Arg-425 of the Citrate Transporter CitP Is Responsible for High Affinity Binding of Di- and Tricarboxylates

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Arg-425 of the Citrate Transporter CitP Is Responsible for High Affinity Binding of Di- and Tricarboxylates*

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The citrate transporter of *Leuconostoc mesenteroides* (CitP) catalyzes exchange of divalent anionic citrate from the medium for monovalent anionic lactate, which is an end product of citrate degradation. The exchange generates a membrane potential and thus metabolic energy for the cell. The mechanism by which CitP transports both a divalent and a monovalent substrate was the subject of this investigation. Previous studies indicated that CitP is specific for substrates containing a 2-hydroxycarboxylate motif, $HO-CR_2-COO^-$. CitP has a high affinity for substrates that have a "second" carboxylate at one of the R groups, such as divalent citrate and (*S*)-malate (Bandell, M., and Lolkema, J. S. (1999) *Biochemistry* **38**, 10352–10360). Monovalent anionic substrates that lack this second carboxylate were found to bind with a low affinity. In the present study we have constructed site-directed mutants, changing Arg-425 into a lysine or a cysteine residue. By using two substrates, *i.e.* (*S*)-malate and 2-hydroxyisobutyrate, the substrate specificity of the mutants was analyzed. In both mutants the affinity for divalent (*S*)-malate was strongly decreased, whereas the affinity for monovalent 2-hydroxyisobutyrate was not. The largest effect was seen when the arginine was changed into the neutral cysteine, which reduced the affinity for (*S*)-malate over 50-fold. Chemical modification of the R425C mutant with the sulfhydryl reagent 2-aminoethyl methanethiosulfonate, which restores the positive charge at position 425, dramatically reactivated the mutant transporter. The R425C and R425K mutants revealed a substrate protectable inhibition by other sulfhydryl reagents and the lysine reagent 2,4,6-trinitrobenzene sulfonate, respectively. It is concluded that Arg-425 complexes the charged carboxylate present in divalent substrates but that is absent in monovalent substrates, and thus plays an important role in the generation of the membrane potential.

In recent years a growing number of secondary transporters have been discovered that generate rather than consume metabolic energy. These transporters have been termed precursor-product exchangers since they catalyze the uptake of a substrate into the cell coupled to the exit of a metabolic end product into the medium (for a review see Ref. 1). An example of such a transporter is the citrate transporter (CitP) found in *Leuconostoc mesenteroides* (2, 3), which catalyzes the uptake of divalent citrate into the cell coupled to the exit of monovalent lactate, a metabolic end product of citrate degradation in lactic acid bacteria (4). The net charge movement over the membrane during the exchange results in a membrane potential of physiological polarity. This, in combination with the consumption of a cytoplasmic proton in the breakdown of citrate, results in a proton motive force and thus generates metabolic energy for the cell. Recovery from acidic stress and resistance against lactate toxicity have recently been suggested as alternative roles for the metabolic pathway (5).

The ability of CitP to transport two such different substrates as citrate and lactate is associated with a high specificity for the 2-hydroxycarboxylate motif present in both substrates (*i.e.* $HO-CR_2-COO^-$), in combination with a high promiscuity toward the two R groups (6). In fact, next to the physiological substrates, CitP is able to transport a wide range of 2-hydroxycarboxylates containing various R groups. The ability to accept both a neutral as well as a negatively charged R group is the basis for membrane potential generation by CitP. CitP is known to have a high affinity for divalent di- and tricarboxylates like citrate and (*S*)-malate, which have a "second" carboxylate at one of the R groups (6). Monocarboxylates like lactate and 2-hydroxyisobutyrate (2-HIB),[1] which lack this second carboxylate, were found to bind with a low affinity but were still transported efficiently. The high affinity of CitP for substrates with a second carboxylate was explained by postulating a strong, possibly electrostatic, interaction between the protein and the second carboxylate. Positively charged residues such as arginine, lysine, or histidine were thought to be able to participate in such an interaction.

CitP belongs to the 2-hydroxycarboxylate transporter (2-HCT) family (7) that contains members found in several lactic acid bacteria as well as in *Bacillus subtilis* and *Klebsiella pneumoniae* (8). The family contains precursor-product exchangers like CitP and the malate transporter MleP of *Lactococcus lactis*, but also the Na^+/citrate symporter CitS of *K. pneumoniae*, suggesting that precursor-product exchangers are "normal" secondary transporters that have been optimized to catalyze exchange. Transporters of the 2-HCT family are believed to consist of 11 transmembrane segments (TMSs), based on topology studies of CitS of *K. pneumoniae* (9–11). The

[1] The abbreviations used are: 2-HIB, 2-hydroxyisobutyrate; RSO, right-side-out; pCMB, *p*-chloromercuribenzoic acid; pCMBS, *p*-chloromercuribenzosulfonate; TNBS, 2,4,6-trinitrobenzene sulfonate; MTSEA, 2-aminoethyl methanethiosulfonate hydrobromide; MTSET, [2-(trimethylammonium)ethyl]methanethiosulfonate bromide; MTSES, sodium (2-sulfonatoethyl)methanethiosulfonate; TMS, transmembrane segment; 2-HCT, 2-hydroxycarboxylate transporter.

C terminus of the proteins resides in the periplasm. A recent study of chimeras between the citrate transporter CitP and the malate transporter MleP indicated that the C-terminal region including TMS XI forms part of the substrate-binding site that interacts with the *R* groups of the substrates (8). The C-terminal region contains two conserved arginine residues, one of which is located in TMS XI. The latter residue was thought to be a good candidate for an interaction with the second carboxylate of divalent substrates.

In the present study Arg-425 in TMS XI of CitP was replaced by lysine and cysteine residues. The mutant transporters were analyzed for their affinity toward the divalent and monovalent substrates (*S*)-malate and 2-HIB, respectively. In addition, the effect of chemical modification of residues at position 425 and the ability of substrate to protect against modification was investigated. It is concluded that Arg-425 is located in the substrate-binding site where it is responsible for a high affinity interaction with the second carboxylate of di- and tricarboxylates.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—*L. lactis* strain NZ9000 is an MG1363 derivative (*pepN::nisRnisK*; (12)) that transports neither citrate nor malate. The *nisR* and *nisK* genes were inserted in the chromosome to allow induced expression of plasmid-encoded genes under control of the tightly regulated *nisA* promoter (12). Cells harboring the expression vector pNZcitP containing the *citP* gene (see below) were grown at 30 °C in closed serum bottles and without shaking in M17 broth (Difco) supplemented with 0.5% (w/v) glucose and 5 μg/ml chloramphenicol. Unless otherwise stated, expression of the transporter was induced by growing the cells to an optical density of 0.6 measured at 660 nm (OD$_{660}$), followed by addition of a 1000-fold dilution of the supernatant of an overnight culture of the nisin producing *L. lactis* strain NZ9700 into the cultures (14). The supernatant contained approximately 10 ng of nisinA/ml (15). Growth was continued for 1 h followed by harvesting of the cells by centrifugation. In some cases a lower level of induction was required to allow kinetic analysis (see "Results"). Then, cells expressing CitP were grown in the presence of a 30,000-fold dilution of the nisin containing supernatant for 0.5 h.

DNA Manipulations—General procedures for cloning and DNA manipulations were performed essentially as described by Sambrook *et al.* (16). Expression vector pNZcitP codes for the CitP protein with 10 additional histidine residues at the N terminus (8). R425C and R425K mutations were introduced in the *citP* gene in two steps by overlap extension polymerase chain reaction (17). All polymerase chain reaction-amplified DNA fragments were sequenced to confirm the nucleotide sequence. Ligation mixtures were transformed to *L. lactis* NZ9000 by electroporation as described by Holo and Nes (18).

Preparation of Right-Side-Out Membrane Vesicles—Right-side-out (RSO) membrane vesicles of NZ9000 cells expressing wild type or mutant CitP were prepared by the osmotic shock lysis procedure in the presence of 5 mM (*S*)-malate, essentially as described previously (6). Membrane vesicles were washed once with 50 mM potassium phosphate, pH 6, containing 5 mM (*S*)-malate and concentrated by centrifugation in an Eppendorf table top centrifuge operated at full speed for 5 min. The internal pool of (*S*)-malate was labeled with (*S*)-[^{14}C]malate by incubating the concentrated membranes with 186.7 μM L-[1,4(2,3)^{14}C]malate for 1 h in the presence of 1 mM valinomycin and 0.5 mM nigericin. Protein concentrations were determined as described by Lowry *et al.* (19). In case the vesicles were used for chemical modification studies, (*S*)-malate was omitted in the preparation procedure, and loading of the vesicles with (*S*)-[^{14}C]malate was done as described under "Chemical Modification."

Exchange Measurements—Aliquots of 2 μl of (*S*)-[^{14}C]malate-loaded vesicles were diluted into 200 μl of 50 mM potassium phosphate, pH 6, containing substrates at the indicated concentrations at 20 °C. Internal radioactivity was determined at different time points by dilution with ice-cold LiCl followed by rapid filtration, as described (6). Final membrane protein concentrations in the assays were between 250 and 350 μg/ml. To evaluate the data, initial rates of exchange were determined by fitting the data to an exponential decay using nonlinear fitting procedures provided by the Sigma Plot software (Jandel Scientific, San Rafael, CA), as described previously (7). The rate of efflux in the absence of external substrate was subtracted from the observed rates. Exchan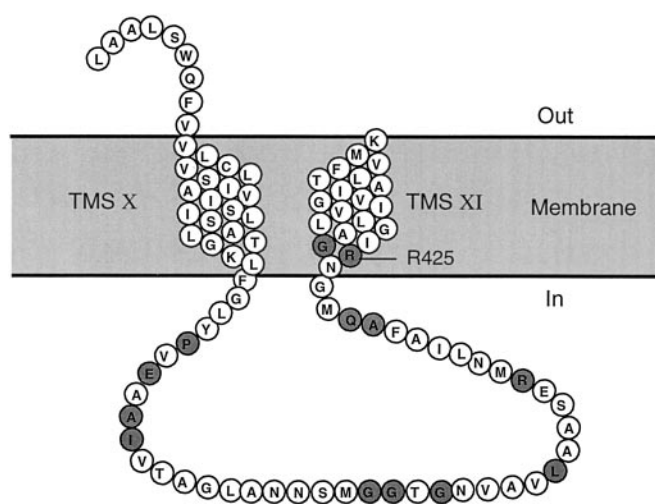ge rates were determined at different external substrate concentrations to estimate the affinity constant K_m^{app}. The data were fitted to an equation describing competitive inhibition, taking into account the effect of (*S*)-[^{14}C]malate in the external buffer caused by the dilution of the (*S*)-[^{14}C]malate-loaded membrane vesicles (6).

Chemical Modification—RSO membrane vesicles (10 mg protein/ml) prepared in the absence of (*S*)-malate were incubated for the indicated times in 50 mM potassium phosphate containing 5 mM of the lysine-specific reagent TNBS (Fluca) or 1 mM of the sulfhydryl reagents MTSEA, MTSES, MTSET (Anatrace), pCMB, and pCMBS (Sigma) at 23 °C. Reactions with the sulfhydryl reagents were performed at pH 6 and with TNBS at a range of pH values. Following incubation, the vesicles were diluted 15 times in 50 mM potassium phosphate, pH 6, and concentrated by centrifugation in an Eppendorf table top centrifuge operated at full speed for 5 min. The membranes were washed 3 times using the same procedure. To load the vesicles with (*S*)-[^{14}C]malate, the concentrated vesicles were incubated overnight at 4 °C in the presence of 5 mM (*S*)-malate, 186.7 μM L-[1,4(2,3)^{14}C]malate, 1 mM valinomycin, and 0.5 mM nigericin. Exchange activity was determined as described above.

To test the effect of substrate on chemical modification, RSO membranes were preincubated for 2 h with and without the substrate in the presence of 1 mM valinomycin and 0.5 mM nigericin followed by reaction with 0.1 mM pCMB for 0.5 min or with 30 mM TNBS for 2 h at pH 7. The membranes were washed twice with 50 mM potassium phosphate, pH 6, in the presence of substrate and 4 times in the absence of substrate. The concentrated vesicles were loaded with 5 mM radiolabeled (*S*)-malate, and exchange activity was determined as described above. To evaluate the efficiency of the washing procedure for removing internal substrate, vesicles were equilibrated with 60 mM 2-HIB, butyrate, (*S*)-malate, (*S*)-citramalate, or succinate and were washed four times. In all cases exchange rates were not significantly different from vesicles that were incubated in the absence of substrate (data not shown).

SDS-Polyacrylamide Gel Electrophoresis and Immunoblot Analysis—Right-side-out membrane vesicles were subjected to SDS-polyacrylamide gel electrophoresis using a 12% polyacrylamide gel matrix (15 μg protein/lane). After electrophoresis, the proteins were transferred to poly(vinylidene difluoride) membranes and analyzed using monoclonal antibodies directed against a His tag (Dianova, Hamburg, Germany). Antibodies were visualized using the Western light chemiluminescence detection kit (Tropix, Bedford, MA).

Chemicals—L-[1,4(2,3)-^{14}C]Malic acid (51 mCi/mmol) was obtained from Amersham Pharmacia Biotech. All other compounds were obtained from Fluka (Buchs, Switzerland) or Sigma.

RESULTS

Construction and Activity of the R425C and R425K Mutant Transporters—Arg-425 is conserved in the transporters of the 2-HCT family and located in the C-terminal putative TMS XI (Fig. 1). The involvement of Arg-425 in the interaction with the second carboxylate present in divalent substrates was investi-

FIG. 1. **Membrane topology and amino acid sequence of the C-terminal part of CitP.** The topology model is based on studies of the Na$^+$-dependent citrate transporter CitS of *K. pneumoniae* which is homologous to CitP (11). Conserved residues in the amino acid sequence alignment of the 2-HCT family (8) were indicated in *gray*.

gated by constructing site-directed mutants of the citrate transporter CitP of *L. mesenteroides*. Arg-425 was replaced with Lys, a conservative mutation that retains the positive charge, and with the neutral Cys residue. Mutant and wild type transporters were N-terminally tagged with 10 histidines and expressed in *L. lactis* NZ9000 cells using the inducible *nisA* promoter system. The expression levels in the membrane were analyzed by immunoblotting using antibodies directed against the His tag. The apparent molecular mass of the proteins was about 40 kDa, and under the same induction conditions, the expression levels were similar for CitP and both mutants (Fig. 2).

Exchange provides a sensitive assay for the activity of CitP (7). Mutant transporters were assayed for homologous exchange in right-side-out membrane vesicles, using the high affinity substrate (*S*)-malate. Vesicles loaded with 5 mM radio-

labeled (*S*)-malate were diluted 100-fold into buffer. In the absence of external substrate, efflux of (*S*)-malate from the membranes down the concentration gradient was a slow process for both wild type and mutant transporters (Fig. 3 (●)). Only when measured for a prolonged period (Fig. 3*C*) significant efflux could be observed. The rate of efflux seemed not significantly different for the wild type and mutant transporters. Dilution of the membranes containing the wild type transporter in buffer containing 5 mM unlabeled (*S*)-malate resulted in very rapid release of the label from the membranes due to CitP-mediated exchange (Fig. 3*A* (■)). Exchange catalyzed by the R425K and R425C mutant was considerably slower but significantly faster than efflux (Fig. 3, *B* and *C* (■)). The Arg to Cys mutation had a more drastic effect on the exchange rate than the conservative Arg to Lys mutation.

Affinity for (S)-Malate and 2-HIB—The kinetic characteristics of the mutant transporters were analyzed with 2-HIB and (*S*)-malate, a mono- and a divalent substrate of CitP, respectively (see Fig. 4). Under standard induction conditions, exchange catalyzed by wild type CitP was so fast that initial rates of transport could not be measured (Fig. 3*A*). To allow the analysis, CitP expression levels were reduced by varying the inducer concentration in the growth medium (see "Experimental Procdures").
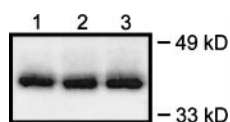

FIG. 2. **Expression levels of CitP, R425K, and R425C.** RSO membrane vesicles prepared from *L. lactis* NZ9000-expressing CitP (*lane 1*), R425K (*lane 2*), and R425C (*lane 3*) were analyzed by Western blotting using antibodies raised against the His tag. The position of the molecular mass markers was indicated on the *right*.
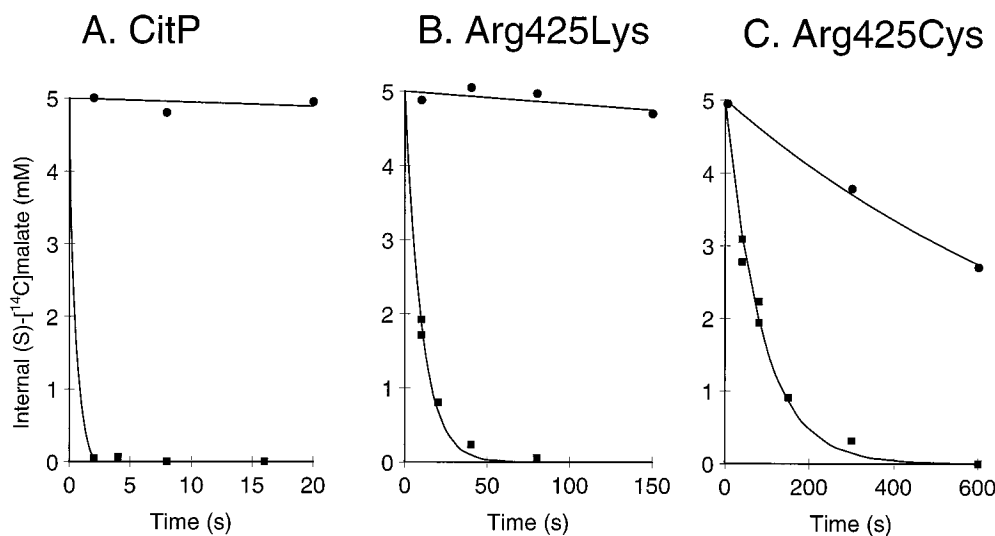

FIG. 3. **Homologous (*S*)-malate exchange catalyzed by CitP, R425K, and R425C.** RSO membrane vesicles prepared from *L. lactis* NZ9000-expressing CitP (*A*), R425K (*B*), and R425C (*C*) were preloaded with 5 mM (*S*)-[14C]malate and diluted 100-fold into buffer containing 5 mM (*S*)-malate (■) or no additions (●).
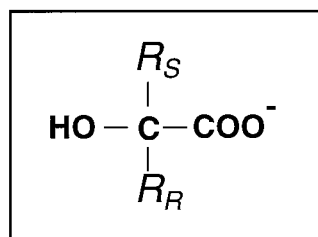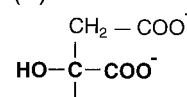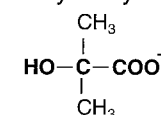

FIG. 4. **CitP substrates.** Chemical structure of the substrates of CitP used in this study. The *R* groups are defined as R_R and R_S to discriminate between the different positions of the *R* groups in chiral compounds. *Inset*, the 2-hydroxycarboxylate motif.
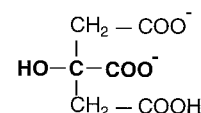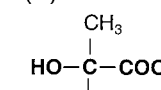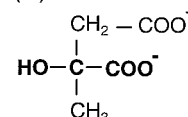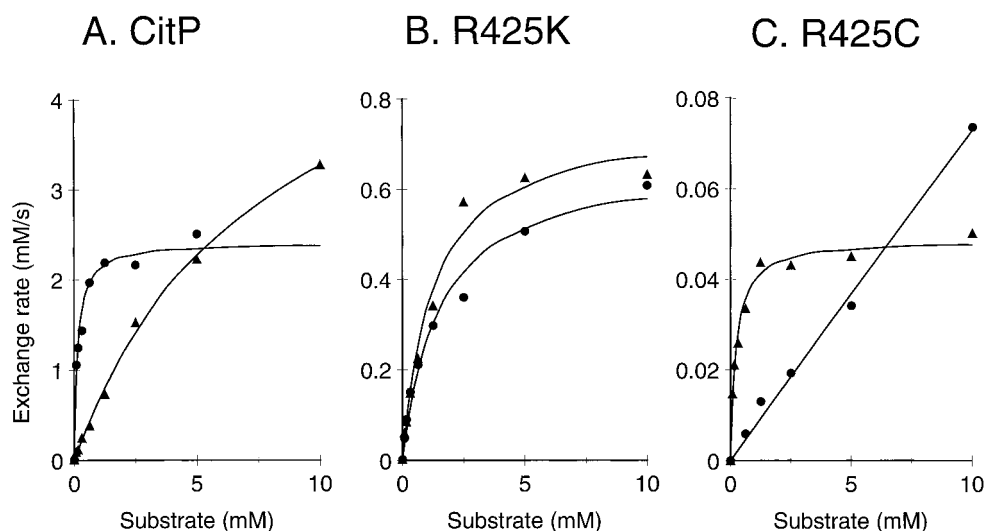
A. CitP   B. R425K   C. R425C

FIG. 5. **Affinity of CitP (*A*), R425K (*B*), and R425C (*C*) for (*S*)-malate and 2-HIB.** RSO membrane vesicles prepared from *L. lactis* NZ9000-expressing CitP (*A*), R425K (*B*), or R425C (*C*) were preloaded with 5 mM (*S*)-[14C]malate and diluted 100-fold into buffer containing the indicated concentrations of (*S*)-malate (●) or 2-HIB (▲). The *curves* were analyzed as described under "Experimental Procedures," and the obtained K_m^{app} and V_{max} values are given in Table I. Expression level for CitP was lower than for R425K and R425C (see text).

The K_m^{app} for external (*S*)-malate at an internal concentration of 5 mM (*S*)-malate was 90 μM for wild type CitP in fair agreement with previous reports (6, 8). In heterologous exchange using the same concentration of internal (*S*)-malate, the K_m^{app} for external 2-HIB was 5 mM (Fig. 5*A* and Table I). Replacing Arg-425 by Lys, thus conserving the positive charge at position 425, reduced the affinity for (*S*)-malate about 10-fold (Fig. 5*B* and Table I). Substitution for the neutral Cys residue had a much more dramatic effect on affinity. No saturation was observed up to a concentration of 10 mM (Fig. 5*C*). In contrast to the decrease in the affinity for divalent (*S*)-malate, the R425K and R425C mutants revealed an increased apparent affinity for monovalent 2-HIB. The affinity of R425K increased 4-fold, whereas R425C had a 20-fold higher affinity for 2-HIB compared with wild type CitP. The maximal rates of exchange decreased in the order CitP > R425K > R425C for both (*S*)-malate and 2-HIB (Fig. 5 and Table I).

The results suggest that Arg-425 is specifically involved in the high affinity toward the divalent (*S*)-malate. The positive charge of the Arg seems to play an important role in the interactions since the changes in (*S*)-malate affinity were much less pronounced when Arg-425 was replaced with the positively charged Lys residue.

Reactivation of the R425C Mutant—The methanethiosulfonate (MTS) derivatives MTSEA, MTSES, and MTSET are small, charged, water-soluble, and cysteine-specific reagents (20). They form a mixed disulfide with the thiol of the cysteine via the addition of –SCH_2CH_2X groups where X is SO_3^-, $N(CH_3)_3^+$, or NH_3^+ for MTSES, MTSET, and MTSEA, respectively. Right-side-out membrane vesicles containing the transporters were treated with the MTS reagents, and (*S*)-malate exchange was measured using non-saturating external (*S*)-malate concentrations (21). Wild type CitP contains one Cys residue at position 361 in putative TMS *X*. Treatment of RSO membranes containing CitP with the MTS reagents had no effect on the exchange activity (Table II). Apparently Cys-361 is not accessible to the reagents or the modification does not affect activity. In contrast, exchange catalyzed by the R425C mutant was severely affected indicating a specific modification of the Cys at position 425 (Table II). The most striking effect was seen upon incubation with MTSEA which restores a positive charge at position 425 (Fig. 6). The homologous exchange rate increased about 50-fold under the conditions of the experiment. In fact, the

TABLE I
Kinetic parameters for CitP, R425K, and R425C in exchange
Values were evaluated from the data shown in Fig. 5 as described under "Experimental Procedures."

	(*S*)-Malate		2-HIB	
	K_m^{app}	V_{max}	K_m^{app}	V_{max}
	mM	*mM/s*	*mM*	*mM/s*
CitP[a]	0.09 ± 0.02	2.4 ± 0.3	4.9 ± 0.6	5.8 ± 0.8
R425K	1.4 ± 0.3	0.67 ± 0.08	1.2 ± 0.3	0.76 ± 0.1
R425C	>10[b]	>0.07[b]	0.22 ± 0.05	0.049 ± 0.004

[a] Expression level for CitP was lower than for R425K and R425C (see text).
[b] Lower limit; no significant saturation was observed.

TABLE II
Exchange (% of control) catalyzed by R425C and CitP after treatment with sulfhydral reagents

RSO membrane vesicles containing R425C CitP were treated with 1 mM of the reagents for the indicated times. After washing away the reagents, vesicles were preloaded with 5 mM (*S*)-[14C]malate and diluted 100-fold into buffer containing 5 mM (*S*)-malate. Rates were given as the percentage of exchange by the untreated membranes.

	R425C		CitP	
	10 min	150 min	10 min	150 min
No addition	100	100	100	100
MTSEA	4800		100	
MTSET	100	49		105
MTSES	98	40		110
PCMB	2		103	
PCMBS	20	2	95	

exchange catalyzed by MTSEA-treated R425C was faster than observed for the R425K mutant (compare Figs. 6 and 3*B*). In contrast to MTSEA, the more bulky positively charged reagent MTSET and the negatively charged MTSES reduced the (*S*)-malate transport activity of the mutant to undetectable levels. The results indicate that the introduction of a small positive group at position 425 in the R425C mutant can largely restore exchange activity.

Chemical Modification of R425K by TNBS—TNBS reacts specifically with lysine residues and/or the N terminus of proteins (22, 23) introducing a covalently linked trinitrophenyl group in the protein. Increased reactivity at higher pH values is characteristic for a specific reaction with an amino group
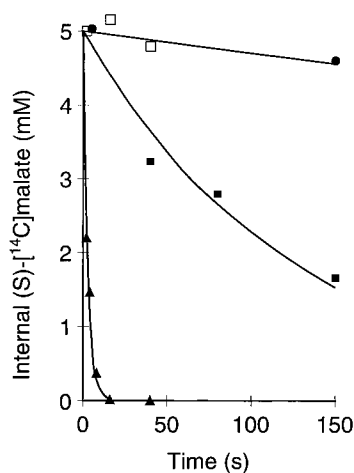
FIG. 6. **Modification of R425C by MTSEA.** RSO membrane vesicles prepared from *L. lactis* NZ9000-expressing R425C were incubated for 10 min in the presence ($\square$ and $\blacktriangle$) or absence ($\bullet$ and $\blacksquare$) of 1 mM MTSEA. After washing, the membranes were preloaded with 5 mM (*S*)-[^{14}C]malate and diluted 100-fold into buffer containing 5 mM (*S*)-malate ($\blacktriangle$ and $\blacksquare$) or no additions ($\square$ and $\bullet$).

(23). Treatment of right-side-out membrane vesicles containing R425K with 5 mM TNBS at pH 6 did not reduce the exchange activity of the transporter. However, the same treatment at pH values of 7 and 8 resulted in the loss of 35 and 80% exchange activity, respectively (Fig. 7A). Under the same reaction conditions, TNBS had no effect on wild type CitP (Fig. 7B) indicating a specific modification of the lysine at position 425 in the R425K mutant. Apparently, modification of Lys-425 in the R425K mutant renders an inactive transporter.

Accessibility of Position 425—The localization of the Cys residue at position 425 in the R425C mutant with respect to the membrane was investigated by the reactivity with membrane-permeable and -impermeable thiol reagents. MTSET is a strong base and MTSES a strong acid, and both are generally regarded to be poorly membrane-permeable. On the other hand, MTSEA is a weak base that equilibrates across the membrane in its undissociated form (24). Chemical modification of the R425C mutant by membrane-impermeable MTSES and MTSET was incomplete even after long incubation times (Table II). The incomplete inactivation was due to low inactivation rates as higher reagent concentrations increased inactivation (not shown). In contrast, the time course of activation with membrane-permeable MTSEA was much shorter. The stimulatory effect was saturated within 10 min.

pCMB and its sulfonic acid derivative pCMBS are organomercurial reagents that react with high specificity with cysteine residues. pCMB is membrane-permeable, and pCMBS is not. Treatment of right-side-out membrane vesicles containing R425C with either pCMB or pCMBS eventually resulted in complete inhibition of homologous (*S*)-malate exchange (Table II). The same treatment had no effect on exchange catalyzed by the wild type transporter, indicating that the Cys at position 425 was modified. Inactivation of the R425C mutant by pCMBS was much slower than observed for of pCMB which fully inactivated the transporter within 3 min (Table II). In fact, 0.5 min of incubation with 0.1 mM pCMB resulted in 80% inhibition (see below), while the same level of inhibition by pCMBS required 10 min of incubation at a 10-fold higher concentration. Taken together these results indicate that Cys-425 reacts much faster with membrane-permeable reagents, suggesting that the residue is accessible at the cytoplasmic side of the membrane.

Substrate Protection against Chemical Modification—The R425C mutant revealed a relatively high affinity for external 2-HIB in heterologous exchange (K_m^{app} = 0.22 mM; Table II) and,
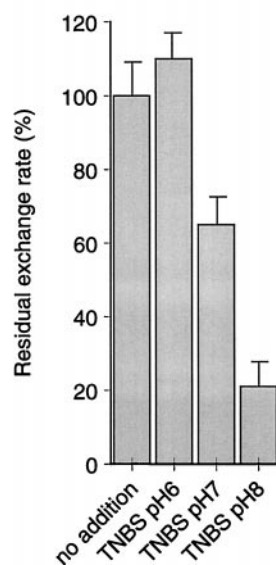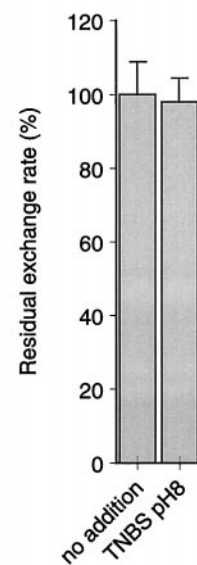

FIG. 7. **TNBS treatment of R425K (*A*) and CitP (*B*).** RSO membrane vesicles prepared from *L. lactis* NZ9000-expressing R425K and CitP were incubated for 2 h with 5 mM TNBS at the indicated pH. After washing, the vesicles were loaded with 5 mM (*S*)-[^{14}C]malate and diluted 100-fold into buffer containing unsaturating (*S*)-malate concentrations, *i.e.* 1.5 mM (*S*)-malate in the case of R425C and 0.15 mM in the case of CitP. Exchange rates were given as a percentage of the exchange rate in the untreated membranes. *Error bars* represent the S.D.

therefore, this substrate was selected to see whether the presence of substrate could protect the mutant against inactivation by pCMB. Right-side-out membrane vesicles were incubated with 2-HIB to equilibrate the substrate over the membrane, followed by treatment with pCMB, and subsequent removal of the substrate and unreacted pCMB (see "Experimental Procedures"). In the absence of 2-HIB, the treatment resulted in 80% inhibition of exchange activity (Fig. 8A). 2-HIB protected R425C against pCMB inactivation in a concentration-dependent manner, but much higher concentrations were required than anticipated. Protection of R425C by 2-HIB was incomplete even at 60 mM which is far above the K_m^{app} for external 2-HIB. In the control experiment, 2-HIB was replaced by butyrate, which is very similar to 2-HIB but lacks the hydroxyl group and therefore is not a substrate of CitP (7). The presence of butyrate did not affect the inactivation by pCMB (Fig. 8A), indicating that the protection by 2-HIB is specific.

(*S*)-Malate and another high affinity substrate of CitP (*S*)-citramalate (6) were tested for their potency to protect the R425K mutant against inactivation by TNBS (Fig. 8B). In the presence of 60 mM (*S*)-malate, the 70% inhibition observed in the absence of substrate was reduced to 30%, whereas 60 mM (*S*)-citramalate completely protected against inactivation under the conditions of the experiment. Similarly as observed for 2-HIB above, much higher concentrations of the substrates were required than would be expected based on the affinity constants for external (*S*)-malate in the homologous exchange reaction. In the control experiment, succinate which lacks the 2-hydroxy group of (*S*)-malate, and therefore is not a substrate of CitP, had no effect on the inhibition by TNBS.

DISCUSSION

In this study, the mechanism by which CitP transports both divalent and monovalent substrates, which is crucial to the physiological function of membrane potential generation by the transporter, was investigated. Previous substrate specificity
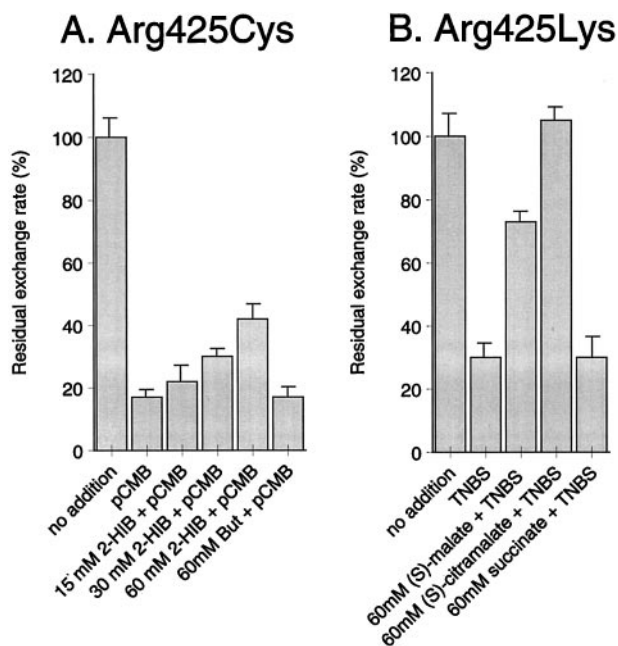
A. Arg425Cys   B. Arg425Lys

FIG. 8. **Substrate protection against modification by pCMB (*A*) and TNBS (*B*).** *A,* RSO membrane vesicles containing R425C were incubated for 0.5 min with 0.1 mM pCMB in the presence or absence of the indicated concentrations of 2-HIB or butyrate. After washing, the membranes were preloaded with 5 mM (*S*)-[¹⁴C]malate and diluted 100-fold into buffer containing 5 mM (*S*)-malate. *B,* RSO membrane vesicles containing R425K were incubated for 2 h with 30 mM TNBS at pH 7 in the presence or absence of 60 mM (*S*)-malate, (*S*)-citramalate, or succinate. After washing, the vesicles were preloaded with 5 mM (*S*)-[¹⁴C]malate and diluted 100-fold into buffer containing 1.5 mM (*S*)-malate. Exchange rates were given as a percentage of the exchange rate without addition of TNBS. *Error bars* represent the S.D.

studies showed that CitP is specific for substrates containing a 2-hydroxycarboxylate motif, HO-CR₂-COO⁻, in which the *R* groups can vary. These *R* groups were defined as R_R and R_S to discriminate between different positions of the *R* groups around the asymmetric C-2 atom in chiral substrates (Fig. 4, *inset*). CitP is known to have a high affinity for substrates that have a second charged carboxylate at the R_S position, like citrate and (*S*)-malate (6). Monovalent substrates that lack this second carboxylate, like lactate and 2-HIB, were found to bind with low affinity but were still transported efficiently. A non-essential interaction between the protein and the second carboxylate of the divalent substrates was postulated that would increase affinity for these substrates. Arg-425 was selected to be a good candidate for the site on the protein involved in this interaction because of the following. (i) Arg-425 is located in the stretch of 46 residues at the C terminus, which has been suggested to be involved in the interaction with the *R* groups (8). (ii) Arg-425 is located in a transmembrane segment of the transporter. (iii) Arg-425 is conserved in the 2-HCT family (8).

It is concluded that Arg-425 indeed interacts with the second carboxylate of the divalent substrates, based on the following observations. First, mutation of Arg-425 decreased the affinity for divalent (*S*)-malate but not for monovalent 2-HIB. Second, Arg-425 is not essential for transporter activity. Third, a positive charge at position 425 resulted in improved transport activity. Fourth, chemical modification of the residue at position 425 significantly affected transporter activity. Finally, the presence of substrate protected the residue at position 425 against chemical modification. The combination of the results strongly suggests that Arg-425 is in the substrate-binding pocket where it interacts with the second carboxylate. The involvement of TMS XI in the binding site is in line with the behavior of chimeric transporters (8).

The affinities of the mutant and wild type transporters were determined by homologous and heterologous exchange using membrane vesicles loaded with a fixed (*S*)-malate concentration and varying the external substrate concentrations. The determined affinities are for the external substrate. In a ping-pong type exchange mechanism, the apparent affinity for the external substrate is dependent on the affinity and concentration of the internal substrate. Non-saturating conditions at the inside result in higher apparent affinities for the external substrate and lower maximal rates. Consequently, a mutation that lowers the affinity for internal (*S*)-malate decreases the K_m^{app} for external substrate and the maximal rate of exchange. The kinetics observed with 2-HIB of the R425K and R425C mutants are consistent with a decrease in internal (*S*)-malate affinity in the order Arg-425, Lys-425, Cys-425, if the binding affinity for 2-HIB would not change significantly. The kinetic affinity for external 2-HIB increases, and the maximal rate for heterologous 2-HIB/(*S*)-malate, as well as for homologous (*S*)-malate exchange, decreases (Fig. 5). The observed decreased affinity of the mutants for external (*S*)-malate in homologous exchange strongly argues in favor of a decreased binding affinity of the protein for (*S*)-malate with a subsequent lowering of the kinetic affinity for internal (*S*)-malate.

The guanidinium group of the Arg-425 residue is not absolutely essential for transport function since even a non-conservative substitution for Cys does not abolish the activity of CitP. However, the affinity for divalent (*S*)-malate was severely affected by this mutation. The drop in affinity was less when Arg-425 was mutated to Lys, and, especially, the reactivation of R425C by MTSEA, which restores a positive charge at position 425, strongly suggests that the positive charge is essential for high affinity binding of divalent substrates. Nevertheless, the positive charge is not the only relevant factor since Arg-425 cannot be replaced with lysine or MTSEA-labeled cysteine without some loss of affinity. The chemistry of the guanidinium group allows for the formation of two hydrogen bonds, which is known to result in an interaction of unusual strength with a carboxylate group (25). Alternatively, only an Arg residue can position its charge accurately relative to the substrate for an optimal interaction. In contrast to the stimulation of exchange by MTSEA, positively charged MTSET inhibited the R425C mutant. The three methyl groups of the quaternary ammonium group of the reagent are likely to interfere sterically with substrate binding and thus inactivate the transporter. MTSES, pCMB, and pCMBS inactivated R425C and TNBS R425K most likely because of the presence of a negative charge on the side chains that is expected to repel the carboxylate of the substrate and/or by steric interference with the substrate.

In the topology model of CitP, Arg-425 is located at the cytoplasmic side of TMS XI (9–11). The present data support this localization. The chemical inactivation of the R425C mutant in right-side-out vesicles was much faster for the membrane-permeable pCMB than for its impermeable counterpart pCMBS (Table II). Since the two probes have similar chemical reactivities, the difference in inactivation rate suggests that the reactive cysteine is only accessible from the inside. Similarly, chemical modification by the membrane-impermeable MTSET and MTSES was very slow, and modification by the permeable MTSEA was fast. These low reaction rates are not due to differences in chemical reactivity with the thiol groups. In fact, MTSET is known to have 2.5-fold higher intrinsic reactivity for sulfhydryls than MTSEA (13). Thus, these results also suggest that the reaction takes place at the internal face of the membrane. The low rate of modification with pCMBS, MTSES, and MTSET may be explained by a slow permeation through the membrane by these reagents followed by reaction

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at the cytoplasmic side or by a low accessibility of the Cys residue from the outside. Accessibility of a binding site residue from either side of the membrane is not unlikely since the transporter has to expose the binding site alternately to the two sides of the membrane during turnover.

The presence of substrate protected against chemical modification of the residue at position 425, which is consistent with the assignment of Arg-425 as a binding site residue. Interestingly, 2-HIB protected R425C against pCMB inactivation in a concentration-dependent manner, but protection was still incomplete at a concentration of 60 mM, indicating a very low affinity for the substrate. This contrasted with the high apparent affinity for external 2-HIB in the exchange assays, $K_m^{app} = 0.22$ mM (Table I). The same situation was observed for the protection of R425K by (*S*)-malate against inhibition by TNBS. Possible explanations for the apparent discrepancy may be the underestimation of the binding affinity for external substrate when deduced from a kinetic experiment as discussed above or, alternatively, the transporter has a much lower affinity for internal substrate than for external substrate. In any case, full protection against chemical modification was shown to be possible by the high affinity substrate (*S*)-citramalate.

In conclusion, the combination of the kinetic characteristics of the mutants and the chemical modification and substrate protection against chemical modification strongly suggest a direct interaction between Arg-425 and the carboxylate at the R_S groups of the divalent substrates. The positive charge of Arg-425 seems to be important in this interaction, but the chemistry of the guanidinium groups may play a role as well. Although Arg-425 is not essential for transport by CitP, its role in specifically interacting with the second carboxylate of di- and tricarboxylate substrates makes it essential for the generation of the membrane potential which is the physiological function of CitP.

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