mM, respectively). The very low rate observed in the absence of added sodium ions confirms that CitS activity is obligatorily coupled to the translocation of sodium ions.

The inactivation of CitS by thiol reagents presented thus far was performed in the absence of added sodium ions. The presence of sodium ions had a dramatic effect on the inactivation. Labeling of wild type CitS with 1 mM NEM was prevented above concentrations of 25 mM Na^+ (Figure 5A, open circles). Importantly, a plot of the residual activity as a function of the Na^+ concentration during NEM treatment revealed the same sigmoidal shape as observed in the relation between activity and Na^+ concentration, indicating that it takes two sodium ions to bind for the cysteine residues to become insensitive to the alkylating reagent (Figure 5C). The Na^+ concentration yielding half of the full protection was 6 mM.

The protection of CitS against labeling by NEM in the presence of sodium ions was also observed for the MTS reagents. In the presence of 70 mM Na^+ , the residual activities were between 80 and 90% for both MTSET and MTSES under the condition of the experiment described in Figure 4A (not shown). The protection by sodium ions requires a reevaluation of the inactivation by MTSES in the absence of added sodium ions (Figure 4A). MTSES comes as the sodium salt (i.e., inactivation down to 35% was obtained in the presence of 10 mM Na^+), a concentration that resulted in 70% protection against NEM labeling. It follows that CitS is much more sensitive to labeling with negatively charged MTSES than positively charged MTSET.

Protection by Citrate Against Inactivation by NEM. Inactivation of wild type CitS with NEM in the absence and presence of 3 mM Na^+ was studied at increasing concentrations of the substrate citrate. A small increase in residual activity was observed in the presence of citrate concentrations up to 10 mM when no sodium ions were added (Figure 6, open circles). At a concentration of 3 mM Na^+ , inactivation by NEM is still significant. Addition of citrate under these conditions resulted in a marked protection against inactivation by NEM. Maximal effect was obtained at a citrate concentration of about 6 mM where the residual activity was 75%. Further increase of the citrate concentration had no further effect on protection against NEM (Figure 6, solid circles). In conclusion, protection by citrate against NEM inactivation was observed only in the presence of sodium ions.

Effect of the Proton Motive Force on CitS Inactivation. The effect of the proton motive force (pmf) on the labeling efficiency of the cysteine residues in CitS was measured in the absence of added sodium ions. Treatment of the membranes at 30 °C in the absence of the proton motive force with 0.3 mM NEM resulted in 50% reduction of the initial citrate uptake rate. In the presence of the proton gradient, the same treatment almost completely inactivated CitS (Figure 7). In a control experiment using membrane vesicles containing the CCCSS mutant, no difference was observed between the two conditions (not shown). Apparently, the presence of the pmf makes CitS much more sensitive to NEM. In contrast, labeling with the membrane

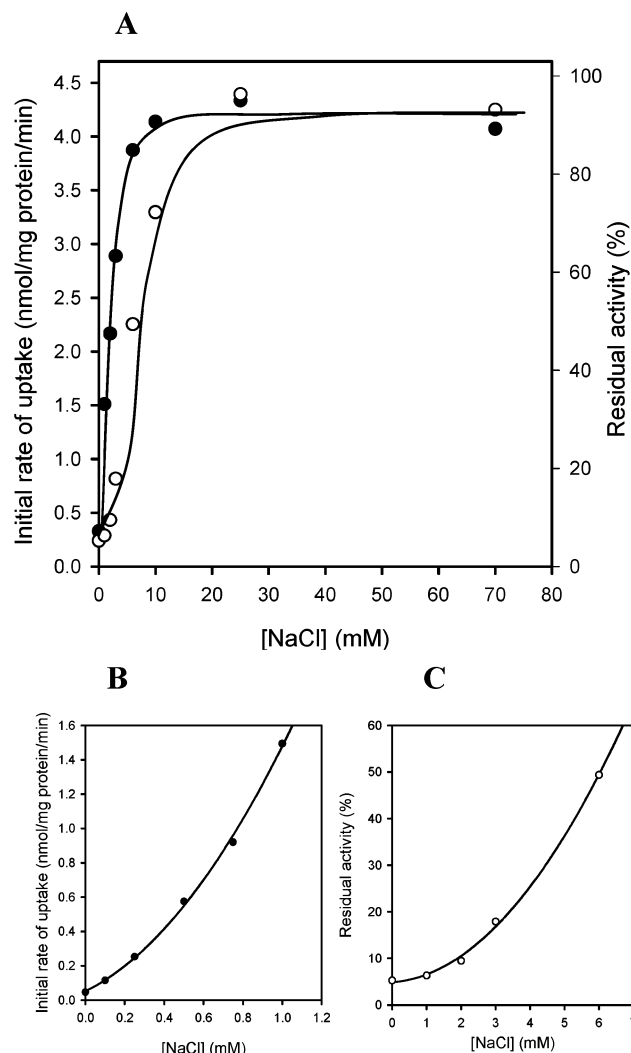


FIGURE 5: Effect of sodium ions on the uptake activity and inactivation by NEM. (A) The initial rate of citrate uptake in RSO membrane vesicles containing CitS (solid circles) and the residual activity after treatment of the same membranes with 1 mM NEM for 10 min (open circles). (B and C) Initial rates (B) and residual activity (C) at low sodium ion concentrations. The experiments were performed using low sodium KPi buffers (see Experimental Procedures). The residual Na^+ concentration in the absence of added sodium ions was estimated to be about 2.5 μM .

impermeable MTS reagents, MTSET and MTSES, appeared to be much less affected by the pmf. Also, the presence of 3 mM Na^+ added as the counterion of the MTSES anion did not appear to significantly change the effect of the pmf on inactivation.

DISCUSSION

The CitS protein contains five cysteine residues, and all are located in the C-terminal half of the transporter. Cys-278 and Cys-347 are located in putative TMSs VII and IX, respectively. Cys-317 is located in the cytoplasmic loop between TMS VIII and IX and the two residues Cys-398 and Cys-414 in the cytoplasmic loop between TMS X and XI (see Figure 1). Labeling of the latter two residues resulted in inactivation of the transporter. Most of the cytoplasmic loop between TMS X and XI forms together with the first turn of putative TMS XI a stretch of about 40 residues that is particularly well-conserved in the 2HCT family (region

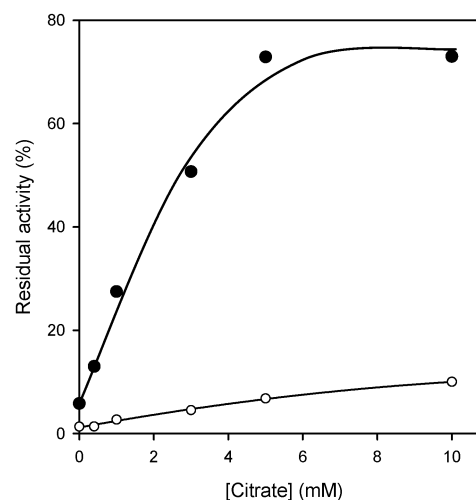


FIGURE 6: Protection by citrate against inactivation by NEM. RSO membranes containing CitS were treated with 1 mM NEM in the absence (open circles) and presence of 3 mM Na^+ (closed circles) and the indicated concentrations of citrate. The residual activity was measured after removal of citrate and plotted as the percentage of an untreated sample.

Xa). Evidence is accumulating that this region is part of the substrate translocation site. (i) Mutation of the conserved arginine residue in the first turn of TMS XI dramatically lowered the activity of the Na^+ -symporter CitS of *K. pneumoniae* (unpublished), the H^+ -symporter CimH of *B. subtilis* (16), and the exchanger CitP of *L. mesenteroides* (19). (ii) The CimH mutant revealed a loss of affinity for citrate and the CitP mutant for the dicarboxylate (*S*)-malate but not for the monocarboxylate (*S*)-2-hydroxyisobutyrate, strongly suggesting a direct interaction between the arginine residue and the second carboxylate of dicarboxylate substrates. (iii) Exchange of the C-terminal 46 residues, containing most of region Xa, between the exchangers CitP and MleP exchanged the affinity for the monocarboxylates (*S*)-mandalate and (*S*)-2-hydroxyisovalerate (18). (iv) Labeling of the cysteine residues in R420C and Q428C mutants of CimH (20) and the SSSCS and SSSSC mutants of CitS (this paper), all located in region Xa, resulted in inactivation of the transporters. (v) Inactivation was prevented by the presence of the substrate (CimH) or by both substrate and co-ion (CitS).

Strong support for the significance of these observations in terms of translocation mechanism follows from the accessibility of region Xa, situated at the cytoplasmic face of the membrane, from the periplasmic side of the membrane. The small, negatively charged, membrane impermeable MTS reagent MTSES was able to reach the cysteine residues in the R420C and Q428C mutants of CimH of *B. subtilis* (20), and here we show that the same is true for the endogenous cysteine residues in region Xa of CitS of *K. pneumoniae*. While the positively charged MTS-derivative MTSET did not inactivate CimH under the conditions tested, it did inactivate CitS albeit to a lower extent than MTSES. More bulky, charged reagents such as Amdis were without effect with both transporters suggesting a clear size limit to the access pathway. The relatively high reactivity with negatively charged MTSES, which is somewhat obscured in the case of CitS because of the protection by Na^+ ions, has been explained before by the resemblance of the molecular properties of MTSES and citrate, the substrate of the

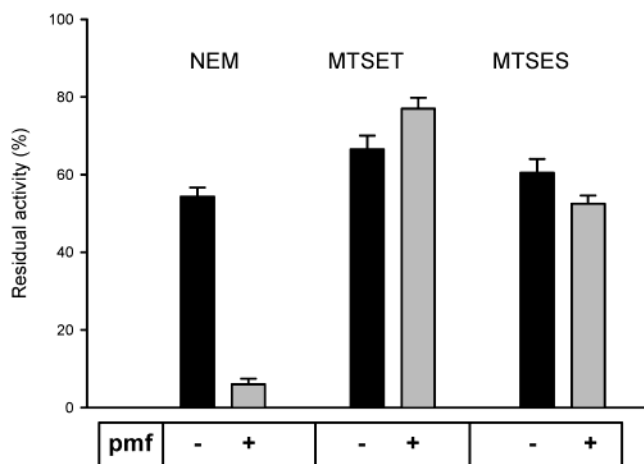


FIGURE 7: Effect of the pmf on inactivation by thiol reagents. RSO membrane vesicles containing CitS were treated at 30 °C with 0.3 mM NEM for 10 min (A), 1 mM MTSET for 20 min (B), and 3 mM MTSES for 20 min (C) in the presence and absence of a pmf as indicated. Sodium ions were not present during the treatment, except for MTSES that contains a stoichiometric amount of Na⁺ (3 mM). The pmf was generated by addition of ascorbate and PMS, which was removed after the treatment by spinning the samples (see Experimental Procedures). The residual activity was plotted as the percentage of a sample not treated with thiol reagents and represents the average and standard deviation from at least three independent measurements.

transporters (30). The access pathway to region Xa would correspond to the substrate entrance pathway that has been optimized for negatively charged substrates.

Transport activity of the CitS protein is strictly coupled to the translocation of two sodium ions demonstrated both in whole cells and RSO membrane vesicles (ref 1; this paper). The protection of CitS by Na⁺ against inactivation by NEM followed a similar relation with increasing Na⁺ concentration linking the conversion to the protected state directly to a transition between two catalytic states of the transporter. The different affinities for Na⁺ obtained in the two types of experiments (2 and 6 mM, respectively) are likely to reflect the different conditions of the transporter in the two types of experiments (i.e., presence vs absence of citrate, presence vs absence of the pmf, and steady state vs equilibrium state). The protection experiment demonstrates that both Na⁺ ions bind to the transporter in the absence of the substrate citrate (i.e., bound citrate is not part of the Na⁺ binding sites (5)) and that both Na⁺ ions have to bind to render the protein in the protected state. The substrate citrate resulted in additional protection of CitS against inactivation by the thiol reagents but only in the presence of subsaturating concentrations of Na⁺. Little protection was observed in the absence of added Na⁺, strongly suggesting that citrate does not bind to the transporter in the absence of Na⁺. The protection by citrate at low concentrations of Na⁺ may be explained by an increased affinity of the protein for Na⁺ upon citrate binding or by a direct protection by the bound citrate molecule. The close vicinity of the target cysteine residues and the conserved arginine residue in TMS XI that is believed to interact with the substrate would favor the latter possibility. The concentration of citrate required to obtain protection against NEM inactivation was 3 orders of magnitude higher than the reported *K_m* value for pmf driven citrate uptake in RSO membrane vesicles (8.3 μM; (6)). Again, the different apparent affinities are likely to be the result of the different

kinetic states of the transporter. During the NEM treatment, in the presence of Na⁺ and citrate, CitS catalyzes equilibrium exchange, and the observed affinity for citrate is an average of the internal and external binding site affinities weighted by the translocation equilibrium (31). Pmf driven uptake represents an unidirectional transport mode for which the (kinetic) affinity is more related to the affinity of the external binding site. Similar differences between pmf driven uptake and exchange modes of transport were observed for the well-studied lactose transport protein LacY of *E. coli* (32).

In a recent study, the CitS molecule was studied by single-molecule fluorescence spectroscopy (33). Cys-398 in SSSCS was labeled with the fluorophores Alexafluor 546/568 C5 maleimide, and the fluorescence of single molecules was monitored. It was demonstrated that the presence of citrate completely quenched the signal and that the quenching was not affected by Na⁺ ions. It was concluded that citrate binding resulted in a conformational change that would be responsible for the quenching. These conclusions appear in marked contradiction with our conclusions (i.e., Na⁺ binding results in a conformational change in which the cysteine residues are protected against thiol reagents and citrate is effective only after Na⁺ binding). Clearly, the conformational changes resulting in the fluorescence quenching and in the thiol protected state are not likely to be the same. An important difference between the experiments may be in the state of the transporter molecule. In the fluorescence studies, CitS was solubilized in detergent solution, while in this study the protein is in its native environment of the cytoplasmic membrane.

The present study suggests the following model for the molecular properties of the catalytic states during turnover of CitS. Residues in region Xa at the membrane/cytoplasm interface are part of or in close vicinity to the citrate binding site. In the absence of citrate and Na⁺, the transporter is in a state in which the two cysteine residues in region Xa are accessible from the periplasmic side of the membrane for the small MTSET and MTSES ions. The coupling of fluxes catalyzed by a symporter like CitS requires that in this unloaded state, the binding site is alternately opened to both sides of the membrane. It is likely that access by the MTS derivatives reflects the site opened to the periplasm, while inactivation by membrane permeable NEM reflects the site opened to the cytoplasm. Importantly, despite the access pathway for MTSES and MTSET, the binding site for citrate would not be formed in this state. Binding of Na⁺ locks the transporter in the conformation with the site opened to the periplasm (or to the cytoplasm) to prevent leakage of Na⁺. In this state, the transporter binds citrate, and the access pathway for MTSES and MTSET is closed. In other words, Na⁺ binding creates the citrate binding site at the membrane/cytoplasm interface and makes the access pathway more specific. This could be envisaged by a narrowing of the translocation pore thereby bringing the residues that interact with the substrate in the correct spatial arrangement. The surprising thing is that in the unloaded state the transport protein seems to be in an open, kind of loose state that changes into a highly specific pore in the presence of Na⁺. The presence of the pmf does not seem to have a significant effect on the access pathway from the periplasmic side of the membrane, neither in the presence or absence of subsaturating concentrations of Na⁺. In contrast, attack from

the cytoplasmic side of the membrane by NEM was significantly more effective, suggesting that the effect of the pmf may be restricted to changes in the domain formed by the Xa region, possibly to the citrate binding site. At any rate, binding of the Na⁺ co-ions and the pmf appear to have quite different effects on the conformation of the transport protein.

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