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Conserved Residues R420 and Q428 in a Cytoplasmic Loop of the Citrate/Malate Transporter CimH of *Bacillus subtilis* Are Accessible from the External Face of the Membrane

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Received September 19, 2002

ABSTRACT: CimH of *Bacillus subtilis* is a secondary transporter for citrate and malate that belongs to the 2-hydroxycarboxylate transporter (2HCT) family. Conserved residues R143, R420, and Q428, located in putative cytoplasmic loops and R432, located at the cytoplasmic end of the C-terminal transmembrane segment XI were mutated to Cys to identify residues involved in binding of the substrates. R143C, R420C, and Q428C revealed kinetics similar to those of the wild-type transporter, while the activity of R432C was reduced by at least 2 orders of magnitude. Conservative replacement of R432 with Lys reduced the activity by 1 order of magnitude, by lowering the affinity for the substrate 10-fold. It is concluded that the arginine residue at position 432 in CimH interacts with one of the carboxylate groups of the substrates. Labeling of the R420C and Q428C mutants with thiol reagents inhibited citrate transport activity. Surprisingly, the cysteine residues in the cytoplasmic loops in both R420C and Q428C were accessible to the small, membrane-impermeable, negatively charged MTSES reagent from the external site of the membrane in a substrate protectable manner. The membrane impermeable reagents MTSET,[1] which is positively charged, and AMdiS, which is negatively charged like MTSES but more bulky, did not inhibit R420C and Q428C. It is suggested that the access pathway is optimized for small, negatively charged substrates. Either the cytoplasmic loop containing residues R420 and Q428 is partly protruding to the outside, possibly in a reentrant loop like structure, or alternatively, a water-filled substrate translocation pathway extents to the cytoplasm-membrane interface.

The 2-hydroxycarboxylate transporter (2HCT) family consists of transporters for L-malate and citrate, some of which have been studied extensively. The family contains Na^+- and H^+-symporters (CitS of *Klebsiella pneumoniae*, MaeN and CimH of *Bacillus subtilis* and MaeP of *Streptococcus bovis* (*1*−*4*)), but also citrate/lactate and malate/lactate exchangers found in lactic acid bacteria (CitP of *Leuconostoc mesenteroides* and MleP of *Lactococcus lactis* (*5*, *6*)). The latter transporters are involved in proton motive force generation under physiological conditions (*7*−*9*). Substrate specificity studies revealed that the symporters in the family are very specific, transporting only citrate and malate, while the exchangers catalyze a wide range of 2-hydroxycarboxylates (*5*). The transporters of the 2HCT family are believed to traverse the cytoplasmic membrane 11 times (*10*, *11*) with the N-terminus and the C-terminus localized inside and outside the cell, respectively (Figure 1).

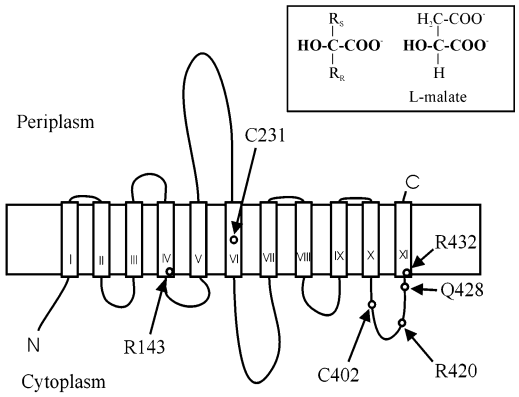

FIGURE 1: Model of the membrane topology of CimH. Boxes indicate putative transmembrane segments. The length of the connecting loops corresponds more or less with the numbers of residues in the loop regions. Conserved residues within the 2HCT family were marked with a grayed dot, the two cysteine residues in the protein with an open dot. The inset shows the 2-hydroxycarboxylate motif that is common to the substrates of the family and L-malate.

Members of the 2HCT family recognize specifically substrates that contain the 2-hydroxycarboxylate motif (Figure 1, inset), indicating essential interactions between sites on the proteins and the hydroxyl and carboxylate groups of the substrates. The difference between the symporters and the exchangers in the family is in their tolerance toward the

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[1] Abbreviations: 2HCT, 2-hydroxycarboxylate transporter; TMS, trans membrane segment; IPTG, isopropylthiogalactopyranoside; RSO, right-side-out; ISO, inside-out; PMS, phenazine methosulfate; NEM, *N*-ethylmaleimide; MTSEA, 2-aminoethyl methanethiosulfonate hydrobromide; MTSET, [2-(trimethylammonium)ethyl]methanethiosulfonate bromide; MTSES, sodium (2-sulfonatoethyl)methanethiosulfonate; AMdiS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid; F-5-M, fluorescein-5-maleimide.

10.1021/bi026874a CCC: $25.00 © 2003 American Chemical Society
Published on Web 12/18/2002

R_S and R_R groups of the substrates, named after the *S*- and *R*-enantiomers of chiral 2-hydroxycarboxylates (*12*). The symporters are strictly stereoselective and accept only CH_2-COOH at R_S and H (in malate) or CH_2COOH (in citrate) at R_R (*2, 12*). In contrast, the exchangers accept groups differing in size and charge. This allows them to translocate both monovalent monocarboxylates (lactate) and divalent di/tricarboxylates (malate/citrate), which is essential for their physiological function of generating membrane potential. The high tolerance of the exchangers to the R groups of the substrates has allowed the identification of the C-terminal transmembrane segment XI (TMS XI) and the preceding cytoplasmic loop, a region that is particularly well conserved in the family, as part of the substrate binding site by analyzing chimeric transport proteins (*13*). More particularly, R425 (CitP numbering) located at the cytoplasmic side of TMS XI was shown to interact directly with the carboxylate at the R_S position resulting in high-affinity binding of di/tricarboxylates (*14*). The essential interactions of the proteins with the hydroxyl and carboxylate of the 2-hydroxycarboxylate motif of the substrates have not been identified. These interactions are likely to be mediated by residues that are conserved within all members of the 2HCT family.

We have previously described the cloning and characterization of CimH in the 2HCT family encoded by the ORF designated *yxkJ* in the *B. subtilis* genome-sequencing project (*2, 15*). CimH is a symporter that transports citrate and L-malate, while it binds but does not transport L-citramalate. Therefore, CimH recognizes 2-hydroxycarboxylates with a carboxylate at the R_S position, suggesting, in addition to essential interactions with the 2HCT motif, an essential role for R432 (the equivalent of R425 of CitP) in substrate recognition and transport. We set out to identify residues involved in the essential interactions with the substrates of CimH. Besides R432, the conserved residues R143 located at the cytoplasmic end of TMS IV (see Figure 1), Q428 located one turn of a helix away from R432, and R420 located in the conserved cytoplasmic loop preceding TMS XI were selected for mutagenesis. R432 is shown to play a similar role as R425 in CitP, but surprisingly, conserved residues R143, R420, and Q428 could be mutated to cysteine without loss of activity and, therefore, are not directly involved in the transport mechanism. Nevertheless, labeling of R420C and Q428C, but not of R143C, with thiol reactive reagents inhibited citrate transport activity. Surprisingly, R420C, Q428C, and also R432C were accessible from the periplasmic site of the membrane by membrane the impermeable thiol reactive reagent MTSES. The data suggest that either the conserved part of the cytoplasmic loop in front of TMS XI containing R420 and Q428 is either partly protruding to the outside, possibly in a reentrant loop like structure, or alternatively, a water filled substrate translocation pathway extents all the way down to the substrate binding site located at the cytoplasm-membrane interface.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions. Escherichia coli strain Top10 harboring the expression plasmid pETCimH that contains the gene coding for CimH with a N-terminal His_6 tag (2) was routinely grown in flasks in Luria Bertani Broth (LB) at 37 °C under continuous shaking at 150 rpm. Ampicillin was added at a final concentration of 50 μg/mL. Expression of CimH was induced for 1.5 h by adding 100 μM isopropylthiogalactopyranoside (IPTG) when the optical density measured at 660 nm (OD_{660}) of the culture was 0.6. The cell culture was cooled on ice and harvested by centrifugation at 10 000*g* for 10 min.

Construction of Site-Directed Mutants of CimH. Site-directed mutants of CimH were prepared by a two-step PCR method using *Pwo* DNA polymerase for high-fidelity amplification (Roche Molecular Biochemicals, Mannheim, Germany). Overlapping primers containing the desired mutation were used to prepare short PCR fragments with either pETCimH or pETC-less as template DNA. These PCR fragments were purified from an agarose gel using the Qiagen gel extraction kit according to the manufacturer protocol, and used as primer in the second PCR step. The final PCR products of the expected size (~1300 bp) were purified from gel and digested with *Nco*I and *Xba*I, ligated into pET302 digested with the same enzymes, and subsequently transformed to *E. coli* DH5α. Transformants were selected for ampicillin resistance on LB agar plates. All mutants were sequenced (Baseclear, Leiden The Netherlands) to confirm the presence of the desired and absence of any undesired mutations.

Preparation of Membrane Vesicles. Right-side-out membrane vesicles (RSO) were prepared by the osmotic lysis procedure, as described previously (16). The membranes were resuspended in 50 mM potassium phosphate (KPi), pH 6.1.

Inside-out (ISO) membrane vesicles were prepared by washing the cells once with 10 mM Tris-HCl pH 8 buffer, followed by suspension in a small volume of 50 mM KPi, pH 7. Subsequently, the cell suspension was passed twice through a French Pressure Cell operated at 13 kpsi. Cell debris and whole cells were removed by a low spin at 8000 rpm for 10 min at 4°C in a Beckman SS34 rotor. Membranes were collected by ultracentrifugation for 20 min at 80 000 rpm at 4°C in a Beckman TLA 100.4 rotor. After washing once with 50 mM KPi, pH 7, the membranes were resuspended in the same buffer.

RSO and ISO membrane vesicles were rapidly frozen in liquid nitrogen and stored at −80 °C. Membrane protein was determined using the *DC* Protein Assay Kit (Bio-Rad Laboratories, USA).

Partial Purification Using Ni−NTA Affinity Chromatography. ISO membranes were solubilized by 4-fold dilution in 50 mM KPi pH 8 buffer containing 10% glycerol, 1% aminoxid, 100 mM NaCl, and 10 mM imidazol, pH 8. The suspension was stirred gently for 30 min while one ice. Nonsolubilized particles were removed by centrifugation at 4 °C for 5 min in an Eppendorf Tabletop centrifuge operated at 13 000 rpm. Ni−NTA columns were prepared according to the manufacturer's instructions. CimH was allowed to bind for 1 h at 4 °C, after which the column was washed once with 5 column volumes of solubilization buffer containing 0.1% aminoxid and once with 10 column volumes containing 0.1% aminoxid and 20 mM imidazol. The protein was eluted by incubating the resin for 30 min at 4 °C with 2 volumes of the same buffer at pH 7 and containing 200 mM imidazol. The protein was stored at −20 °C. Purification of all mutants was equally successful as observed for the wild-type CimH. In all cases the protein running at a molecular weight of 48 kDa was the most abundant protein band on a silver- or

Coomassie Brilliant Blue (CBB) stained SDS−PAGE (Sodiumdodecylsulfate Polyacrylamide Gel Electrophoresis) gel.

Immunoblot Analysis. Following SDS−PAGE of the membranes, the proteins were transferred to poly(vinylidene difluoride) (PVDF) membranes (Boehringer Mannheim) using semi dry electroblotting. The blots were analyzed using monoclonal antibodies directed against a His_6 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 Diagnostics GmbH, Mannheim, Germany).

Citrate Uptake in RSO Membranes. Uptake of [1,5-^{14}C]-citrate (114 mCi/mmol, Amersham Pharmacia, The Netherlands) was measured using the rapid filtration method essentially as described before (*2*). RSO membranes (final membrane protein concentration of 0.5 mg/mL) were incubated in 50 mM KPi, pH 6.1, for 2 min at 30 °C with 200 μM phenazine methosulfate (PMS) and 10 mM potassium ascorbate under a flow of water-saturated air while stirring magnetically. Initial rates of uptake were determined from the uptake during the first 10 s at least in duplicate. The concentration of [1,5-^{14}C]-citrate in the assay was 4.4 μM.

Labeling of Cysteine Residues. RSO membranes were treated with sulfydryl reactive agents in 50 mM KPi, pH 6.0, at room temperature. ISO vesicles were treated in 50 mM KPi, pH 7.0, also at room temperature. The treatment was stopped by the addition of an excess of DTT. In substrate protection experiments, trisodium citrate was added at a 1 mM final concentration 5 min prior to treatment with the sulfhydryl reagent. Following the treatment, the RSO membranes were washed three times in 50 mM KPi, pH 6.0, to remove the substrate. Subsequently, the membranes were resuspended to a protein concentration of 0.5 mg/mL and assayed for uptake activity. Control samples, without added substrate and with and without treatment with the thiol reagent, were treated in the same way. No influence of residual substrate could be detected indicating that the washing step was sufficient.

Labeling of whole cells was done essentially as described by Slotboom et al. (*27*). In short, cells were resuspended at a high cell density in 50 mM KPi, pH 6, and stored on ice until use. After addition of the thiol reagent, one part of the suspension was sonicated 5 times for 15 s with intermittent cooling on ice for 45 s, while the other part was kept on ice. Subsequently, the samples were immediately incubated for 30 min at 37 °C. Unreacted thiol reagent was quenched by the addition of 1 mM DTT. Membranes were collected by ultracentrifugation for 20 min at 80 000 rpm at 4°C in a Beckman TLA 100.4 rotor followed by partial purification and labeling with F-5-M as described below.

Partially purified protein was treated with 250 μM fluorescein-5-maleimide (F-5-M) in 50 mM KPi pH 7.0 containing 0.1% aminoxid. When indicated, labeling was preceded by treatment with 1 mM *N*-ethylmaleimide (NEM) for 15 min at room temperature. The samples were run on a 12% SDS−PAGE gel, and after rinsing the gel with water, fluorescence was detected using a Lumi-Imager F1 imager (Roche Diagnostics GmbH, Mannheim, Germany). The gel was irradiated by UV light, and the emitted light was filtered with a 520 nM filter. All samples containing F-5-M were kept in the dark until the gel was exposed. After fluorescence analysis the gel was CBB stained to estimate protein concentrations.

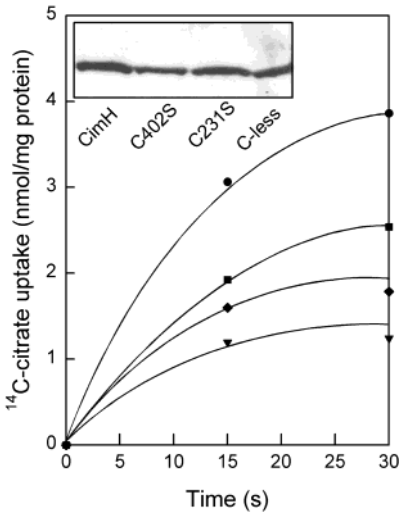

FIGURE 2: Citrate uptake activity of cysteine mutants of CimH. Proton motive force driven ^{14}C-citrate uptake in RSO membrane vesicles containing CimH (●), C231S (▼), C402S (■), or the C-less mutant (◆). The insert shows the expression levels of the proteins determined by Western-blotting using an antibody raised against the His tag.

Materials. Na (2-sulfonatoethyl) methanethiosulfonate (MTSES), 2-(trimethylammonium)ethyl methanethiosulfonate bromide (MTSET), and 2-ethylammonium methanethiosulfonate hydrobromide (MTSEA) were purchased from Anatrace Inc. (Ohio, USA). NEM was purchased from Sigma-Aldrich BV (Zwijndrecht, The Netherlands) and F-5-M and 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMdiS) were purchased from Molecular Probes Europe BV (Leiden, The Netherlands).

RESULTS

Construction of C-Less CimH. CimH contains two cysteine residues at positions 231 and 402. C231 is located in the middle of TMS VI and C402 is located just upstream of the conserved region in the cytoplasmic loop between TMS X and TMS XI (Figure 1). Two single cysteine mutants, C231S and C402S, and the C-less mutant (C231S−C402S) were constructed. The two single cysteine mutants and the C-less mutant were expressed to similar levels as the wild-type, as shown by Western blotting using an antibody directed against the His tag (Figure 2, inset). Uptake activity in right-side-out (RSO) membrane vesicles using [1,5-^{14}C]-citrate as the substrate indicated that all mutants showed ^{14}C-citrate transport activity (Figure 2). Corrected for expression levels, the C231S, C402S, and C-less mutants showed specific activities of 75%, 55%, and 52% relative to wild-type, respectively.

Treatment of RSO membrane vesicles containing CimH or one of the mutants with 5 mM of the membrane permeable thiol reagent *N*-ethylmaleimide (NEM) for 15 min did not result in any inhibition of ^{14}C-citrate uptake (data not shown). The following procedure was followed to show whether the cysteine residues were actually labeled with NEM. CimH and the C231S, C402S, and C-less mutants were partially purified by Ni^{2+}−NTA affinity chromatography and treated

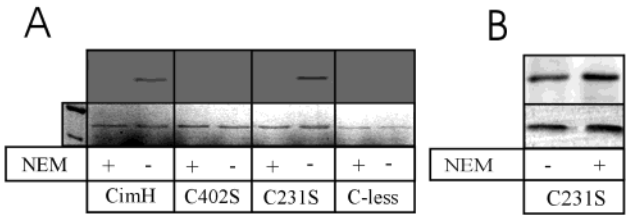

FIGURE 3: Labeling of the endogenous cysteine residues of CimH by thiol reactive reagents. (A) Partial purified CimH (lanes 1 and 2), C231S (lanes 3 and 4), C402S (lanes 5 and 6), and C-less (lanes 7 and 8) was treated with F-5-M with or without prior treatment with 1mM NEM for 5 min as indicated. (B) ISO membranes containing the C231 mutant were or were not treated with 1 mM NEM for 15 min as indicated, followed by partial purification and treatment with F-5-M. The upper lanes show the fluorescence image of the gel and the lower lanes the protein content after CBB staining. The marker proteins shown in the most left lane in panel A represent proteins of 50 and 37 kDa.

in the detergent solubilized state with the fluorescent label fluorescein-5-maleimide (F-5-M). As expected the C-less mutant did not label, while CimH was readily labeled with F-5-M (Figure 3A). Labeling of CimH could be completely prevented by prior incubation of the solubilized protein with 1 mM NEM for 5 min (Figure 3A).

Treatment of the two single cysteine mutants upon purification with F-5-M resulted in labeling of C231S, but not of C402S. Apparently, the labeling of the wild-type reflects labeling of C402 and not of C231. Similar to wild-type CimH, pretreatment of solubilized C231S with NEM prevented labeling with F-5-M. In contrast, treatment of the mutant with NEM prior to purification, i.e., while the protein was still embedded in the membrane, did not prevent labeling with F-5-M after purification (Figure 3B).

In conclusion, the C-less version of CimH is functional, indicating that the two cysteine residues present in CimH are not essential for citrate transport activity. Furthermore, the two cysteine residues in the wild-type protein are not accessible to NEM when the protein is embedded in the membrane.

Construction and Activity of the R143C, R420C, Q428C, R432C, and R432K Mutants. Mutants R143C, R420C, Q428C, R432C, and R432K were constructed in both the wild-type and the C-less genetic background. Unexpectedly, R420C in the C-less genetic background was not expressed to levels detectable by Western blotting, and uptake studies using RSO vesicles showed no ^{14}C-citrate uptake activity (not shown). All other mutants were expressed to similar levels in the wild-type and C-less background (see Figure 4, inset).

Uptake activities of the R143C, R420C, and Q428C mutants in RSO membrane vesicles with ^{14}C-citrate as the substrate were comparable to wild type (Figure 4). Kinetic analysis indicated that the affinity and maximal rates of uptake of these mutants for the substrates citrate, L-citramalate and L-malate were not dramatically changed (Table 1). Apparently, residues R143, R420, and Q428 which are conserved in the 2HCT family do not play an important role in the catalytic activity of CimH.

In contrast, replacement of R432 with Cys resulted in the loss of significant citrate uptake activity (but see below), while the conservative replacement with Lys severely reduced the activity to about 5% of the wild type (Figure

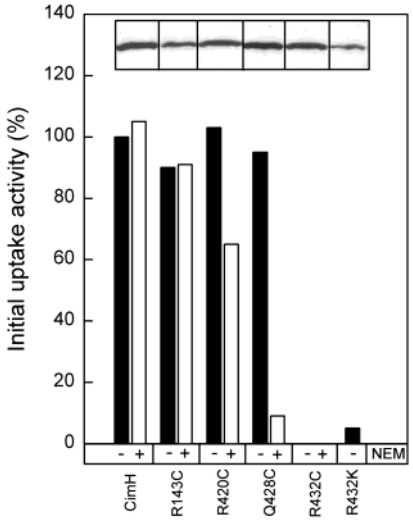

FIGURE 4: Effect of *N*-ethylmaleimide on citrate uptake activity catalyzed by cysteine mutants. Initial rate of ^{14}C-citrate uptake in RSO vesicles containing the indicated CimH alleles before (closed bars) and after (open bars) treatment with 5 mM NEM for 10 min. The rates were expressed as the percentage of the initial rate of the untreated wild-type CimH. The insert shows the expression levels of the proteins determined by Western blotting.

Table 1: Kinetic Analysis of ^{14}C-Citrate Uptake Catalyzed by Wild-Type and Mutant CimH Transporters

	citrate		L-malate	L-citramalate
	V_{max} (nmol min^{-1} mg^{-1})	K_m (μM)	K_I[a] (μM)	K_I[a] (μM)
wild type	37	10	1.5	100
R143C	50	10	1	100
R420C	36	14	1	55
Q428C	33	12.5	2	35
R432K	31	300	nd[b]	nd[b]

[a] Inhibition constants determined from the inhibition of the initial rate of citrate uptake. [b] Not determined.

4). Kinetic analysis showed that the affinity constant K_m of the R432K mutant for citrate was around 300 μM, which is 1 order of magnitude higher than observed for the wild type. The maximal rate of transport V_{max} was not significantly altered compared to the wild-type (Table 1). It follows that the arginine residues at position 432 in the symporter CimH and at the equivalent position 425 in the citrate/lactate exchanger CitP are involved in high affinity binding of di/tricarboxylates in both transporters.

Reactivity of R143C, R420C, Q428C, and R432C with NEM. Inside-out (ISO) membrane vesicles containing the single Cys-mutants R143C, Q428C, and R432C mutants (C-less background) were treated with NEM, followed by purification and labeling with F-5-M to assay for the accessibility of the cysteine residues in the mutants (Figure 5). Mutant R420C was omitted from these experiments since it does not express in the C-less background. All three mutants readily labeled with F-5-M in the solubilized state, and in contrast to the C231S mutant, labeling was prevented by pretreatment of the ISO membranes with NEM. The cysteine residues at positions 143, 428 and 432 were labeled with NEM when CimH was embedded in the membrane.

Treatment of RSO membranes containing R143C with NEM had no effect on the citrate uptake activity (Figure 4). A small but significant inhibition was observed for mutant

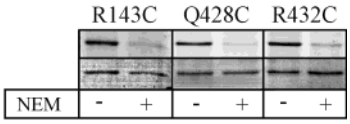

FIGURE 5: Accessibility of cysteines to thiol reactive reagents. ISO vesicles expressing R143C (lanes 1 and 2), Q428C (lanes 3 and 4), and R432C (lanes 5 and 6) were treated with and without 1 mM NEM for 15 min as indicated, followed by partial purification and treatment with F-5-M. The upper lanes show the fluorescence image of the part of the gel containing the CimH mutants. The lower lanes show the same part of the gel after staining for protein with CBB.

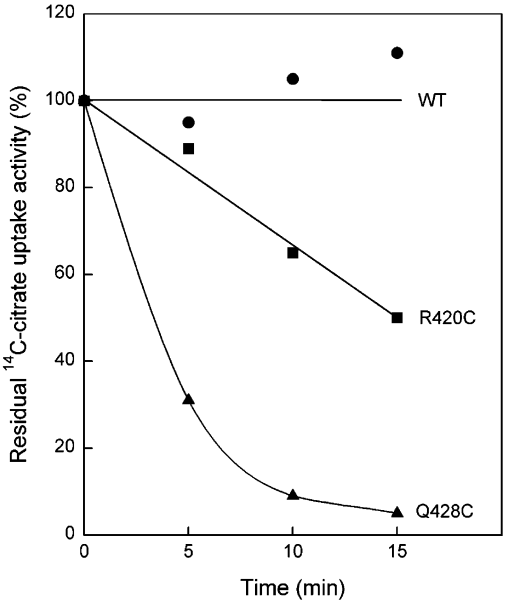

FIGURE 6: Kinetics of NEM labeling. RSO vesicles containing CimH (●), R420C (■) or Q428C (▲) were incubated for increasing periods of time with 5 mM NEM after which ^{14}C-citrate uptake activity was determined. The remaining activity was plotted relative to the activity of the untreated membranes.

R420C (wild type background), showing that the cysteine residue at position 420 is accessible to NEM. The most severe effect of NEM labeling was observed for the Q428C mutant that was almost completely inactivated. The different effects on the R420C and Q428C mutants were due to different kinetics of labeling (Figure 6). The Q428C mutant labeled with NEM with a half time of about 3−4 min, while under identical conditions, the R420C mutant retained about 50% residual activity after 15 min of treatment. Prolonged incubation of the R420C eventually completely inhibited citrate transport (data not shown).

It is concluded that labeling of a cysteine at position 143 in CimH leaves the activity of the protein unaltered, while labeling of cysteines at positions 420 and 428 completely inhibit the protein. The reactivity of the cysteine residue in R420C was significantly lower than observed for the cysteine in Q428C. Furthermore, the cysteine in the inactive mutant R432C was shown to be accessible to NEM.

Reactivation of R432C with MTS Reagents. The methanethiosulfonate (MTS) derivatives MTSEA, MTSET, and MTSES represent a set of thiol reactive reagents that differ in size and charge of the groups attached to the reactive MTS moiety. They react with cysteine residues on proteins to form disulfides, thereby introducing a positive (MTSEA, MTSET) or negative (MTSES) charge on the protein at the site of the

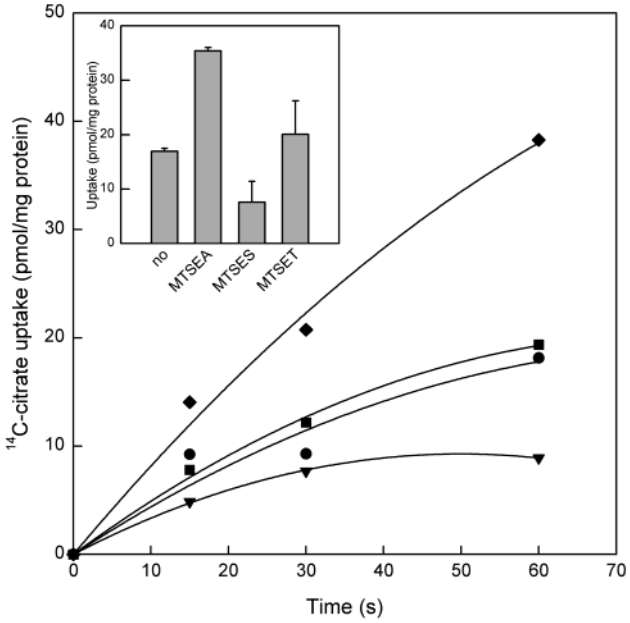

FIGURE 7: Reactivation of R432C with MTS reagents. ^{14}C-citrate uptake activity was measured in RSO membranes containing the R432C mutant without any treatment (●) or after treatment with MTSET (■), MTSES (▼), and MTSEA (◆) at 1 mM concentrations. (inset) The uptake was measured at $t = 1$ min in triplicate for the same conditions. Bars and error bars represent the average uptake levels and standard deviations, respectively.

cysteine residue (*17*, *18*). RSO vesicles containing the inactive R432C mutant were incubated for 15 min with 1 mM of each of the three reagents (Figure 7). A very low uptake activity of about 0.5% of the wild-type activity was observed in the untreated membranes suggesting that the R432C is not completely inactive. When R432C was treated with MTSES (negative), the initial uptake rate was decreased by a factor of 2. The positively charged MTSET had no effect, but treatment with MTSEA which is less bulky than MTSET resulted in an increase of activity of about a factor of 2 (Figure 7). Since the uptake activities in these experiments were very low, the uptake of citrate was measured in triplicate after 1 min of incubation. The results justified the conclusion (Figure 7, insert) that the positive charge of MTSEA linked to the cysteine introduced at position 432 reactivated the mutant, while the negative charge of MTSES further lowered the activity. The activation of the R432C mutant by the MTSEA reagent is in line with the activity observed for the R432K mutant and suggests that a positive charge at position 432 improves substrate binding.

Accessibility of R420C and Q428C. Depending on their variable group, MTS reagents are either membrane permeable (MTSEA) or impermeable (MTSES, MTSET). The accessibility of the cysteine residues at positions 420 and 428 in the C-terminal cytoplasmic loop for the reagents was investigated in RSO membrane vesicles. Both the wild-type CimH protein and the R143C mutant were not inhibited by treatment with any of the MTS reagents (not shown). The R420C mutant in the wild type background showed severe inhibition when treated with MTSES and MTSEA, but not with MTSET (Figure 8, left). The effect of MTSES (impermeable) was more pronounced than the effect of MTSEA (permeable). Similarly, the single-Cys mutant Q428C was inhibited by both MTSES and MTSEA and not

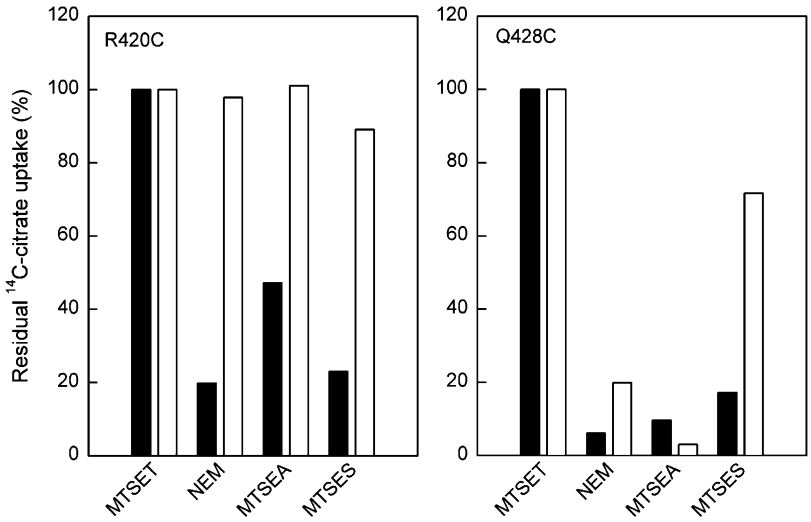

FIGURE 8: Substrate protection against inactivation by thiol reactive reagents. RSO membranes containing the R420 (left) and Q428 (right) mutants were treated with the indicated thiol reagents in the presence (open bars) and absence (closed bars) of 1 mM citrate. The R420C mutant was treated with 10 mM NEM for 15 min and the Q428 mutant with 5 mM NEM for the same period of time. The concentration of the MTS reagents was 1 mM. Activity was plotted as the percentage of the activity of untreated membranes.

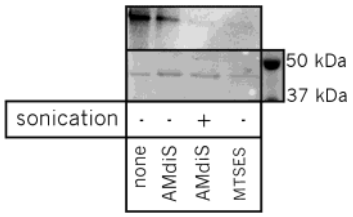

FIGURE 9: Accessibility of Q428C in whole cells to AMdiS and MTSES. *E. coli* strain TOP10 expressing the Q428C mutant was treated with 0.5 mM AMdiS with and without sonication as indicated and 1 mM MTSES without sonication (see Methods). The upper lanes show the fluorescence image, the lower lanes the protein stained gel. The lane on the right shows two marker proteins.

by MTSET (Figure 8, right). It should be noted that in these experiments the MTS reagent were used at the same concentration of 1 mM, while the reactivity of the reagents decreases in the order of MTSET > MTSEA > MTSES (17). The results suggest that the cysteine residues of both R420C and Q428C are accessible in RSO membranes to the membrane impermeable, negatively charged MTSES reagent. AMdiS is a membrane impermeable, negatively charged maleimide based thiol reagent that is more bulky than MTSES (MW of 536 and 219 Da, respectively. In contrast to MTSES, treatment of RSO membranes containing Q428C (C-less background) with AMdiS did not effect the activity of the mutant (not shown). Treatment of whole cells expressing the Q428C mutant with AMdiS followed by partial purification and labeling with F-5-M showed that the lack of inactivation by AMdiS was due to lack of reaction with the reagent (Figure 9). In the same experiment, MTSES did prevent labeling with F-5-M. Importantly, sonication of the cells in the presence of AMdiS also prevented subsequent labeling with F-5-M showing that the C428 residue was accessible for AMdiS, but only from the cytoplasmic side of the membrane.

The inactivation of mutants R420C and Q428C by the thiol reactive reagents NEM, MTSEA and MTSES was studied in the presence of substrate (Figure 8). Labeling of R420C with the reagents was completely prevented by the presence of 1 mM of citrate during the treatment. In case of R428C, MTSES labeling was largely prevented in the presence of the same concentration of citrate, but labeling with NEM and MTSEA, both of which are membrane permeable, was not influenced. The results suggest that in the presence of citrate, R420C is no longer accessible from either side of the membrane while in the case of Q428C substrate blocks the accessibility from the outside, but not from the inside.

DISCUSSION

Members of the 2HCT family recognize substrates that contain a 2-hydroxycarboxylate motif. Binding of the substrates is mediated by essential interactions between sites on the protein and the hydroxyl and carboxylate groups of the motif. The hydroxyl group was speculated to form hydrogen bonds with the protein (*5*), while the carboxylate is likely to interact via electrostatic interactions in combination with hydrogen bonding. In search for the sites on the proteins that interact with the 2-hydroxycarboxylate motif, we mutated four conserved residues in CimH of *B. subtilis*, R143, R420, Q428, and R432, that are positively charged and/or capable of forming hydrogen bonds. Mutation of R143, R420, and Q428 to Cys did not affect the kinetic parameters of the transporter, indicating that none of these residues are essential for transport activity. Substitution of R432 for a cysteine residue greatly reduced citrate transport activity, which was in line with previous results obtained for the same mutation of R425, the corresponding residue of R432 in CitP of *Lc. mesenteroides*. R425 in CitP was shown to be responsible for the nonessential interaction with the carboxylate group at the R_S position in di/tricarboxylates (see below; *14*). The results for the R143 and R420 mutations were unexpected since the two arginine residues are the only conserved positive charges (in addition to R432) in the 2HCT family, which to date contains 10 different proteins. Possibly, electrostatic interactions are not involved in the binding of the carboxylate group of the 2-hydroxycarboxylate motif, or the group interacts with residues that are not conserved.

The 2HCT family contains H^+ or Na^+ symporters and exchangers that couple the uptake of the substrate to the exit of a structurally related metabolic end product of the

substrate. CitP of *Lc. mesenteroides* belongs to the latter group and exchanges citrate for L-lactate. The affinity of CitP for the tricarboxylate citrate is 3 orders of magnitude higher than that for the monocarboxylate lactate. It was demonstrated that R425 of CitP is responsible for the high-affinity binding of citrate by interacting directly with the carboxylate at the R_S position (Figure 1), which is absent in L-lactate (*14*). R425 of CitP is conserved throughout the 2HCT family and corresponds to R432 of CimH, which belongs to the symporters in the family. CimH only transports substrates containing a carboxylate group at the R_S position, i.e., citrate, L-malate, and L-citramalate, and it was anticipated that the interaction of R432 of CimH with the substrates would be more critical than in the case of R425 of CitP (*2*). Nevertheless, the properties of the R432 mutants of CimH suggest a similar role in substrate binding. In both transporters, replacement of the arginine residue with Cys reduced transport activity by 2 orders of magnitude. The conservative arginine to lysine mutation resulted in a lowering of the affinity for the substrate by 2 orders of magnitude. Remarkably, the maximal rate of the R432K mutant of CimH was unchanged, while the R425K mutant of CitP showed a strongly reduced maximal rate. It should be noted though that the kinetic modes were different, unidirectional uptake and exchange, respectively. Positioning of a positive charge at the cysteine residues in the arginine-to-cysteine mutants of the transporters via labeling with MTSEA enhanced transport activity 2-fold and 48-fold for CimH and CitP, respectively. In contrast, introducing a negative charge by labeling with MTSES reduced transport 2-fold in both transporters. Apparently, the interaction between the conserved arginine and the carboxylate group at the R_S position of the substrates is not responsible for the lack of binding of L-lactate by CimH. The most prominent difference between R432 in CimH and R425 in CitP is the accessibility of the site from the outside of the cell. Using membrane-permeable and -impermeable thiol reagents, it was concluded that R425 in CitP was not accessible from the outside (*14*), while in this study it is concluded that R432 in CimH is accessible from the outside (see below). This difference may reflect the different physiological functions of symport and exchange of the transporters in the family.

Wild-type CimH and the single-Cys mutants were treated with thiol reactive reagents both in the detergent solubilized state and in the membrane embedded state. Three different labeling behaviors were observed. (i) Cysteine residues at positions 143, 420, 428, and 432 were readily labeled both in the detergent solubilized and in the membrane embedded state, indicating that the residues are exposed to the aqueous phase in both states. (ii) The cysteine at position 231, located in the middle of TMS VI, was not labeled with F-5-M in either state. F-5-M is believed to react with thiols in a hydrophilic environment, but has also been reported to be able to penetrate into lipophilic environments and react with cysteine residues within a detergent micel (*19*, *20*). The complete lack of reactivity of C231 suggests that this position is buried in the protein structure. (iii). The cysteine at position 402 is readily labeled when solubilized in detergent but not when embedded in the membrane. The latter observation is in agreement with data obtained for CitS of *K. pneumoniae* showing that the cysteine residue at the corresponding position was not accessible to the thiol reagents AMdiS and 3-(N-maleimido-propinyl) biocytin (MBP) in everted membrane vesicles, nor in whole cells (*21*). Possibly, the transporters are present in a higher oligomeric state in the membrane (*22*, *23*), in which the cysteine at position 402 (CimH numbering) is shielded. In the detergent solubilized state, the oligomeric state may be lost, thereby exposing C402 to the water phase. Alternatively, local conformational changes or increased flexibility upon solubilization may be responsible for the observed labeling in detergent solution.

Cysteine residues at positions 420 and 428 in a putative cytoplasmic loop of CimH reacted with the membrane impermeable MTSES reagent in right-side-out membrane vesicles, indicating that the residues are accessible through the protein from the exterior side of the cell membrane. The access pathway is likely to be the substrate translocation pathway since, (i) positions 420 and 428 are close to R432 that interacts directly with the substrate, (ii) labeling of the cysteines at positions 420 and 428 resulted in an inactive transporter, and (iii) substrate binding protected against inactivation. The membrane permeable thiol reagent NEM reacted considerably faster with Q428C than with R420C. NEM was found to react much faster with water accessible cysteines than with cysteines in the lipid phase (*24*), suggesting that R420C is only poorly accessible from the water phase. Surprisingly, membrane impermeable MTSES, which is believed to react only from the water phase, reacted faster with R420C than NEM. Moreover, at the same concentrations, MTSES reacted faster with R420C than the positively charged MTS derivatives MTSEA and MTSET, the latter of which was not reactive at all. The difference is not caused by differences in reactivity, as MTSES is about 4 times less reactive than MTSEA and 10 times less than MTSET (*17*). It is tempting to explain the different labeling kinetics by assuming that the entrance pathway for the substrate from the outside in CimH would be optimized for negatively charged substrates such as citrate and malate. The translocation pathway would favor negatively charged MTSES over the positively charged MTSEA and MTSET, and neutral NEM to penetrate to R420C. Possibly, membrane-permeable MTSEA and NEM react with R420C (and Q428C) from the inside, which would explain why membrane-impermeable MTSET does not react at all. Interestingly, molecular modeling studies have shown that MTSES and citrate have very similar molecular volumes and it was suggested that negatively charged MTSES might be a substrate analogue (*25*). The inaccessibility of C428 in the Q428C mutant for the negatively charged, but more bulky reagent AMdiS supports this hypothesis.

Labeling of the R420C mutant was prevented by the presence of substrate, irrespective of the nature of the thiol reactant, suggesting that R420 is close to the substrate in the three-dimensional structure of CimH. In contrast, substrate protected the Q428C mutant against labeling by membrane impermeable MTSES and not against membrane permeable MTSEA and NEM, suggesting that the substrate blocks the access pathway to C428 from the outside and not from the inside (Figure 10).

Similar results as those reported here were obtained for residues in a cytoplasmic loop of the related glutamate transporters GltT of *B. stearothermophilus* and GLT-1, found in the central nervous system (*26*, *27*). It was concluded that the cytoplasmic loop in part traverses the membrane and

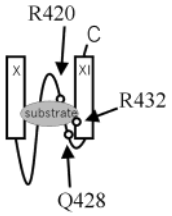

FIGURE 10: Model for the membrane topology of the C-terminal region of CimH. The two boxes represent putative transmembrane segments X and XI. The putative location of the substrate relative to the residues substituted with cysteine is indicated in gray. The top of the scheme is the periplasmic side of the protein.

forms a reentrant loopike structure as commonly observed in channels (*28*). Possibly, the cytoplasmic loop between TMS X and XI in the transporters of the 2HCT family is folded in a similar structure. A more systematic Cys scanning of this region in CimH is necessary to confirm this hypothesis.

ACKNOWLEDGMENT

This research was supported by the Ministry of Economic Affairs, in the framework of the IOP Milieutechnologie/ Zware metalen IZW97404.

REFERENCES

1. Kawai, S., Suzuki, H., Yamamoto, K., and Kumagai, H. (1997) *J. Bacteriol. 179*, 4056−4060.
2. Krom, B. P., Aardema, R., and Lolkema, J. S. (2001) *J. Bacteriol. 183*, 5862−5869.
3. van der Rest, M. E., Siewe, R. M., Abee, T., Schwarz, E., Oesterhelt, D., and Konings, W. N. (1992) *J. Biol. Chem. 267*, 8971−8976.
4. Wei, Y., Guffanti, A. A., Ito, M., and Krulwich, T. A. (2000) *J. Biol. Chem. 275*, 30287−30292.
5. Bandell, M., Ansanay, V., Rachidi, N., Dequin, S., and Lolkema, J. S. (1997) *J. Biol. Chem. 272*, 18140−18146.
6. Marty-Teysset, C., Lolkema, J. S., Schmitt, P., Divies, C., and Konings, W. N. (1995) *J. Biol. Chem. 270*, 25370−25376.
7. Lolkema, J. S., Poolman, B., and Konings, W. N. (1996) Secondary Transporters and Metabolic Energy Generation in Bacteria, in *Transport Processes in Eukaryotic and Procaryotic Organisms*, (Konings, W. N., Kaback, H. R., and Lolkema, J. S., Eds.) Elsevier, Amsterdam.
8. Marty-Teysset, C., Posthuma, C., Lolkema, J. S., Schmitt, P., Divies, C., and Konings, W. N. (1996) *J. Bacteriol. 178*, 2178−2185.
9. Poolman, B., Molenaar, D., Smid, E. J., Ubbink, T., Abee, T., Renault, P. P., and Konings, W. N. (1991) *J. Bacteriol. 173*, 6030−6037.
10. van Geest, M., Nilsson, I., von Heijne, G., and Lolkema, J. S. (1999) *J. Biol. Chem. 274*, 2816−2823.
11. van Geest, M., and Lolkema, J. S. (2000) *Biochim. Biophys. Acta 1466*, 328−338.
12. Bandell, M., and Lolkema, J. S. (1999) *Biochemistry 38*, 10352−10360.
13. Bandell, M., and Lolkema, J. S. (2000) *Biochemistry 39*, 13059−13067.
14. Bandell, M., and Lolkema, J. S. (2000) *J. Biol. Chem. 275*, 39130−39136.
15. Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., Bertero, M. G., Bessières, P., Bolotin, A., Borchert, S., Borriss, R., Boursier, L., Brans, A., Braun, M., Brignell, S. C., Bron, S., Brouillet, S., Bruschi, C. V., Caldwell, B., Capuano, V., Carter, N. M., Choi, S. K., Codani, J. J., Connerton, I. F., and Danchin, A. (1997) *Nature 390*, 249−256.
16. Kaback, H. R. (1983) *J. Membr. Biol. 76*, 95−112.
17. Karlin, A., and Akabas, M. H. (1998) *Methods Enzymol. 293*, 123−145.
18. Seal, R. P., Leighton, B. H., and Amara, S. G. (1998) *Methods Enzymol. 296*, 318−331.
19. Joseph-Liauzun, E., Delmas, P., Shire, D., and Ferrara, P. (1998) *J. Biol. Chem. 273*, 2146−2152.
20. Zhou, J., Fazzio, R. T., and Blair, D. F. (1995) *J. Mol. Biol. 251*, 237−242.
21. van Geest, M., and Lolkema, J. S. (1999) *J. Biol. Chem. 274*, 29705−29711.
22. Heuberger, E. H., Veenhoff, L. M., Duurkens, R. H., Friesen, R. H., and Poolman, B. (2002) *J. Mol. Biol. 317*, 591−600.
23. Pos, K. M., Bott, M., and Dimroth, P. (1994) *FEBS Lett. 347*, 37−41.
24. Kimura, T., Suzuki, M., Sawai, T., and Yamaguchi, A. (1996) *Biochemistry 35*, 15896−15899.
25. Kaplan, R. S., Mayor, J. A., Brauer, D., Kotaria, R., Walters, D. E., and Dean, A. M. (2000) *J. Biol. Chem. 275*, 12009−12016.
26. Grunewald, M., Bendahan, A., and Kanner, B. I. (1998) *Neuron 21*, 623−632.
27. Slotboom, D. J., Sobczak, I., Konings, W. N., and Lolkema, J. S. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 14282−14287.
28. MacKinnon, R. (1995) *Neuron 14*, 889−892.

BI026874A