Conformationally Sensitive Residues in Transmembrane Domain 9 of the Na\(^{+}\)/dicarboxylate Co-transporter*

Ana M. Pajor‡

From the Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, Texas 77555

The Na\(^{+}\)/dicarboxylate co-transporter, NaDC-1, couples the transport of sodium and Krebs cycle intermediates, such as succinate and citrate. Previous studies identified two functionally important amino acids, Glu-475 and Cys-476, located in transmembrane domain (TMD) 9 of NaDC-1. In the present study, each amino acid in TMD-9 was mutated to cysteine, one at a time, and the accessibility of the membrane-impermeant reagent [2-(trimethylammonium)ethyl]methanethiosulfonate (MTSET) to the replacement cysteines was determined. Cysteine substitution was tolerated at all but five of the sites: the A461C mutant was not present at the plasma membrane, whereas the F473C, T474C, E475C, and N479C mutants were inactive proteins located on the plasma membrane. Cysteine substitution of four residues found near the extracellular surface of TMD-9 (Ser-478, Ala-480, Ala-481, and Thr-482) resulted in proteins that were sensitive to inhibition by MTSET. The accessibility of MTSET to the four substituted cysteines was highest in the presence of the transported cations, sodium or lithium, and low in choline. The four mutants also exhibited substrate protection of MTSET accessibility. The MTSET accessibility to S478C, A481C, and A480C was independent of voltage. In contrast, T482C was more accessible to MTSET in choline buffer at negative holding potentials, but there was no effect of voltage in sodium buffer. In conclusion, TMD-9 may be involved in transducing conformational changes between the cation-binding sites and the substrate-binding site in NaDC-1, and it may also form part of the translocation pathway through the transporter.

The mammalian Na\(^{+}\)/dicarboxylate co-transporter, NaDC-1, is found on the apical membrane of the renal proximal tubule and small intestine (1, 2). NaDC-1 has a broad substrate selectivity for a wide range of di- and tricarboxylates, including succinate and citrate. Previous studies identified two functionally important amino acids, Glu-475 and Cys-476, located in transmembrane domain (TMD) 9 of NaDC-1. In the present study, each amino acid in TMD-9 was mutated to cysteine, one at a time, and the accessibility of the membrane-impermeant reagent [2-(trimethylammonium)ethyl]methanethiosulfonate (MTSET) to the replacement cysteines was determined. Cysteine substitution was tolerated at all but five of the sites: the A461C mutant was not present at the plasma membrane, whereas the F473C, T474C, E475C, and N479C mutants were inactive proteins located on the plasma membrane. Cysteine substitution of four residues found near the extracellular surface of TMD-9 (Ser-478, Ala-480, Ala-481, and Thr-482) resulted in proteins that were sensitive to inhibition by MTSET. The accessibility of MTSET to the four substituted cysteines was highest in the presence of the transported cations, sodium or lithium, and low in choline. The four mutants also exhibited substrate protection of MTSET accessibility. The MTSET accessibility to S478C, A481C, and A480C was independent of voltage. In contrast, T482C was more accessible to MTSET in choline buffer at negative holding potentials, but there was no effect of voltage in sodium buffer. In conclusion, TMD-9 may be involved in transducing conformational changes between the cation-binding sites and the substrate-binding site in NaDC-1, and it may also form part of the translocation pathway through the transporter.

The mammalian Na\(^{+}\)/dicarboxylate co-transporter, NaDC-1, is found on the apical membrane of the renal proximal tubule and small intestine (1, 2). NaDC-1 has a broad substrate selectivity for a wide range of di- and tricarboxylates, including succinate, citrate, and \(\alpha\)-ketoglutarate. The transport cycle involves the ordered binding of three sodium ions followed by a divalent anion substrate, with the net movement of one positive charge across the membrane (3, 4). Lithium can substitute for sodium in NaDC-1, but the substrate affinity in lithium is lower than in sodium. The four mutants also exhibited substrate protection of MTSET accessibility. The MTSET accessibility to S478C, A481C, and A480C was independent of voltage. In contrast, T482C was more accessible to MTSET in choline buffer at negative holding potentials, but there was no effect of voltage in sodium buffer. In conclusion, TMD-9 may be involved in transducing conformational changes between the cation-binding sites and the substrate-binding site in NaDC-1, and it may also form part of the translocation pathway through the transporter.
EXPERIMENTAL PROCEDURES

Oligonucleotide-directed Mutagenesis—Site-directed mutagenesis was done using the method of Kunkel (13) with reagents from the Muta-gene kit from Bio-Rad, according to the manufacturers instructions. The template for mutagenesis was 3C, a mutant of the rabbit NaDC-1 (GenBank™ U12186) which contained only 3 out of 11 native cysteines (10). Single-stranded DNA was rescued using helper phage RF499 and precipitated using polyethylene glycol. Threoleitidines used for mutagenesis contained mutations for changing the codon to a cysteine codon as well as silent mutations to introduce or remove a restriction site. The restriction sites were used to identify positive mutants, which were then verified by sequencing.

Western Blots of Cell-surface Biotinylated Proteins—Cell surface biotinylation and Western blotting were used to determine relative expression of the mutants, as described previously (15). The oocytes were labeled with the membrane impermeant biotin reagent, Sulfo-NHS-LC-Biotin (Pierce), and the biotinylated proteins were precipitated using ImmunoPure-immobilized streptavidin beads (Pierce). The proteins were blotted onto nitrocellulose membranes and probed with an anti-NaDC-1 antibody (15) applied at 1,500 dilution for 2 h followed by incubation with horseradish peroxidase-linked anti-rabbit Ig (Amerham Pharmacia Biotech) at a 1,500,000 dilution for 1 h. Antibody binding to NaDC-1 was detected with the Supersignal CL-HPR substrate system (Pierce). In previous studies using the same batch of oocytes under identical conditions, the H106A mutant of NaDC-1 was not detected at the plasma membrane but it was found in microsomal membranes.

Oligonucleotide-directed Mutagenesis—Site-directed mutagenesis was done using the method of Kunkel (13) with reagents from the Muta-gene kit from Bio-Rad, according to the manufacturers instructions. The template for mutagenesis was 3C, a mutant of the rabbit NaDC-1 (GenBank™ U12186) which contained only 3 out of 11 native cysteines (10). Single-stranded DNA was rescued using helper phage RF499 and precipitated using polyethylene glycol. Threoleitidines used for mutagenesis contained mutations for changing the codon to a cysteine codon as well as silent mutations to introduce or remove a restriction site. The restriction sites were used to identify positive mutants, which were then verified by sequencing.

Western Blots of Cell-surface Biotinylated Proteins—Cell surface biotinylation and Western blotting were used to determine relative expression of the mutants, as described previously (15). The oocytes were labeled with the membrane impermeant biotin reagent, Sulfo-NHS-LC-Biotin (Pierce), and the biotinylated proteins were precipitated using ImmunoPure-immobilized streptavidin beads (Pierce). The proteins were blotted onto nitrocellulose membranes and probed with an anti-NaDC-1 antibody (15) applied at 1,500 dilution for 2 h followed by incubation with horseradish peroxidase-linked anti-rabbit Ig (Amerham Pharmacia Biotech) at a 1,500,000 dilution for 1 h. Antibody binding to NaDC-1 was detected with the Supersignal CL-HPR substrate system (Pierce). In previous studies using the same batch of oocytes under identical conditions, the H106A mutant of NaDC-1 was not detected at the plasma membrane but it was found in microsomal membranes.

Transport Experiments—Uptake of [3H]succinate (PerkinElmer Life Sciences) was measured in groups of 5 oocytes between 4 and 6 days after injection. Culture dishes and medium were changed daily.

Scrambled Sequences—Female Xenopus laevis were obtained from Nasco or Xenopus I. Stage V and VI oocytes were dissected and collagenase treated K409 and precipitated using polyethylene glycol. The oligonucleotides used for mutagenesis contained mutations for changing the codon to a cysteine codon as well as silent mutations to introduce or remove a restriction site. The restriction sites were used to identify positive mutants, which were then verified by sequencing.

Xenopus Oocytes—Female Xenopus laevis were obtained from Nasco or Xenopus I. Stage V and VI oocytes were dissected and collagenase treated K409 and precipitated using polyethylene glycol. The oligonucleotides used for mutagenesis contained mutations for changing the codon to a cysteine codon as well as silent mutations to introduce or remove a restriction site. The restriction sites were used to identify positive mutants, which were then verified by sequencing.

RESULTS

Cysteine Scan of Transmembrane Domain 9—The sequence alignment of amino acids 461–482 in the rabbit NaDC-1 with the other members of the SLC13 family is shown in Fig. 1. This region is predicted to form TMD-9. The amino acids in TMD-9 are highly conserved, particularly at the extracellular half of the helix between amino acids 473 and 482. For this study, each amino acid in TMD-9 of the rabbit NaDC-1 was changed to cysteine. The parental transporter for the cysteine mutants was a mutant of NaDC-1 called 3C, which contained only 3 out of the 11 native cysteines (Cys-38, Cys-50, and Cys-65) (10). Although the wild-type NaDC-1 is not sensitive to inhibition by MTSET, the 3C mutant was used for the initial screen of the mutants to ensure that possible changes in the shape of the protein as a result of mutagenesis would not expose the endogenous cysteine. The 3C mutant contained the minimal number of cysteines which would allow measurable transport activity in Xenopus oocytes. Our previous study had shown a correlation between the number of cysteines in NaDC-1 and the amount of protein expressed at the plasma membrane (10).

Transport Activity of Cysteine Mutants—The cysteine mutants were expressed in Xenopus oocytes and the succinate transport activity was compared with the parental 3C mutant expressed in the same batch of oocytes. As shown in Fig. 2, the substitution of cysteines for many of the amino acids in TMD-9 had little effect on transport activity. Two of the mutants, S478C and A480C, had transport activities that were reduced by ~75% relative to the parental 3C transporter activity. Five of the mutants, A461C, P473C, T474C, E475C, and N478C, exhibited no transport activity.

Cell-surface Expression of the Cysteine Mutants—The cell-surface distribution of the mutants was tested by biotinylation of the oocytes with a membrane impermeant biotin analog, Sulfo-NHS-LC-biotin, followed by Western blotting. Most of the mutants were found on the plasma membrane (Fig. 3). One of the inactive mutants, A461C, was found to be absent from the plasma membrane, suggesting that this residue is either involved in the gating of the protein or that the mutation at the beginning of the helix alters the folding of the protein.

MTSET Sensitivity of Cysteine Mutants—The sensitivity of
the cysteine mutants to the membrane-impermeant reagent MTSET was tested using a high concentration, 2.5 mM, in order to identify all reactive cysteines. Most of the mutants and the parental 3C transporter were insensitive to inhibition by MTSET (results not shown). However, cysteine replacement of S478C, A480C, A481C, and T482C, four residues found near the extracellular surface of helix 9, resulted in inhibition of transport activity in the parental NaDC-1 mutant, 3C, which contained only 3 cysteines. The activity of 3C was 242 ± 41 pmol/oocyte h (n = 19 frogs). The data shown are the mean ± S.E. of two to five experiments.

The time course of inactivation of transport by 1 and 10 μM MTSET is shown in Fig. 4. The T482Cwt mutant was less sensitive to inhibition than the other mutants, and therefore 10 μM was used for experiments with T482Cwt. All four mutants reacted very quickly to the reagent and more than 50% inhibition was seen after 1 min of preincubation.

Effects of Cations and Substrates on the Accessibility of MTSET to Substituted Cysteines—The inhibition of succinate transport activity in S478Cwt, A480Cwt, A481Cwt, and T482Cwt by MTSET was dependent on the buffer used, suggesting that the accessibility of these cysteines depends on the conformation of the transporter. Approximately 15–40% of the control activity remained (i.e. 60–85% inhibition) after preincubation of the S478Cwt, A480Cwt, and A481Cwt mutants with 1 μM MTSET and the T482Cwt mutant with 10 μM MTSET in sodium buffer (Fig. 5). In contrast, the inhibition was much lower after preincubation with MTSET in choline, a non-transported cation (5). Lithium is a transported cation in NaDC-1 but the substrate-dependent currents in lithium are much smaller than in sodium (17, 5). The K_s for succinate in lithium in NaDC-1 is ~3 mM, 10-fold greater than in sodium (5, 17). However, preincubation with MTSET in lithium buffer resulted in a similar inhibition of transport as preincubation with MTSET in sodium (Fig. 5).

All four cysteine-substituted mutants showed substrate pro-
tested. The S478Cwt, A480Cwt, A481Cwt, and T482Cwt mutants were
mutant (Fig. 5).

since lithium does not produce the optimal configuration change for substrate binding (5), the presence of succinate in lithium also reduced the inhibition by MTSET in the T482C mutant (Fig. 5D).

Properties of the Cysteine Mutants — The kinetic properties of the S478Cwt, A480Cwt, A481Cwt, and T482Cwt mutants were tested. The K_m for succinate in all four mutants was similar to the wild-type NaDC-1 and the parental C476S transporter, between 160 and 420 μM but the V_max in the four mutants was lower than in the C476S parental transporter (Table I). The cell surface expression of S478Cwt, A480Cwt, A481Cwt, and T482Cwt was similar to that of the parental C476S transporter (Fig. 3).

The sodium kinetics of the four MTSET-sensitive mutants were determined by measuring the activation of succinate transport with increasing concentrations of sodium. The sodium activation curves in the S478Cwt and A480Cwt mutants were very similar to those of the C476S parental and the NaDC-1 wild type (18) with K_m values between 28 and 42 mM and Hill coefficients greater than 1 (Fig. 6, A and B). In contrast, the A481Cwt and T482Cwt mutants had altered sodium activation curves (Fig. 6C). The curves were sigmoidal in shape and the apparent sodium affinity was decreased in these mutants, but it was not possible to obtain accurate estimates of the kinetic constants because the curves did not show saturation.

Effect of MTSET on Substrate-dependent Currents — The coupled transport of three sodium ions and a divalent anion substrate molecule in NaDC-1 produces an inward current (5). The S478Cwt, A480Cwt, A481Cwt, and T482Cwt mutants had substrate-dependent currents between −30 and −300 nA at −50 mV (not shown), with IV curves that were similar in shape to those of the parental NaDC-1. The substrate-dependent currents produced by the four mutants were sensitive to inhibition by MTSET under voltage clamp conditions, whereas the parental transporter, C476S, was insensitive to MTSET (not shown). Since the substrate-dependent currents were measured using 10 mM succinate, which estimates the V_max (5), the results indicate that the inhibition of transport by MTSET occurs, at least in part, by a decrease in V_max. The inhibition of substrate-dependent currents by MTSET could be reversed by incubation for 10 min in 10 mM dithiothreitol (not shown). Several oocytes expressing the inactive mutant, N479C, were tested by two-electrode voltage clamp but no substrate-dependent currents or leak currents were evident (not shown).

Effect of Holding Potential on Accessibility of Substituted Cysteines — Oocytes expressing the S478Cwt, A480Cwt, A481Cwt, and T482Cwt mutants were held at different voltages during the superfusion with MTSET to determine whether the holding potential affects the accessibility of these substituted cysteines. Time and concentration combinations of MTSET in sodium buffer were used that would produce −50–60% inhibition of substrate-dependent currents. The substrate-dependent currents in S478Cwt, A480Cwt, and A481Cwt were inhibited more than 50% by a 15-s exposure to 1 μM MTSET in sodium buffer, whereas the T482C mutant required 30 s exposure to 5 μM MTSET in sodium buffer to produce a similar inhibition (Fig. 7). In all four mutants, the accessibility of the MTSET to the substituted cysteine was much lower in choline buffer compared with sodium. There was no significant effect of holding potential on the accessibility of the substituted cysteine to MTSET when the experiment was done in the presence of sodium (Fig. 7). Although the S478Cwt mutant appears less sensitive to MTSET in sodium at more negative potentials (Fig. 7A), the difference is not statistically significant (−150 mV versus 0 mV, p < 0.058). Similarly, in three of the mutants (S478Cwt, A480Cwt, and A481Cwt), there was no significant effect of holding potential on the amount of inhibition produced by MTSET in choline buffer. However, the T482Cwt mutant exhibited voltage-dependent MTSET accessibility in choline...
buffer. There was greater inhibition of T482Cwt by MTSET at more negative holding potentials, but relatively little inhibition at 0 mV (Fig. 7D).

**DISCUSSION**

The key finding of this study is that four amino acids (Ser-478, Ala-480, Ala-481, and Thr-482) found in TMD-9 near the extracellular surface of NaDC-1 are alternately accessible and inaccessible from the outside of the cell during the transport cycle. Replacement of any of the four amino acids by cysteine produces a transporter that is sensitive to inhibition by the membrane-impermeant reagent, MTSET. Interestingly, the accessibility of these sites to MTSET parallels the exposure of the substrate-binding site in NaDC-1. The accessibility of these residues is highest in the presence of sodium, and lower in the presence of substrate. The results suggest that TMD-9 participates in the conformational changes seen during transport and it likely forms part of the translocation pathway.

A total of eight residues near the extracellular surface of TMD-9 in NaDC-1 appear to be functionally important (Fig. 8A). Cysteine mutations of the four outermost residues (Ser-478, Ala-480, Ala-481, and Thr-482), produce transporters that are sensitive to MTS reagents and cysteine mutations of four additional residues (Phe-473, Thr-474, Glu-475, and Asn-479) produce inactive transporters that are found on the plasma membrane. When viewed in a helical wheel projection, six of the functionally important amino acids (Asn-479, Glu-475, Thr-482, Ser-478, Thr-474, and Ala-481), are located on one face of the helix (Fig. 8B). The opposite face of the helix contains Ala-480 and Phe-473, located close to the endogenous cysteine at position 476 that mediates inhibition by pCMBS (10). Interestingly, Cys-476 is sensitive to inhibition by pCMBS but not MTSET. The four residues that were sensitive to inhibition with MTSET (Ser-478, Ala-480, Ala-481, and Thr-482), do not appear to be critical for function because substitutions are tolerated at those sites. However, the A481C and T482C mutants had lower sodium affinity, which further supports previous suggestions that TMD-9 is involved in cation binding or translocation (9). Ser-478, Ala-481, and Thr-482 are absolutely conserved residues (Fig. 1) whereas Ala-480 is only found in the rabbit NaDC-1.

Our previous study showed that the conserved residue, Glu-475, in TMD-9 is critical for determining the affinity of NaDC-1 for both cations and substrate, and is also involved in determining cation selectivity (9). NaDC-1 mutants with alanine or

**TABLE I**

**Succinate kinetics in cysteine substitution mutants**

| Mutant  | $K_m$ ($\mu$M) | $V_{max}$ (pmol/oocyte h) |
|---------|---------------|--------------------------|
| C476S   | 238 ± 57      | 2901 ± 1980              |
| S478Cwt | 314 ± 74      | 363 ± 21                 |
| A480Cwt | 424 ± 109     | 517 ± 55                 |
| A481Cwt | 192 ± 36      | 1014 ± 387               |
| T482Cwt | 168 ± 80      | 1892 ± 189               |

FIG. 5. **Effect of preincubation conditions on inhibition by MTSET.** Oocytes expressing the mutants were preincubated for 1 min in 1 $\mu$M MTSET/S478Cwt, A480Cwt, and A481Cwt or 10 $\mu$M MTSET (T482Cwt), after which 15-min uptakes of 100 $\mu$M [3H]succinate were measured. The preincubation buffers consisted of sodium, choline, or lithium transport buffer with or without 5 mM succinate. The data are expressed as a percentage of the uptakes measured in the same mutant incubated without MTSET. Each bar represents the mean ± S.E. of two to four experiments.
glutamine in place of Glu-475 exhibit altered cation and substrate affinities, but replacement by aspartate (9) or cysteine (this study) results in inactive transporters that are present on the plasma membrane. Although the T474C mutant was inactive, replacement of Thr-474 with serine or asparagine results in functional transporters.2 However, Asn-479 appears to be one of the few irreplaceable residues in NaDC-1. In addition to the cysteine replacement made in this study, substitutions of Asn-479 by alanine, glutamine, and aspartate produce inactive transporters that are present at the plasma membrane.2 By comparison, the lactose permease contains only six irreplaceable amino acids, two of which are directly involved in substrate translocation and four of which are involved in proton binding and translocation (19).

The membrane-impermeant reagent, MTSET, was used to identify functionally important residues in NaDC-1 since substrate and cation binding likely occurs in a crevice or water-filled pore. MTSET reacts preferentially with thiolate anions, which are more likely to be found in an aqueous environment (20). Changes in the apparent accessibility of the thiolate anions can be produced by changes in the concentration of the MTSET in the vicinity of the thiol group, or by physical exposure or occlusion of the thiol to the reagent (11, 21). The four sensitive cysteine mutants (S478C, A480C, A481C, and T482C) were inhibited by chemical modification with both MTSET, which adds a positive charge to the protein, and MTSES, which adds a negative charge. Therefore, it is likely that chemical modification by MTS reagents produces transport inhibition by steric hindrance or reduced mobility.

The current model of NaDC-1 function, based on kinetic studies with isolated membranes and cloned transporters (3–5), follows an ordered binding alternate access mechanism. In this model, three sodium ions bind first to NaDC-1, in a cooperative fashion, which produces a conformational change in the protein and an increased affinity for substrate. The binding of substrate follows sodium binding. The fully loaded carrier undergoes an additional conformational change which reorients the binding sites from the outside to inside, allowing the substrate and cations to be released inside the cell. Finally, the empty carrier reorients to face the outside of the cell. In the

2 D. A. Griffith and A. M. Pajor, unpublished results.

FIG. 6. Sodium activation of succinate uptakes in mutants expressed in Xenopus oocytes. The transport of 100 µM [3H]succinate was measured for 5 min in buffers containing 0–100 mM Na⁺ (Na⁺ was replaced by choline). The data have been normalized relative to the V_max and were fit by the Hill equation. The kinetic constants were as follows: A, C476S mutant (K_M 36 ± 4 mM, n 1.7 ± 0.2); B, S478Cwt mutant (K_M 58 ± 8 mM, n 2.6 ± 1.8); A480Cwt mutant (K_M 42 ± 9 mM, n 2.2 ± 0.6); C, A481Cwt mutant (not possible to fit data); T482Cwt (K_M 78 ± 33 mM, n 1.5 ± 0.3). The error is the standard error of the fit. Each data point represents the mean ± S.E., n = 5 oocytes.

FIG. 7. Effect of holding potential on accessibility of MTSET. Oocytes were held at voltages between −150 and 0 mV during the incubation with MTSET and the 2-min washout with sodium or choline buffer afterward. The voltage was then returned to −50 mV and substrate-dependent currents were measured using 10 mM succinate. The data are expressed as a percentage of control measured in the absence of MTSET. The S478Cwt (A), A480Cwt (B), and A481Cwt (C) mutants were treated with 1 µM MTSET for 10 s in either sodium or choline buffer. D, the T482Cwt mutant was treated with 5 µM MTSET for 30 s in either sodium or choline buffer. Each data point represents the mean ± S.E. of 2–3 oocytes. In some cases the error bars are smaller than the size of the data points.
In the present study, the accessibility of the membrane-impermeant MTSET to the four replacement cysteines in TMD 9 (at Ser-478, Ala-480, Ala-481, and Thr-482) mirrors the accessibility of the binding site for succinate (Fig. 9). In the absence of sodium, i.e., in choline, the transporter is in a conformational state that prevents accessibility of MTSET to the cysteine by placing the cysteine in a lipid environment or between two helices that do not have an aqueous pore open to the outside of the cell. The binding of sodium triggers a conformational change in the protein which increases the substrate affinity. This is also the conformation in which the cysteine is most accessible, suggesting that the binding of sodium triggers a movement of TMD-9 which exposes the cysteine. The binding of lithium, a transported cation that does not produce the optimal conformational change for substrate binding (5), also allows increased access to MTSET, which indicates that the movement of TMD9 (or the rest of the protein relative to TMD 9) does not have to be very large to allow access by MTSET. The binding of substrate produces yet another conformational change in the protein which again makes the cysteine inaccessible to the MTSET. During this conformational change, the helices may tilt or rotate to allow exposure of the substrate-binding site to the inside of the cell. If the occlusion of the cysteine occurs as a result of helix tilting then one would predict that the cysteine would be accessible to MTS reagents from the inside of the cell in the presence of extracellular substrate. A similar result has been observed for the γ-aminobutyric acid transporter, GAT-1, in which the endogenous Cys-399 is alternately accessible from the inside and outside of the cell (22). In the present study, substrate protection was seen in the S478C, A480C, and A481C mutants in sodium buffer, whereas the T482C mutant exhibited substrate protection in both lithium and sodium. Therefore, it seems likely that a smaller movement around Thr-482, compared with the other conformationally sensitive amino acids, is enough to occlude the residue.

In the H+–coupled lactose transporter, the lactose permease, transport produces widespread changes in the tertiary structure of the protein, which includes changes in helix rotation and tilt (19). Transmembrane domain VIII in the lactose permease contains the key residue Glu-269, which plays an essential role in coupling between substrate and H+ translocation (23). The face of the helix in TMD VIII that contains Glu-269 also contains substrate-protectable residues that are sensitive to inhibition by the cysteine-specific reagent, NEM. Helix VIII is thought to couple the conformational change induced by substrate binding in helices IV and V to the interface between helices IX and X, which is involved in H+ binding. Furthermore, the face of helix VIII near TMD X and IX, and near V and IV appears to form part of the permeation pathway for H+ and substrate (23). By analogy with the lactose permease, it is possible that TMD-9 in NaDC-1 is also involved in coupling conformational changes between sodium and substrate bind-
ing. Mutations in Glu-475 affect both cation and substrate affinity, while Asn-479 appears to be an irreplaceable residue. The face of the helix that contains both Glu-475 and Asn-479 also contains the substrate protectable residues (Ser-478, Ala-480, and Thr-482) that are sensitive to inhibition by MTSET (Fig. 8B). The results indicate that TMD-9 may mediate a conformational change during the transport cycle that transduces changes in conformation between the cation-binding sites and the substrate-binding site, and also suggests that TMD-9 may form part of the permeation pathway.

The ordered binding mechanism of transport and the effect of membrane voltage in NaDC-1 is similar to that of the Na\(^+\)/glucose co-transporter, SGLT1 (5). However, there also appear to be differences between the two transporters. Gln-457 in SGLT1, located near the extracellular surface of helix 11, is a conformationally-sensitive residue and the accessibility of MTSEA to the Q457C mutant is strongly dependent on voltage (24). In contrast, no effects of voltage were evident in three of the conformationally sensitive mutants of NaDC-1 (S478C, A480C, and A481C). In the T482C mutant, the accessibility of MTSET in choline buffer was dependent on voltage, which could indicate that a change in membrane voltage produces enough of a conformational change to make Thr-482 accessible from the outside. Although it is possible that the predominant effect of membrane potential is to change the concentration of the reactive cysteine, the different effects of voltage in sodium and choline buffer argue against this. The results again support the idea that T482C is very sensitive to conformational changes in TMD-9 and relatively small movements of the protein are enough to expose or occlude this residue, although this will need to be tested further.

The current secondary structure model of NaDC-1 contains 11 transmembrane domains (1). As is the case for almost all of the sodium-cotransport proteins, no crystal structures are available, and the structural models must be inferred from hydrophobicity analysis or location of epitopes or glycosylation sites. For example, the C terminus of NaDC-1 contains the N-glycosylation site (25), and the other members of the SLC13 family have one or two N-glycosylation sites at this location (1), which places the C terminus on the outside of the cell. There is also immunological evidence that both the N terminus and the loop between TMD-4–5 of NaDC-1 are located intracellularly, which also supports the present model (26). It is likely that the key residues involved in transport function are located within the transmembrane domains, as has been shown for the lactose permease (19). Therefore, the proposed location of the functionally important residues accessible from the outside of the cell (Ser-478, Ala-480, Ala-481, and Thr-482) at the top of TMD-9 is consistent with our model. Cys-476 is accessible to the water-soluble reagent, pCMBS (10), which supports the hypothesis that aqueous pores in NaDC-1 extend into the membrane (Fig. 8A).

In conclusion, the results of this study suggest that TMD-9 in NaDC-1 contains residues which exhibit differences in accessibility to the outside of the protein depending on the conformational state of the transporter. Our previous study identified Glu-475 in TMD-9 as a residue involved in determining the affinity for both cations and substrate (9). The present study shows that four amino acids found near the extracellular surface of TMD9 (Ser-478, A479, Ala-481, and Thr-482) are exposed or occluded depending on the conformational state of the transporter. When replaced by cysteine, these four residues are sensitive to inhibition by MTSET in the presence of sodium. These residues also show substrate protection from MTSET inhibition, suggesting that they are not accessible to the MTSET after substrate binding. The results indicate that TMD 9 may mediate a conformational change during the transport cycle that transduces alterations in conformation in the cation-binding sites to the substrate-binding site, and also suggests that portions of TMD-9 may alternately form part of the permeation pathway for substrate and cations.

Acknowledgments—We thank N. Sun, R. Gangula, C. Hudgins, and A. Wang for excellent technical assistance at different stages of the project. We also thank Dr. Bruce Hirayama for discussions throughout the study.

REFERENCES
1. Pajor, A. M. (2000) J. Membr. Biol. 175, 1–8
2. Pajor, A. M. (1999) Annu. Rev. Physiol. 61, 663–682
3. Yao, X., and Pajor, A. M. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 739–743
4. Griffith, D. A., and Pajor, A. M. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 739–743
5. Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 488–492
6. Pajor, A. M. (1995) J. Biol. Chem. 270, 5779–5785
7. Pajor, A. M., Sun, N., and Valmone, H. G. (1998) Biochem. J. 331, 257–264
8. Pascual, J. M., and Karlin, A. (1998) J. Gen. Physiol. 111, 717–729
9. Wright, E. M., and Pajor, A. M. (1998) Biochemistry 37, 7524–7531
10. Pajor, A. M., Krajeski, S. J., Sun, N., and Gangula, R. (1998) Biochim. Biophys. Acta 1370, 98–106
11. Karlin, A., and Akabas, M. H. (1998) Methods Enzymol. 293, 123–145
12. Danielson, M. A., Bass, R. B., and Falke, J. J. (1997) J. Biol. Chem. 272, 32678–32688
13. Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 488–492
14. Pajor, A. M. (1995) J. Biol. Chem. 270, 5779–5785
15. Pajor, A. M., Sun, N., and Valmone, H. G. (1998) Biochem. J. 331, 257–264
16. Pascual, J. M., and Karlin, A. (1998) J. Gen. Physiol. 111, 717–729
17. Wright, E. M., Wright, S. H., Hirayama, B., and Kippen, I. (1992) Am. J. Physiol. Renal Fluid Electrolyte Physiol. 261, F1099–F1106
18. Kabaeb, H. R., and Wu, J. (1999) Biochim. Biophys. Acta 1370, 98–106
19. Akabas, M. H., Stauffer, D. A., Xu, M., and Karlin, A. (1992) Science 258, 307–310
20. Horn, R. (1996) Methods Enzymol. 263, 145–155
21. Frillingos, S., and Kabaeb, H. R. (1997) Protein Sci. 6, 438–443
22. Loo, D. D., Hirayama, B. A., Gallardo, E. M., Lam, J. T., Turk, E., and Wright, E. M. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 7789–7794
23. Pajor, A. M., and Sun, N. (1996) Am. J. Physiol. Cell Physiol. 271, C1808–C1816
24. Zhang, F. F., and Pajor, A. M. (2001) Biochim. Biophys. Acta 1511, 80–89
Conformationally Sensitive Residues in Transmembrane Domain 9 of the Na+/dicarboxylate Co-transporter
Ana M. Pajor

J. Biol. Chem. 2001, 276:29961-29968.
doi: 10.1074/jbc.M011387200 originally published online June 8, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M011387200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 26 references, 12 of which can be accessed free at
http://www.jbc.org/content/276/32/29961.full.html#ref-list-1