Site-Directed Sulfhydryl Labeling of the Oxaloacetate Decarboxylase Na^+ Pump of *Klebsiella pneumoniae*: Helix VIII Comprises a Portion of the Sodium Ion Channel†

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ABSTRACT: Helix VIII of the β-subunit of the oxaloacetate decarboxylase of *Klebsiella pneumoniae* contains the functionally important residues βN373, βG377, βS382, and βR389. Using a functional oxaloacetate decarboxylase mutant devoid of Cys residues in the β-subunit, each amino acid residue in helix VIII was replaced individually with Cys. Structural and dynamic features of this region were studied by using site-directed sulfhydryl modification of 20 single-Cys replacement mutants with methanethiosulfonate (MTS) reagents in the absence or presence of Na^+ ions. The pattern of accessibility of the MTS reagents from the periplasmic side of helix VIII shows a periodicity which suggests that this region is α-helical. In particular, a water-accessible face comprising βN373, βG377, βS382, βM386, and βV390 may be part of a Na^+ channel. Cys residues introduced in the cytoplasmically oriented part of helix VIII were accessible to three different water-soluble MTS compounds and therefore believed to be exposed to water on this side of the membrane. Most residues located in the upper part of helix VIII (residues βN373−βV381C) were protected by Na^+ ions for inactivation by the MTS reagents. The distinct results on accessibility toward the different MTS reagents obtained in the presence or absence of Na^+ ions may suggest a conformational change upon binding of Na^+ in this region. The βR389C mutant had a reduced activity and a pH optimum at pH 9, which could be restored to a wild-type pH optimum of 6.5 and to a 400% gain in activity upon chemical modification with 2-aminoethyl methanethiosulfonate.

Oxaloacetate decarboxylase (OAD)[1] of *Klebsiella pneumoniae* exemplifies the sodium ion transport decarboxylase family of enzymes, which also includes methylmalonyl-CoA decarboxylases, malonate decarboxylase, and glutaconyl-CoA decarboxylases from various anaerobic bacteria. These enzymes use the free energy of decarboxylation to pump Na^+ ions across the membrane (*1*−*3*). The oxaloacetate decarboxylase Na^+ pump consists of subunits α, β, and γ (Figure 1). The α-subunit is a peripheral membrane protein, harboring carboxyltransferase activity in the N-terminal domain; its C-terminal domain functions as a biotin carrier (*4*, *5*). The β-subunit is an integral membrane protein, composed of nine transmembrane α-helices, a hydrophobic segment (segment IIIa, proposed to insert into the membrane without traversing it), and connecting loops of various lengths (Figure 2) (*6*). The β-subunit catalyzes the decarboxylation of carboxybiotin bound to the α-subunit, an event coupled to

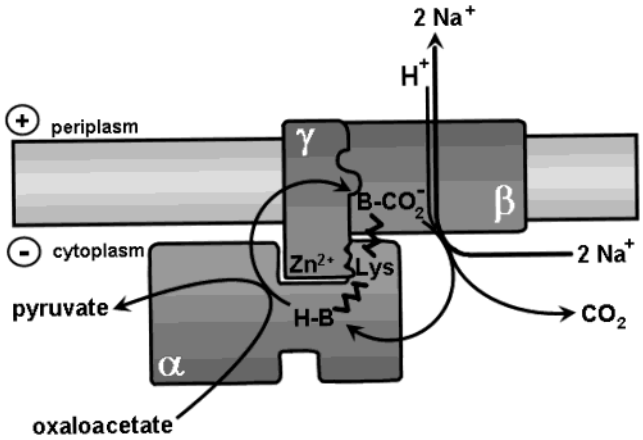

FIGURE 1: Cartoon depicting the structure and function of the oxaloacetate decarboxylase Na^+ pump. The catalytic cycle of oxaloacetate decarboxylase involves carboxyl transfer from oxaloacetate to the prosthetic biotin group located on the α-subunit, movement of the carboxybiotin into the decarboxylase site on the β-subunit, and its decarboxylation. Decarboxylation of carboxybiotin consumes a periplasmically derived proton and pumps two Na^+ ions from the cytoplasmic to the periplasmic reservoir. B represents biotin.

Na^+ pumping and consumption of a periplasmically derived proton (*1*, *7*). The γ-subunit is anchored in the membrane with a single N-terminal α-helix and has a hydrophilic C-terminal portion with an attached Zn^{2+} ion (*7*, *8*). The γ-subunit is responsible for complex stability, and accelerates the carboxyl-transfer reaction, presumably by polarizing the

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[1] Abbreviations: IPTG, isopropyl β-D-thiogalactopyranoside; LB, Luria Bertani; OAD, oxaloacetate decarboxylase; OadA, oxaloacetate decarboxylase α-subunit; OadB, oxaloacetate decarboxylase β-subunit; OadG, oxaloacetate decarboxylase γ-subunit; PCR, polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; MTSEA, 2-aminoethyl methanethiosulfonate; MTSET, 2-(trimethylammonium)ethyl methanethiosulfonate; MTSES, 2-sulfonatoethyl methanethiosulfonate; MMTS, methyl methanethiosulfonate; MTSPA, 2-aminopropyl methanethiosulfonate.

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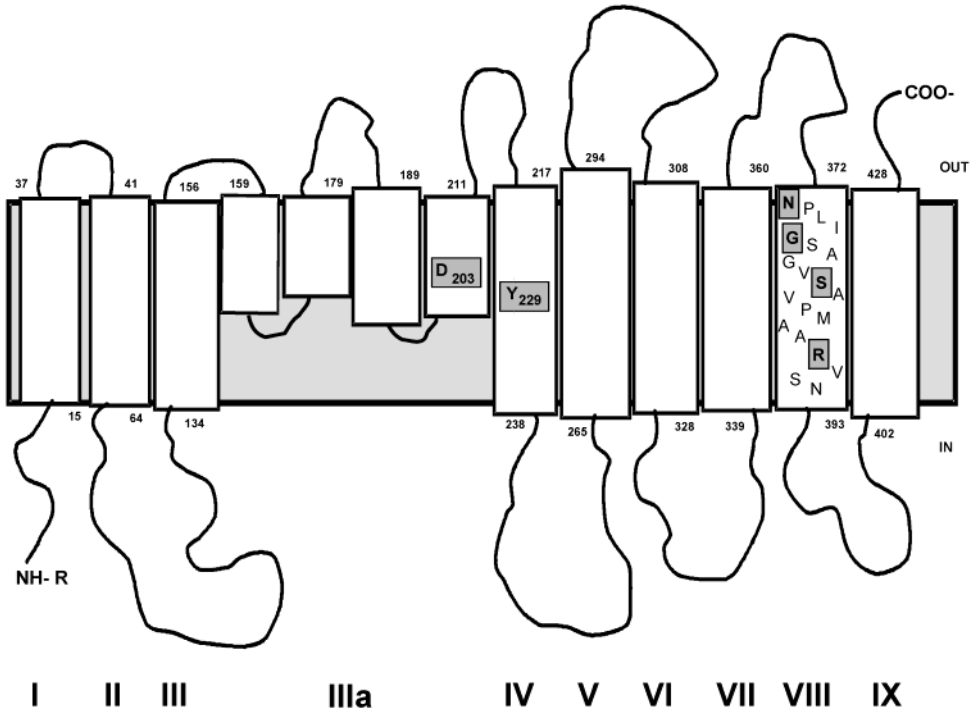

FIGURE 2: Topology model of the β-subunit of the oxaloacetate decarboxylase deduced from alkaline phosphatase and β-galactosidase fusion analyses and by studies of the accessibility of thiol reagents to selected cysteine sites (*6*). Also shown are the 20 amino acid residues of helix VIII which were individually replaced with cysteine in this study. Functionally important residues are marked with a gray background.

carbonyl oxygen bond of oxaloacetate with the Zn^{2+} ion (*8*, *9*).

The most conserved regions of the β-subunit are region IIIa and transmembrane helix VIII. Functionally important residues are D203 (region IIIa), Y229 (helix IV), and N373, G377, S382, and R389 (helix VIII), which are believed to be involved in the translocation of Na^+ and H^+ through the membrane (*10*). In this work, we subjected the functionally important helix VIII to cysteine scanning mutagenesis in a Cys-less β-subunit background. The effect of various hydrophilic MTS compounds on the activity of every single-cysteine mutant was examined, and the results suggest that helix VIII is part of the Na^+ translocating pathway.

EXPERIMENTAL PROCEDURES

Materials. MTS reagents were purchased from Toronto Research Chemicals (Toronto, ON).

Bacterial Strains and Plasmids. Escherichia coli DH5α (Bethesda Research Laboratories) and *E. coli* C43(DE3) (*11*) were routinely grown at 37 °C in Luria Bertani (LB) medium (*12*). The transformed *E. coli* C43(DE3) strains were inoculated with 1% of an overnight culture, grown under aerobic conditions at 37 °C until and OD_{600} of 0.5−0.6 was reached, and induced with 0.5 mM IPTG (final concentration). Cells were grown for an additional 4 h at 30 °C before being harvested. Media inoculated with plasmid-containing strains were supplemented with the selective antibiotic kanamycin (50 μg/mL).

Recombinant DNA Techniques. Standard recombinant DNA techniques were performed essentially as described by Sambrook *et al*. (*12*). Oligonucleotides used for mutagenesis were custom-synthesized by Microsynth (Balgach, Switzerland). All inserts derived from polymerase chain reaction (PCR) and ligation sites were checked by DNA sequencing according to the dideoxynucleotide chain-termination method (*13*) using a *Taq* Dye-Dideoxy Terminator Cycle Sequencing Kit and the ABI PRISM 310 genetic analyzer from Applied Biosystems.

Construction of the Cys-Less Mutant and Single-Cys Replacement Mutants in OadB. Primers used for site-directed mutagenesis are listed in Table 1. The Cys-less mutant of OadB was obtained as follows. PCR fragments containing C87A and C291M mutations were constructed in a two-step protocol. For the 5′ part of the PCR fragments of the β-subunit, primer prNKpnI+ and primers with the affix *rev* were used. For the corresponding 3′ part of the PCR, primer prCBamHI− and primers with the affix *for* were used. In the case of the C87A mutant, pET-GAB (*10*) served as the template. In the case of the C291M mutation, pET-GAB-C87A served as the template. The purified mutagenic PCR fragments were used as a template for the subsequent PCR with primers prNKpnI+ and prCBamHI−. Resulting PCR products were digested with *Kpn*I and *Bam*HI and cloned into pET-GAB or in the case of the C291M mutation into pET-GAB-C87A digested with the same restriction enzymes. The PCR fragment containing the C351A mutation was constructed in a one-step protocol. The PCR fragment was synthesized with primers prNBamHI+/C351A and prCStyI−, and the resulting PCR products were digested with *Bam*HI and *Sty*I and cloned into pET-GAB-C87A/C291M digested with the same restriction enzymes, yielding the pET-GAB-βCL plasmid. Single-Cys replacement mutants of the β-subunit were obtained as follows. The PCR fragments containing the mutations were constructed in a two-step protocol. For the 5′ part of the PCR fragments of the β-subunit, primer prNBamHI+ and primers with the affix *rev* were used. For the corresponding 3′ part of the PCR, primer prCStyI− and primers with the affix *for* were used.

Table 1: Primers Used for Mutagenesis[a]

primer	sequence (5′−3′)	restriction site
prNKpnI+	AGCCTTTGAGCC**GGTACC**GCAGGCGGAAGC	*Kpn*I
prCBamHI−	CAGCACCA**GGATCC**CCAGCGTCTG	*Bam*HI
prBamHI+/C351A	AGACGCTGG**GGATCC**TGGTGCTGGGGGTGATCGCCTTC*GCG*GTGGGGACCGCCGCCGGG	*Bam*HI
prCStyI−	CCGTTTAGAGGCC**CCAAGG**GGTTATGC	*Sty*I
prNBamHI+	AGCCGCAGACGCTGGGGATC	
C87Afor	GCCGCGAAGCTCCAC*GCC*GCGCCGGACGTCCACGCC	
C87Arev	GGCGTGGACGTCCGGCGC*GGC*GTGGAGCTTCGCGGC	
C291Mfor	GCGCCGCTGCTGGGGATGTTC*ATG*TTCGGCAACCTGATGCGC	
C291Mrev	GCGCATCAGGTTGCCGAA*CAT*GAACATCCCCAGCAGCGGCGC	
N373Cfor	GAACGTGTTC**TCGC*GA***CACAAAATC*TGC*CCGCTGATCGGC	*Bsp*68I
N373Crev	GCCGATCAGCGG*GCA*GATTTTGTG***TC*GCGA**GAACACGTTC	*Bsp*68I
P374Cfor	GATGAACGTGTTC**TCGCGA**CACAAAATCAAC*TGC*CTGATCGGC	*Bsp*68I
P374Crev	GCCGATCAG*GCA*GTTGATTTTGTG**TCGCGA**GAACACGTTCATC	*Bsp*68I
L375Cfor	CGTGTTC***TCGCGA***CACAAAATCAACCCG*TGC*ATCGGCTCG	*Bsp*68I
L375Crev	CGAGCCGAT*GCA*CGGGTTGATTTTGTG***TCGCGA***GAACACG	*Bsp*68I
I376Cfor	CAACCCGCTG*TGC*GGCTCGGCGGGGGTGTCGGCGGTGC**CCATGG**CGGC	*Nco*I
I376Crev	GCCG**CCATGG**GCACCGCCGACACCCCCGCCGAGCC*GCA*CAGCGGGTTG	*Nco*I
G377Cfor	CAACCCGCTGATC*TGC*TCGGCGGGGGTGTCGGCGGTGC**CCATGG**CGGC	*Nco*I
G377Crev	GCCG**CCATGG**GCACCGCCGACACCCCCGCCGA*GCA*GATCAGCGGGTTG	*Nco*I
S378Cfor	CCGCTGATCGGC***TGCGCA***GGGGTGTCGGCG	*Nsb*I
S378Crev	CGCCGACACCCC***TGCGCA***GCCGATCAGCGG	*Nsb*I
A379Cfor	GATCGGCTCG*TGC*GGGGTGTCGGCGGTGC**CCATGG**CGGCGC	*Nco*I
A379Crev	GCGCCG**CCATGG**GCACCGCCGACACCCC*GCA*CGAGCCGATC	*Nco*I
G380Cfor	GATCGGCTCG**GC*ATGC***GTGTCGGCGGTGC	*Pae*I
G380Crev	GCACCGCCGACAC***GCATGC***CGAGCCGATC	*Pae*I
V381Cfor	GCTCGGCGGGG*TGC*TCGGCGGTGC**CCATGG**CGGCG	*Nco*I
V381Crev	CGCCG**CCATGG**GCACCGCCGA*GCA*CCCCGCCGAGC	*Nco*I
S382Cfor	GCTCGGCGGGGGTG*TGC*GCGGTGC**CCATGG**CGGCG	*Nco*I
S382Crev	CGCCG**CCATGG**GCACCGC*GCA*CACCCCCGCCGAGC	*Nco*I
A383Cfor	CGGCGGGGGTGTCG*TGC*GTGC**CCATGG**CGGCGCGG	*Nco*I
A383Crev	CCGCGCCG**CCATGG**GCAC*GCA*CGACACCCCCGCCG	*Nco*I
V384Cfor	GGGGGTGTCGGCA*TGC*CCGATGGCGGCG	
V384Crev	CGCCGCCATCGG*GCA*TGCCGACACCCCC	
P385Cfor	GGGGTGTCGGCGGT***ATGCAT***GGCGGCGCGG	*Mph*1103I
P385Crev	CCGCGCCGCC**AT*GCAT***ACCGCCGACACCCC	*Mph*1103I
M386Cfor	GTCGGCGGTGCCG*TGT*GCCGCGCGGGTGTCG	
M386Crev	CGACACCCGCGCGGC*ACA*CGGCACCGCCGAC	
A387Cfor	GCGGTGCCGATG***TGCGCA***CGGGTGTCGAAC	*Nsb*I
A387Crev	GTTCGACACCCG***TGCGCA***CATCGGCACCGC	*Nsb*I
A388Cfor	GGTGCCGATG**GC*ATGC***CGGGTGTCGAAC	*Pae*I
A388Crev	GTTCGACACCCG***GCATGC***CATCGGCACC	*Pae*I
R389Cfor	CGGCGGTGC**CCATGG**CGGCG*TGC*GTGTCGAACAAG	*Nco*I
R389Crev	CTTGTTCGACAC*GCA*CGCCG**CCATGG**GCACCGCCG	*Nco*I
V390Cfor	CGGTGC**CCATGG**CGGCGCGG*TGC*TCGAACAAGGTG	*Nco*I
V390Crev	CACCTTGTTCGA*GCA*CCGCGCCG**CCATGG**GCACCG	*Nco*I
S391Cfor	CGATGGCGGCGCGCGTG*TGT*AACAAGGTGGG	
S391Crev	CCCACCTTGTT*ACA*CACGCGCGCCGCCATCG	
N392Cfor	GGCGGCGCGGGTCTCG*TGC*AAGGTGGGTCTGG	
N392Crev	CCAGACCCACCTT*GCA*CGAGACCCGCGCCGCC	

[a] Restriction sites in bold and mutations in italics.

In both reactions, pET-GAB-βCL served as the template. The purified mutagenic PCR fragments were used as the template for the subsequent PCR with primers prNBamHI+ and prCStyI−. Resulting PCR products were digested with *Bam*HI and *Sty*I and cloned into pET-GAB-βCL digested with the same restriction enzymes.

Purification of Oxaloacetate Decarboxylase Mutants and Enzyme Assays. Oxaloacetate decarboxylase and mutant derivatives were purified by affinity chromatography of a solubilized membrane extract on a SoftLink monomeric avidin−Sepharose column (Promega). Large-scale purification was performed according to the method from ref *14* but using 20 mM Tris-HCl (pH 8.0) and 50 mM KCl as buffer A and adding 20% glycerol to all buffers used following sedimentation of membrane vesicles. The decarboxylation activity was determined with the simple spectrophotometric assay at 265 nm as described previously (*5*). Activities are presented as a percentage of the activity of the single-Cys mutant before incubation with the MTS reagent and after correction of the inactivation due to the remaining Cys residues on the α- and γ-subunit (Cys-less mutant with the MTS reagent).

Preparation and Labeling of Membrane Vesicles with an Inside-Out Orientation. E. coli C43(DE3) cells synthesizing oxaloacetate decarboxylase variants were washed with buffer A and resuspended in the same buffer containing 1 mM $MgCl_2$ and 10 μg/mL DNase. The suspension was passed once through a French press cell operated at 84 MPa. Removal of debris and collection of the membranes was carried out as described previously (*8*). Membrane vesicles were washed once and resuspended in buffer B [20 mM Tris-HCl (pH 8.0), 50 mM KCl, and 20% glycerol] to a protein concentration of 25 mg/mL. Vesicles were preincubated with or without NaCl for 15 min at 0 °C, and then incubated with

the following MTS reagents for 5 min at 25 °C: MTSEA, MTSES, and MTSET (at final concentrations of 2.5, 10, and 1 mM, respectively). The reaction was stopped by diluting the sample (2 μL) into the spectrophotometric activity assay (*14*).

Labeling of Cells and Preparation of Mixed Membrane Vesicles. An induced culture (800 mL) was divided into several aliquots (89 mL) before harvesting. Cells were resuspended in buffer A (2 mL) and incubated with or without NaCl for 5 min at 0 °C prior to incubation with MTS reagents for 20 min at 25 °C (final concentration as described above). The reaction was quenched with freshly prepared L-cysteine (22 mM) and the mixture washed three times with buffer A. Membranes were prepared as described previously (*8*). Membrane vesicles were washed once and resuspended in buffer B to a final protein concentration of 30 mg/mL. Decarboxylation activity was determined with the simple spectrophotometric assay at 265 nm as described previously (*5*). In this series of measurements, we added 0.05% Triton X-100 to the assay, to measure activities of both inside-out and right-side-out membrane vesicles.

Determination of Oxaloacetate Decarboxylase Activity at Various Na^+ Concentrations and Various pH Values. The decarboxylase activities of wild-type and mutant oxaloacetate decarboxylases were measured at Na^+ concentrations ranging between 0.2 and 15 mM in 100 mM Tris-HCl (pH 7.5) containing 1 mM oxaloacetate. The kinetic data sets were collected for each mutant. Experimental data were fitted to the Hill equation, $V = v_{max}[s^n/(s^n + k)]$, where V represents the initial velocity, v_{max} the maximal velocity, s the Na^+ concentration in the assay, k the Na^+ concentration yielding half-maximal velocity, and n the Hill coefficient describing the dimension of cooperativity. The activities of the Cys-less mutant and the R389C mutant were measured at pH values ranging between 5.5 and 9.0 in a 20 mM MES/MOPS/Tris, 1 mM oxaloacetate, 20 mM NaCl buffer system.

Labeling of the Purified Single-Cys Replacement Mutants. Labeling of purified single-cysteine mutant decarboxylase was carried out using the protocol for inside-out membrane vesicles as described above.

Analytical Procedures. The protein content of samples was determined using the BCA protein assay (Pierce) with bovine serum albumin as the protein standard.

RESULTS

In a recent model, the highly conserved helix VIII of the oxaloacetate decarboxylase β-subunit was proposed to be part of the ion channel (*10*). To probe this hypothesis, we analyzed the accessibility of single-cysteine replacement mutants within the helix VIII domain by three different methanethiosulfonate reagents by measuring their effect on oxaloacetate decarboxylase activity. In the first step, the naturally occurring cysteines at positions 87, 291, and 351 of OadB were replaced with alanine, methionine, and alanine, respectively. The decarboxylase with Cys-less OadB retained 36% of the wild-type activity and was therefore suitable for further mutagenesis. For our purpose, it was not necessary to replace the four naturally occurring cysteines of OadA and the one cysteine of OadG. However, upon modification of (part of) these residues by MTS reagents, the decarboxylase activity was approximately 24% reduced. This inhibition was therefore taken into account when calculating the effect of MTS reagents on the single-Cys replacement mutants of OadB (see below).

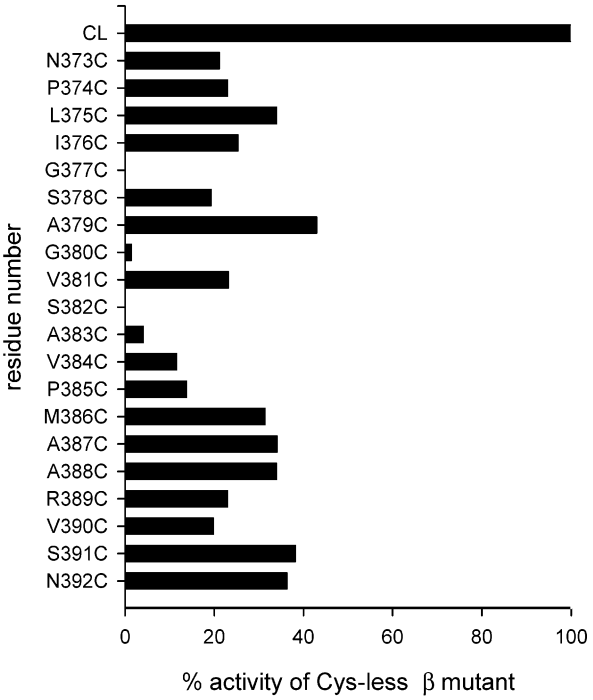

FIGURE 3: Specific oxaloacetate decarboxylase activities of purified single-Cys replacement mutants of OadB recorded as a percentage of activity with Cys-less OadB. This Cys-less mutant exhibits 36% of the activity of wild-type oxaloacetate decarboxylase.

Synthesis, Purification, and Characterization of Single-Cys Replacement Mutants. Each of the 20 amino acid residues predicted to form helix VIII of OadB was individually replaced with cysteine as described in Experimental Procedures. The mutant oxaloacetate decarboxylases were synthesized in *E. coli* C43(DE3) and purified by monomeric avidin−Sepharose affinity chromatography. Approximately the same amount of protein was isolated from each mutant, indicating that similar amounts had been synthesized by the *E. coli* cells.

Figure 3 compiles specific oxaloacetate decarboxylase activities of single-Cys OadB replacement mutants. Of the 20 mutants, 14 exhibited activities between 20 and 63% of that of the enzyme with Cys-less OadB, and for four mutants, this activity dropped below 20%. Upon replacement of the functionally important S382 residue with cysteine, the residual decarboxylase activity was 0.3% of that with Cys-less OadB and the βG377C mutant was completely inactive, consistent with the previous findings (*15*). For all mutants, the Na^+ activation profiles at pH 7.5 were determined to detect possible effects on Na^+ binding properties. The K_{Na^+} of the wild-type enzyme or the Cys-less OadB mutant was 0.32 or 1.4 mM, respectively, and the K_{Na^+} values of most single-Cys replacement mutants were within the same range (0.3−2.5 mM, data not shown). Slightly elevated K_{Na^+} values were found for the βV384C (3.1 mM) and βP385C (6.9 mM) mutants.

Probing the Accessibility of Single-Cys OadB Mutants by Methanethiosulfonate Derivatives. Methanethiosulfonate reagents react with the SH group of cysteine residues by liberating sulfinic acid and forming a mixed disulfide

between the cysteine residue and the reagent. Two positively charged MTS reagents (MTSEA and MTSET) and one negatively charged MTS reagent (MTSES) have been routinely used to probe the accessibility of single-cysteine mutants introduced into helix VIII of OadB. The accessibility approach with negatively or positively charged MTS reagents is widely employed to probe the existence of aqueous channels within proteins (*16*). The rationale for this approach is the assumption that charged reagents can only penetrate into the membrane interior and react with cysteine residues via an aqueous access channel. MTSEA, however, is also membrane-permeable, which has to be taken into account (*17*). Moreover, modification of cysteine residues by positively or negatively charged bulky compounds could affect the structure and/or activity of the enzyme. For practical reasons, we studied the effect of MTS reagents on the oxaloacetate decarboxylase activity. This widely used approach could have certain limitations: if the incubation with the MTS reagents is without effect on the decarboxylase activity, one cannot distinguish whether the cysteines are not accessible for the reagent or whether the modification has no effect on the activity. This ambiguity can be resolved, however, if a different experimental setup causes inhibition by the MTS reagents. Such inhibition eliminates the possibility that the modification has no effect on the activity of the enzyme. We have therefore investigated the effect of MTS reagents on the oxaloacetate decarboxylase activity of single-cytseine mutants in both orientations of the membrane, in detergent micelles and in the presence or absence of 100 mM NaCl. Sodium ions are known to induce a conformational change in the β-subunit which might affect the accessibility of a specific thiol site to the MTS reagent (*15*). As each cysteine mutant was inhibited under specific experimental conditions by the MTS reagent, we conclude that the lack of inhibition under a different set of conditions reflects the inaccessibility of the thiol group to the MTS reagent under these conditions. The results depicted in Figure 4 show the degree of inhibition of oxaloacetate decarboxylase mutants by MTS reagents in bacterial membrane vesicles with an inside-out orientation. The most pronounced inhibition was observed in the C-terminal half of helix VIII. With the positively charged MTSEA or MTSET reagents or with the negatively charged MTSES reagent, the activity was completely eliminated upon addition to single-cysteine replacement mutants between residues βV384 and βA388. These results indicate that this part of OadB can be readily accessed by the three different water-soluble MTS reagents and that substitution of cysteine residues within this region is not compatible with catalytic activity. Significant inhibition of oxaloacetate decarboxylase activity was further observed for cysteine mutants in the region between the essential βG377 and βS382 residues in the case of MTSEA. With MTSES, only the mutant βG380C was strongly inhibited within this region. The presence of Na^+ ions significantly protected the cysteine mutants in this region from inactivation by MTSEA and less severely from inactivation by MTSES and MTSET. An exception is the βV381C mutant which was not protected against modification by MTSET in the presence of Na^+. These results might suggest that in the Na^+-bound state the channel portion between residues βG377 and βS382 is less accessible for the water-soluble MTS reagents. Inhibition of the oxaloacetate decarboxylase activity was also

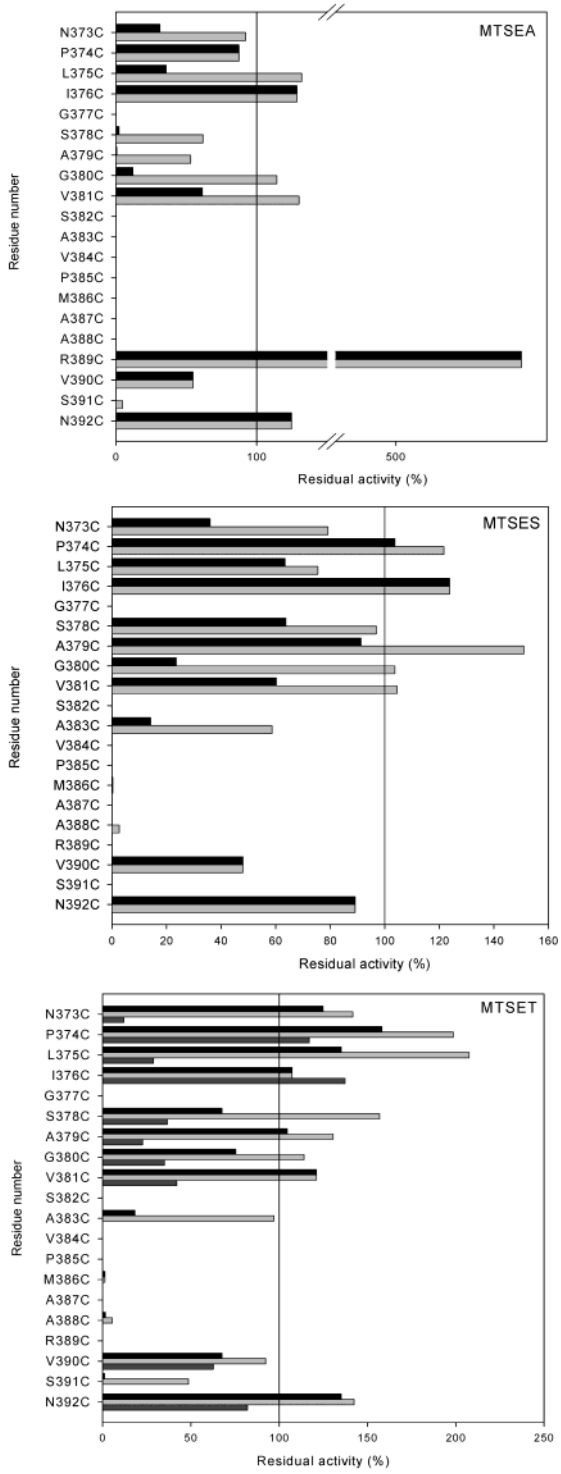

FIGURE 4: Effect of MTS reagents on oxaloacetate decarboxylase activity of inside-out membrane vesicles. Membrane vesicles were prepared from each *E. coli* clone by synthesizing a mutant oxaloacetate decarboxylase and incubated with or without NaCl (100 mM) for 15 min at 0 °C. MTSEA, MTSES, and MTSET (2.5, 10, and 1 mM, respectively) were subsequently added to samples of these vesicles, and the mixtures were incubated 5 min at 25 °C. The reactions were stopped by dilution of the samples (2 μL) into the assay mixtures for the spectrophotometric activity measurements (*5*). The Cys-less β mutant exhibited a specific activity of 1 unit/mg. The top, middle, and bottom panels show the results obtained with MTSEA, MTSES, and MTSET, respectively. The results obtained in the presence of NaCl are presented with light gray bars, and those without NaCl addition are presented with black bars. Residual activities obtained with 3 mM MTSET are presented with dark gray bars.

observed for cysteine mutants at both ends of helix VIII. In the case of MTSEA, mutants βN373C, βL375C, and βV390C were more strongly inhibited, and for the two former mutants, a protective effect of Na^+ ions against modification was observed. In the case of MTSES, mutants βN373C and βV390C were more severely inhibited and the presence of Na^+ ions could not protect mutant βV390C against modification by MTSES. The βS391C mutant was completely inactivated by all three MTS reagents that were tested with strong protection from inactivation by Na^+ ions in the case of the MTSET reagent. Further of interest is mutant βR389C which is completely inactivated by MTSES and MTSET but activated more than 5-fold by MTSEA (see below).

The accessibility of cysteine mutants to MTS reagents from the periplasmic side was also studied. For this purpose, the *E. coli* C43(DE3) cells synthesizing the oxaloacetate decarboxylase mutants were incubated with either MTSEA, MTSES, or MTSET, after which the cells were disrupted in a French press and the membranes were isolated by fractionated centrifugation. The results depicted in Figure 5 show the effect of the MTS reagents on residual oxaloacetate decarboxylase activities of all 20 single-cysteine mutants within helix VIII. In general, the inhibitory effect of the MTS reagents is less pronounced than with inside-out vesicles (see above, Figure 4), especially in the C-terminal portion of the helix. This observation is most prominent with the bulky reagent MTSET. Like with inside-out vesicles, the decarboxylase activity of the cysteine mutants was protected to various degrees from inactivation by MTS reagents in the presence of Na^+ ions. This protective effect of Na^+ ions was found for all three MTS reagents and for individual cysteine mutants positioned over the entire length of the helix. In contrast to the investigations with inside-out vesicles, we observed for several mutants a more severe inhibition in the presence of Na^+ than in the absence of the cation. The mutants βV384C, βP385C, βA387C, and βA388C showing this effect with MTSEA and MTSET are residing on one face of the helix in the C-terminal part as shown by a helical wheel presentation (Figure 9). Also, a stronger inhibition in the presence of Na^+ was observed for mutant βS391C in the case of MTSEA and for mutant βG380C in the case of MTSET. Hence, once Na^+ binds, a conformational change may be induced by which these residues become more readily accessible to these MTS reagents.

The results depicted in Figure 6 show inhibition of the purified detergent-solubilized oxaloacetate decarboxylase mutants by the three different MTS reagents used above to probe the accessibility of cysteine residues in the membrane-bound enzyme specimens. The overall inhibition pattern is quite similar to the results obtained with inside-out membrane vesicles showing the most significant inhibition between residues βV384 and βA388 in the C-terminal part of the helix. Most mutants were in the N-terminal portion of the helix, also strongly inhibited by MTSEA and MTSES, whereas MTSET was less effective. In this region of the protein, the first two compounds more severely inhibited the decarboxylase mutants in the detergent than in inverted vesicles. This finding is easily rationalized by a single access route for the inhibitors from the cytoplasm in inverted membrane vesicles and access routes from both sides in the detergent-solubilized enzyme specimens. Like with inverted

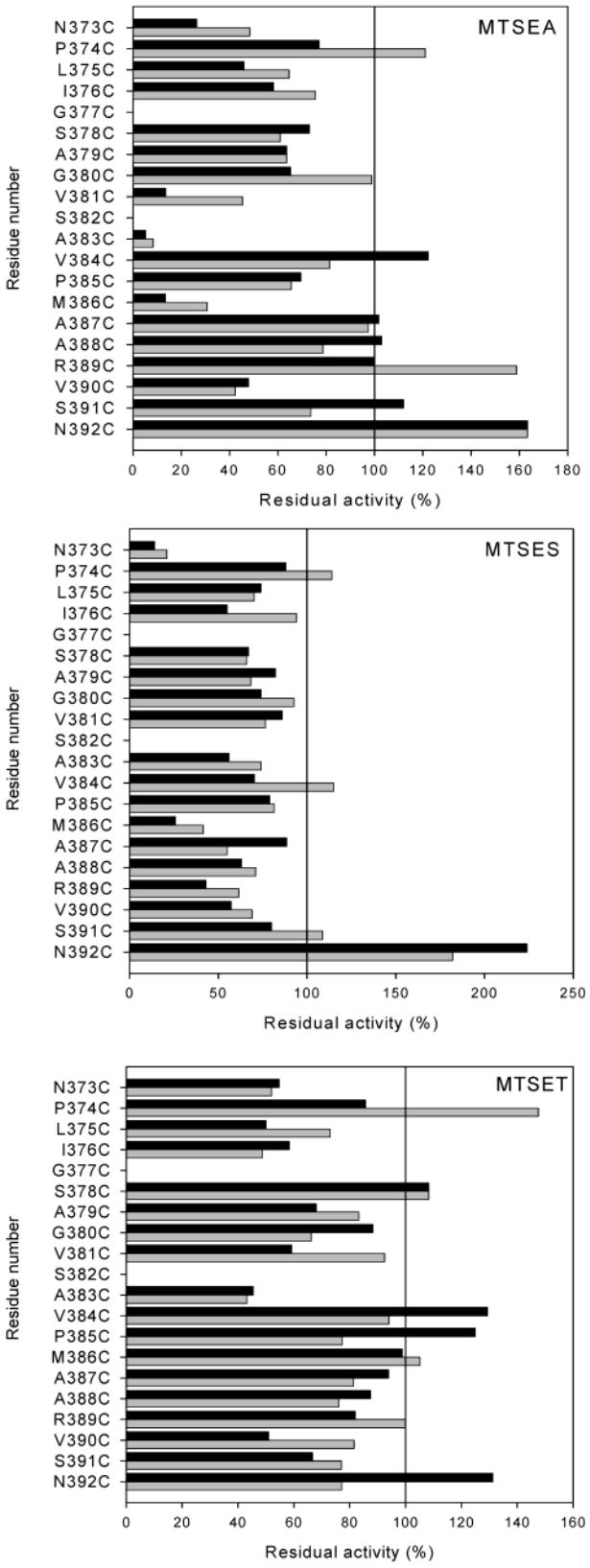

FIGURE 5: Oxaloacetate decarboxylase activity after incubation of cells with MTS reagents. Cells from *E. coli* clones synthesizing mutant oxaloacetate decarboxylase were incubated with (light gray bars) or without 100 mM NaCl (black bars) for 5 min at 0 °C prior to incubation with MTS reagents for 20 min at 25 °C. The reaction was quenched with freshly prepared L-cysteine. Membrane vesicles were prepared, and the oxaloacetate decarboxylase activitiy was determined in the presence of 0.05% Triton X-100. The Cys-less β mutant exhibited a specific activity of 2 units/mg. Details are described in the legend of Figure 4 and in Experimental Procedures.

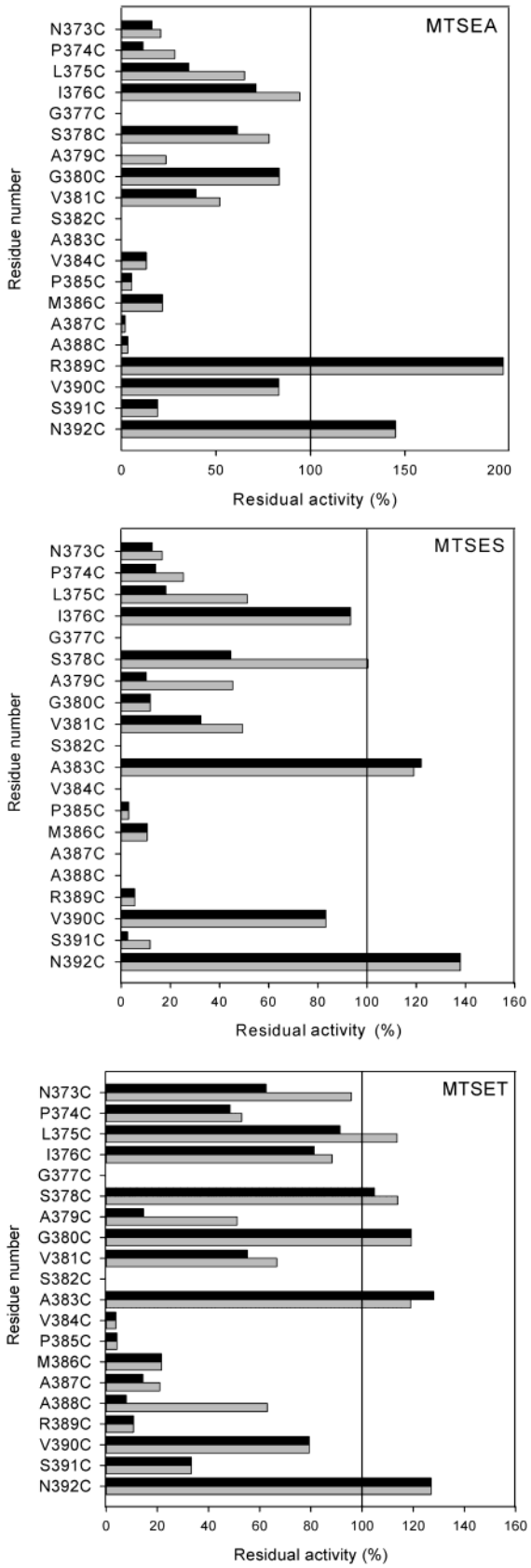

FIGURE 6: Effects of MTS reagents on detergent-solubilized purified oxaloacetate decarboxylase with single-Cys replacement mutants. The purified oxaloacetate decarboxylase was incubated with (light gray bars) or without 100 mM NaCl (black bars) for 15 min at 0 °C and then for 5 min with the indicated MTS reagents. The Cys-less β mutant exhibited a specific activity of 14 units/mg. Further experimental details are described in the legend of Figure 4 and in Experimental Procedures.

vesicles and unlike in intact cells, the protective effect of Na^+ was found mainly in the N-terminal portion in experiments with detergent-solubilized oxaloacetate decarboxylase mutants.

Functional Characterization of the βR389C Mutant. The positively charged βR389 residue near the cytoplasmic surface has been shown to have a functional role in the ion translocation mechanism. Upon substitution of βR389 with cysteine in the Cys-less OadB background, 75% of the oxaloacetate decarboxylase activity was lost. This βR389C mutant was completely or severely inhibited by addition of MTSES or MTSET to inverted vesicles or the detergent-solubilized enzyme, respectively. These compounds were much less inhibitory with the bacterial cell system which indicates that residue βR389 is reached more readily from the cytoplasmic than from the periplasmic surface. The most remarkable results were obtained with MTSEA which significantly increased the oxaloacetate decarboxylase activity of the βR389C mutant. The activation was more than 5-fold with inside-out vesicles and approximately 2-fold with the detergent-solubilized enzyme, irrespective of whether Na^+ ions were present. With the bacterial cells, the activity increased 1.6-fold in the presence of Na^+ but was not affected in the absence of this alkali ion. These results indicate ready modification of the cysteine at position 389 from the cytoplasmic surface independent of the binding of Na^+ to the enzyme. On the other hand, access of this site for MTSEA from the periplasmic surface appears to be Na^+-dependent. This result is consistent with a Na^+-induced conformational change of OadB that affects the channel aligned by helix VIII. The activation of the βR389C mutant after modification with MTSEA further emphasizes the importance of the positively charged side chain at position 389. Upon removal of this charge by mutation of βR389 to cysteine, the decarboxylase activity was recovered by modifying the SH group of this cysteine with the positive charge introduced with MTSEA. Figure 7 shows the pH profile for oxaloacetate decarboxylase with Cys-less OadB and the βR389C mutant with and without modification with MTSEA. The Cys-less mutant has a pH optimum between 6 and 7 comparable to that of the wild-type enzyme. In the βR389C mutant, the activity drops 78% in the neutral range and the pH optimum shifts by approximately 2 pH units to the alkaline range, findings similar to those with the βR389A mutant (*15*). Part of the activity could be restored by modifying the cysteine at position 389 with the positively charged MTSEA, and this modification shifts the pH optimum back into the neutral range.

DISCUSSION

Oxaloacetate decarboxylase is a perfectly coupled vectorial catalyst which performs Na^+ ion translocation across the membrane only in linkage to the decarboxylation of carboxybiotin (*10*). The rationale for this strict coupling between the chemical and vectorial reaction is the consumption of a periplasmically derived proton in the chemical event and the dependence of this proton transport on the countertransport of Na^+ in the vectorial event. Hence, any effect on Na^+ (or H^+) transport across the membrane-intrinsic β-subunit will automatically affect the oxaloacetate decarboxylase activity. This property of the enzyme has previously been used to identify amino acid residues of the β-subunit with putative

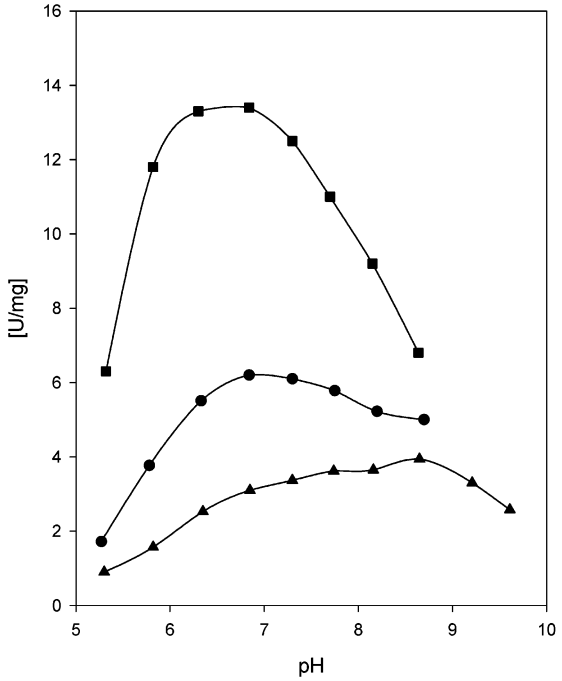

FIGURE 7: Dependence of oxaloacetate decarboxylase activity of mutants with or without modification with MTSEA on pH: (■) Cys-less mutant (βR389) preincubated with MTSEA, (▲) mutant βR389C, and (●) mutant βR389C, preincubated with MTSEA. The purified mutants were incubated with 2.5 mM MTSEA for 5 min at 25 °C. Further experimental details are described in Experimental Procedures.

roles in Na^+ (or H^+) binding or translocation and is now taken to indicate critical sites within the highly conserved helix VIII domain of this protein by cysteine scanning mutagenesis and subsequent modification studies of the single-cysteine mutants with water-soluble MTS reagents. On the basis of numerous mutagenesis studies and other biochemical data, a model for translocation of Na^+ and H^+ through the β-subunit was proposed which involves two Na^+ binding sites in the interior portion of the membrane (*10*). Na^+ binding center I is located in the periplasmic portion of the protein and involves functionally indispensable residue βD203 (on segement IIIa, Figure 2) and βN373 (on helix VIII). Na^+ binding center II is located near the center of the membrane and includes functionally essential amino acids βY229 on helix IV and βS382 on helix VIII. Another important residue is βR389, which has been shown to play a role in decreasing the p*K* of the phenolic hydroxyl group of βY229 so that upon Na^+ binding to center II the phenolic proton dissociates and catalyzes the H^+-dependent decarboxylation of the carboxybiotin. This central step in the chemical catalysis is succeeded by a conformational change by which the access of Na^+ to the binding sites switches to the periplasmic surface. Releasing the Na^+ ions to this side and restoring the βY229 hydroxyl with a periplasmically derived proton completes the vectorial events of the catalytic cycle. Helix VIII which is involved in both Na^+ binding centers and has the highest number of conserved residues which cannot be replaced without loss of function has been proposed in the model to be part of the ion channel for translocation of Na^+ or H^+ across the membrane. This hypothesis has now been probed by cysteine scanning mutagenesis and the accessibility of each mutant cysteine residue by charged thiol reactive methanethiosulfonate

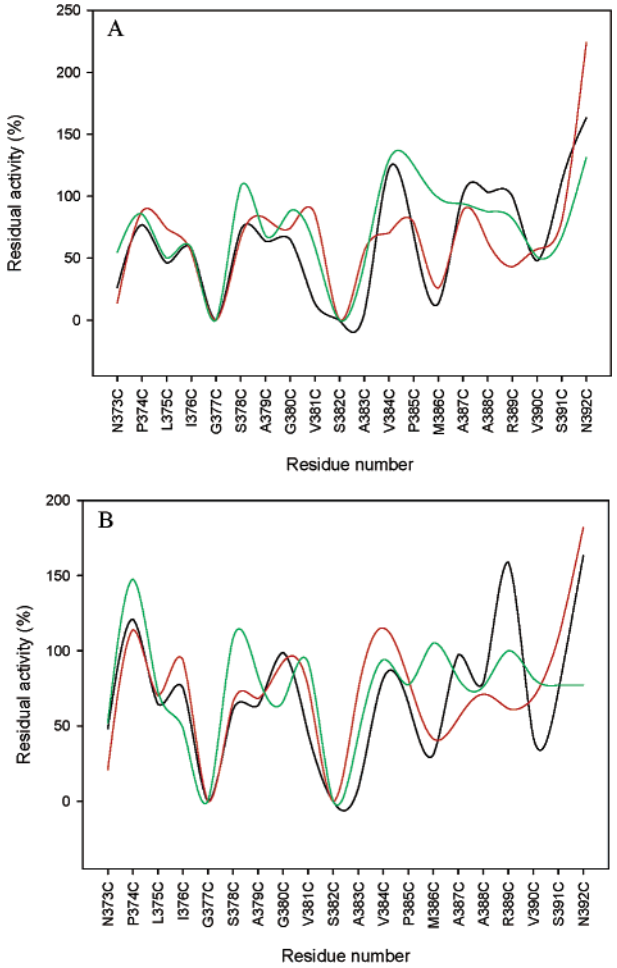

FIGURE 8: Periodicity of the accessibility pattern with MTSEA (black line), MTSES (red line), and MTSET (green line) over the entire helix VIII in right-side-out membrane vesicles (A) without NaCl addition and (B) after preincubation with 100 mM NaCl for 5 min at 0 °C. Residues G377 and S382 are inactive without modification.

reagents. From the general appearance of the inhibition patterns, the N-terminal and C-terminal half of helix VIII are clearly distinct, and it is also evident that more complete inhibition is found for inside-out vesicles especially with mutants where the cysteine is located in the C-terminal portion of the helix. Here a stretch of cysteine mutants spanning five consecutive residues became completely inactivated by any of the three charged MTS reagents that were tested. These results indicate that the C-terminal region of helix VIII is readily accessible for these probes from the cytoplasmic side of the membrane and is much less accessible from the periplasmic side. The C-terminal portion of helix VIII may thus align an aqueous channel into which ions can easily penetrate from the cytoplasmic surface while the access from the periplasmic surface is restricted. Generally, cysteine mutants in the N-terminal part of helix VIII are also inhibited but to a minor degree compared to those in the C-terminal part, which might indicate that this region of the protein is less accessible to the MTS reagents, or that the modifications affect the decarboxylase activity to a lesser extent. The second option seems less likely, however, since mutants βS378C, βA379C, and βG380C became almost completely inactivated in inverted membrane vesicles with MTSEA. Modification of these residues from the *trans* side

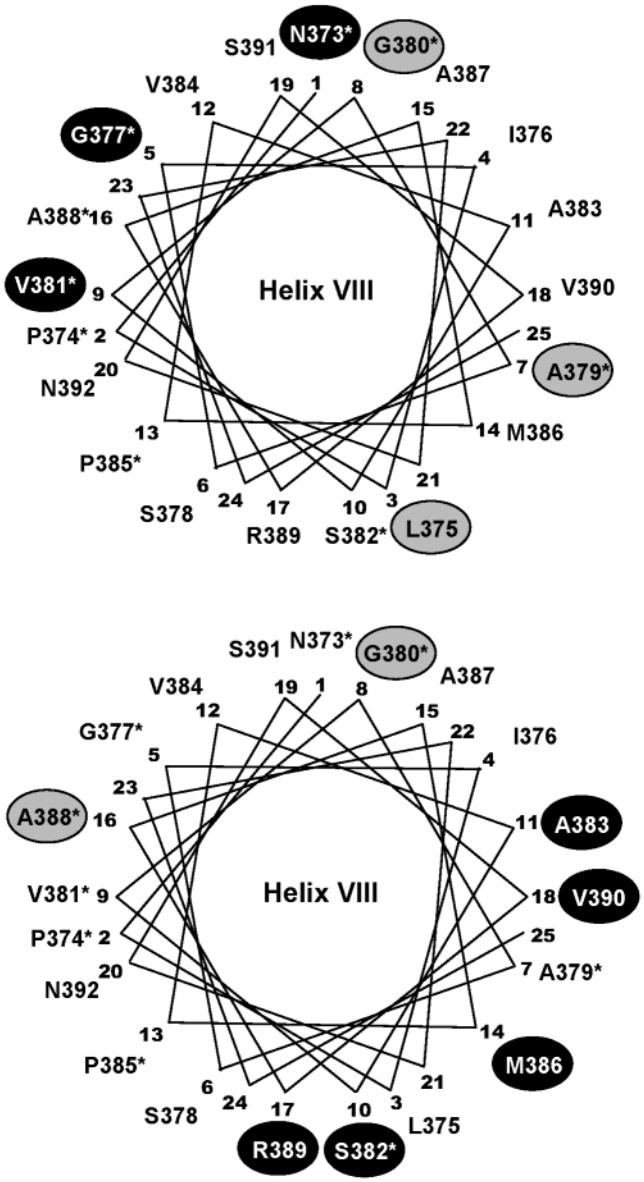

FIGURE 9: Helical wheel analysis of helix VIII as viewed from the periplasmic side. The top panel shows the situation of the upper half-channel, which is accessible from the periplasmic side. The helical wheel in the bottom panel shows the situation of the half-channel, which is opened toward the cytoplasmic side. Positions sensitive to hydrophilic MTS reagents are highlighted against a black background. Positions which are thought to be involved in hydrophobic contacts between helices are highlighted against a gray background. Conserved residues are denoted with asterisks. Small numbers mark the positions in the helix.

cannot be ruled out, because MTSEA is somewhat membrane-permeable (*17*). Furthermore, the presence of Na^+ strongly protected these mutant enzymes from inactivation. Taken together, these results indicate that the probes penetrate easily from the cytoplasmic surface into the C-terminal channel aligned by helix VIII, but penetration into the N-terminal part from either side is more restricted. Subtle differences in the inhibition of oxaloacetate decarboxylase mutants are particularly apparent in experiments with the right-side-out membrane system where the inhibition pattern may reflect predominantly the degree of accessibility of the cysteine mutants by the MTS reagents. The transmodification by extracellular MTSEA is in this case probably restricted, because soluble thiol compounds (e.g., glutathione) inside the cell are scavenging MTSEA. As shown in Figure 8, there is a clear periodicity of the inhibition pattern over the entire helix VIII. From a helical wheel presentation of this pattern (Figure 9), it becomes evident that mutant residues yielding the most severe inhibition are located on one face of the helix in the N-terminal portion and on the other face of the helix in the C-terminal portion. As cysteine residues facing the channel are probably more easily modified than those facing the hydrophobic part of the membrane, we propose that residues βN373, βG377, and βV381 align the channel in the N-terminal portion of helix VIII and that residues βS382, βA383, βM386, βR389, and βV390 align the channel in the C-terminal portion of the helix. A special case is mutant βR389C which was strongly (re)activated after exposure to MTSEA, but completely inactivated in the presence of the other MTS reagents (Figures 4−6). MTSEA is likely mimicking the guanidine group of the arginine residue and furthermore has a volume almost identical to that of arginine (125 $Å^3$) (*18*). Modification by MTSET or MTSPA (data not shown) does not restore the activity, and therefore, it appears that both the volume and charge are critical factors at this position. A potential connection of the two proposed half-channels defined by helix VIII is located in the region of residues βV381, βS382, and βA383, because only in this region is there strong inhibition, especially by the smallest MTSEA of mutant βV381C oriented to the left side of helix VIII as well as of mutant βA383C oriented to the right side of the helix. Please note that the βS382C mutant is inactive without modification. According to previous results, βS382 is part of Na^+ binding center II and therefore almost certainly exposed to the aqueous channel. Hence, the channel may turn around helix VIII in the region of residues βV381−βA383. This switch in the orientation of the channel may be necessary because each of the two Na^+ binding centers includes a residue on a different domain of the β-subunit and a residue in helix VIII. To form this interdomain site, i.e., center I between βN373 on helix VIII and βD203 on segment IIIa and center II between βS382 on helix VIII and βY229 on helix IV, segment IIIa and helix IV may have to face helix VIII from two opposing sides.

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