Cysteine residues in the Na⁺/dicarboxylate co-transporter, NaDC-1

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The role of cysteine residues in the Na⁺/dicarboxylate co-transporter (NaDC-1) was tested using site-directed mutagenesis. The transport activity of NaDC-1 was not affected by mutagenesis of any of the 11 cysteine residues, indicating that no individual cysteine residue is necessary for function. NaDC-1 is sensitive to inhibition by the impermeant cysteine-specific reagent, *p*-chloromercuribenzenesulphonate (pCMBS). The pCMBS-sensitive residues in NaDC-1 are Cys-227, found in transmembrane domain 5, and Cys-476, located in transmembrane domain 9. Although cysteine residues are not required

for function in NaDC-1, their presence appears to be important for protein stability or trafficking to the plasma membrane. There was a direct relationship between the number of cysteine residues, regardless of location, and the transport activity and expression of NaDC-1. The results indicate that mutagenesis of multiple cysteine residues in NaDC-1 may alter the shape or configuration of the protein, leading to alterations in protein trafficking or stability.

Key words: citrate, mutagenesis, succinate, *Xenopus* oocytes.

INTRODUCTION

The absorption of tricarboxylic acid cycle intermediates, such as succinate and citrate, across the apical membrane of the kidney proximal tubule and the small intestine is mediated by a sodium-coupled dicarboxylate transporter called NaDC-1 [1]. NaDC-1 has a broad substrate specificity for di- and tricarboxylates, but the preferred substrates are carried in bivalent-anion form. One important function of NaDC-1 in the kidney is to help regulate the concentration of urinary citrate, which acts as an endogenous chelator of calcium and prevents the formation of kidney stones. NaDC-1 belongs to a distinct gene family of sodium-coupled transporters for organic anions that includes the Na⁺/sulphate co-transporter, NaSi-1 [1].

In studies with rabbit renal brush border membrane vesicles, the activity of the NaDC-1 was sensitive to inhibition by the cysteine-specific reagent, *p*-chloromercuribenzenesulphonate (pCMBS) [2]. Therefore, the goal of this study was to identify the binding sites for pCMBS in the cloned NaDC-1 using site-directed mutagenesis. Since pCMBS is water soluble, it should help to identify cysteine residues that are either in a water-filled pore or accessible from the outside of the cell. The results show that no single cysteine residue is required for transport activity in NaDC-1 and that Cys-227 and -467 are the inhibitory pCMBS binding sites. However, an unexpected and remarkable finding of this study is that the removal of cysteine residues in NaDC-1 is correlated with the loss of protein expression in the plasma membrane, suggesting a role for cysteine residues in targeting or stability of the NaDC-1 protein.

EXPERIMENTAL

Xenopus oocytes

Stage V and VI oocytes from *Xenopus laevis* were dissected and collagenase treated as described previously [3]. Oocytes were cultured at 18 °C in Barth's medium supplemented with 5%

(v/v) heat-inactivated horse serum, 2.5 mM sodium pyruvate and gentamicin at 50 mg/l [3]. Culture dishes and medium were changed daily.

In vitro cRNA transcription

The wild-type and mutant NaDC-1 cDNAs in pSPORT plasmids were used as templates for cRNA synthesis [3]. Plasmids were linearized with *Xho*I and *in vitro* cRNA transcription was done using the T7 mMessage mMachine Kit (Ambion, Austin, TX, U.S.A.). The cRNAs were resuspended in water to a final concentration of 0.5–1 µg/µl.

Transport experiments

Transport of [¹⁴C]succinate (DuPont–NEN) was measured 5–7 days after oocyte injections, as previously described [3]. Sodium and choline buffers were as follows: 100 mM NaCl or choline chloride, 2 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM Hepes/Tris, pH 7.5. The oocytes were rinsed briefly with choline buffer to remove sodium and serum. Transport was initiated by replacement of the choline rinse with 0.4 ml of the appropriate transport buffer as described in the figure legends. Transport was stopped by the addition of 4 ml ice-cold choline buffer followed by removal of extracellular radioactivity with three additional washes in cold choline buffer. Individual oocytes were transferred to scintillation vials and dissolved in 0.5 ml 10% (w/v) SDS. Econo-safe scintillation cocktail (Research Products, Int., Mount Prospect, IL, U.S.A.) was added and radioactivity was counted. Counts in control uninjected oocytes were subtracted from the counts in cRNA-injected oocytes. Data are presented as means ± S.E.M. Statistical analysis was done using the SigmaStat Program (Jandel Scientific, Madera, CA, U.S.A.).

Inhibition experiments

Xenopus oocytes were pre-incubated at room temperature in sodium buffer containing 2.5 mM pCMBS or sodium buffer alone (control condition) for 20 min. The pCMBS solutions were

Abbreviations used: NaDC-1, rabbit renal Na⁺/dicarboxylate co-transporter; pCMBS, *p*-chloromercuribenzenesulphonate

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Table 1 Oligonucleotides used for mutagenesis

Mutation	Restriction site	Sequence (5' to 3')
C4S	<i>SpeI</i>	CAGGCCCTGCCAACTAGTGCCATGGT
C38S		GATGGCGTATGCCGAGTAGGCTTCCTT
C50S	<i>SalI</i>	CAGAGCATCAGTCGACCAAGACAG
C65S	<i>HindIII</i>	CATGGGAACAAGCTTAGGGCAGGAG
C225,227S	<i>AflII</i>	TGAGTAGCACAGCATAAGCTCATGCC
C225S	<i>AflII</i>	TGAGTAGCTCAGCATAAGCTCATGCC
C227S	<i>AflII</i>	TGAGTAGCACAGCTTAAGCTCATGCC
C467S	<i>HindIII</i>	GGTGGCAACCAGCAAGCTTATGATGAAGAC
C476S	<i>SpeI</i>	GGCCGCATTACTAGTGTCTCAGT
C498S	<i>EcoRV</i>	CGGGTGCAGGATATCGCTGAGCCAT
C498A		CAGCGGGTGCAGGGCGATGGGCTG
C508S	<i>SalI</i>	CGAGGCCAGGGTCGACGGCAGCATGAC
C583S	<i>NheI</i>	GGGGGGGCTGGCTAGCGAGTGGGTGGT

Table 2 Nomenclature of NaDC-1 mutants containing multiple mutations

Name	Characteristics
C-less	All 11 Cys mutated to Ser or Ala (C498)
C-less (C50A)	Like C-less but C498 and C50 mutated to Ala
1C(476)	Contains 1 Cys at position 476
3C(C4S)	Contains 3 Cys (38, 50 and 65)
3C(C38S)	Contains 3 Cys (4, 50 and 65)
3C(C65S)	Contains 3 Cys (4, 38 and 50)
4C(Nterm)	Contains 4 N-terminal Cys (4, 38, 50, 65)
4C(Cterm)	Contains 4 C-terminal Cys (467, 498, 508, 583)
5C(Cterm)	Contains 5 C-terminal Cys (467, 476, 498, 508, 583)

prepared just before use and kept in the dark. After the incubation period, the pCMBS-containing solution was removed with four washes of choline buffer at room temperature, after which the transport buffer containing [14 C]succinate was added for measurement of succinate uptake.

Site-directed mutagenesis: single mutations

The 11 cysteine residues in NaDC-1 [3] were altered by site-directed mutagenesis using the oligonucleotide-directed method [4] with reagents from the Muta-Gene plasmid kit from BioRad, according to manufacturer's directions. The sequences of the oligonucleotides used in the mutagenesis procedure are shown in Table 1. In addition to the cysteine mutation, the mutagenic oligonucleotides introduced restriction sites by silent mutagenesis to facilitate identification. Mutants identified by the presence of the introduced restriction site were verified by sequencing. The mutants are designated using the single letter amino acid code followed by the number indicating the position of the residue in wild-type NaDC-1. The second letter following the sequence number indicates the amino acid replacement at that position.

Preparation of NaDC-1 mutants containing multiple mutations

Table 2 lists the nomenclature for cysteine mutants of NaDC-1 containing multiple mutations. The C-less (cysteine-less) NaDC-1 was prepared by a combination of site-directed mutagenesis using the oligonucleotides listed in Table 1 and by subcloning of mutants lacking single or multiple cysteines. The final C-less construct was completely sequenced in both directions. Mutants of NaDC-1 containing between 1 and 5 cysteine residues were

also made using a combination of site-directed mutagenesis and chimaera formation between the C-less and wild-type NaDC-1. The nomenclature of these mutants lists the number of cysteine residues, followed by information on their location (Table 2).

Cell-surface biotinylation of oocytes

The cell-surface expression of wild-type and mutant NaDC-1 was tested using a membrane-impermeant reagent, Sulpho-NHS-LC-Biotin (Pierce, Rockford, IL, U.S.A.), as described [5]. For each biotinylation experiment, groups of five oocytes were rinsed three times with 4 ml ice-cold PBS, pH 8 [5]. The oocytes were then incubated 10 min at room temperature in 0.5 ml PBS containing Sulpho-NHS-LC-Biotin (0.5 mg/ml). The biotinylation reagent was removed with four washes of 4 ml ice-cold PBS. The oocytes were then dissolved in lysis buffer (150 mM NaCl, 20 mM Tris/HCl, pH 7.6, 1% Triton X-100) for 30 minutes on ice. The solubilized oocytes were centrifuged 15 min at 14000 *g* and the supernatants transferred to new tubes. The biotinylated proteins were incubated with 50 μ l ImmunoPure Immobilized Streptavidin beads (Pierce) for 1 h at 4 °C. The biotin-streptavidin-agarose bead complexes were washed four times by suspension in lysis buffer followed by centrifugation. The final pellets were resuspended in the appropriate sample buffer and boiled 5 min before being loaded on to a polyacrylamide gel for SDS/PAGE [6].

Western blots of cell-surface proteins

The transporters precipitated by the biotinylation-streptavidin-precipitation procedures were identified by Western blotting, as described previously [6]. Briefly, oocyte proteins were separated by SDS/PAGE and blotted onto nitrocellulose [6]. The nitrocellulose membranes were blocked for a minimum of 1 h in PBS/TM (PBS containing 0.05 % Tween 20 and 0.5 % Carnation instant dried milk). The Western blots were probed with an antibody directed against a fusion protein of NaDC-1, which was raised in rabbits [7]. The primary antibody was diluted 1:10000 in PBS/TM, and applied for 1 h, after which the secondary antibody, horseradish peroxidase-linked anti-(rabbit IgG) (Amersham), was diluted 1:10000 in PBS/TM and applied for 1 h. All incubations and washes were done at room temperature. Antibody binding was visualized by enhanced chemiluminescence using the Supersignal CL-HRP substrate system (Pierce). Molecular masses were estimated by comparison with pre-stained protein standards (BioRad). The Western blots were scanned with a Microtek Scanmaker E3 scanner (Microtek Int., Redondo Beach, CA, U.S.A.), and peak mass and intensity were calculated using SigmaGel software (Jandel Scientific).

RESULTS

Previous studies have suggested that the NaDC-1 contains functionally important cysteine residues that are sensitive to inhibition by reagents such as pCMBS [2]. The cloned NaDC-1 expressed in oocytes is also sensitive to inhibition by pCMBS. Figure 1 shows the inhibition of succinate transport in NaDC-1 with increasing concentrations of pCMBS. The IC_{50} is approx. 0.7 mM, and the maximal inhibition was 95 % at 2.5 mM pCMBS. For this reason, all subsequent experiments were done using 2.5 mM pCMBS. There was no effect of substrate or cations on the inhibition by pCMBS (results not shown), similar to previous studies using rabbit renal brush border membranes [2].

The sequence of the NaDC-1 contains 11 cysteine residues, 7 of which are predicted to be in transmembrane domains (Figure

Figure 1 Dose-response of pCMBS inhibition

Oocytes expressing NaDC-1 were preincubated with concentrations of pCMBS up to 2.5 mM for 20 min as described in the Experimental section. The solutions were washed away and uptake of 100 μ M succinate was measured for a 15 min time period. The results shown are means $\pm$ S.E.M. ($n = 5$).

2). To identify functionally important cysteine residues, site-directed mutagenesis was used to replace each of the cysteine residues in turn with serine residues. Most of the replacements had little effect on transport function, with the exception of the C498S mutant, which had no measurable activity (Table 3). However, replacement of Cys-498 by alanine produced a transporter that had wild-type transport activity (Table 3). All of the functionally active mutant transporters were expressed at the plasma membrane, as shown by cell-surface-biotinylation experi-

Figure 2 Secondary-structure model of NaDC-1 showing the positions of the 11 cysteine residues

The positions of the 11 cysteine residues are indicated (●). The 'Y' represents the N-glycosylation site at the carboxy-terminus.

Table 3 Transport of succinate by cysteine mutants expressed in *X. laevis* oocytes

Uptake is expressed as the percentage of uptake compared with the wild-type (WT) NaDC-1 expressed in the same batch of oocytes. The data shown are means $\pm$ S.E.M. The n refers to the number of experiments using oocytes from different donor frogs.

Mutant	Succinate uptake (% of WT)	<i>n</i>
C4S	96.5 $\pm$ 18.4	6
C38S	67.0 $\pm$ 6.9	4
C50S	58.7 $\pm$ 13.9	9
C65S	93.2 $\pm$ 13.5	6
C225S	127.4 $\pm$ 11.9	3
C227S	92.9 $\pm$ 3.2	3
C467S	47.4 $\pm$ 4.2	5
C476S	66.0 $\pm$ 16.4	10
C498S	0	2
C498A	90.6 $\pm$ 11.6	5
C508S	78.7 $\pm$ 10.9	3
C583S	78.2 $\pm$ 7.0	7

Figure 3 Western blots of cell-surface biotinylated proteins from oocytes expressing wild-type NaDC-1 (WT) or the single cysteine-residue mutants

X. laevis oocytes were labelled using a membrane-impermeant derivative of biotin, Sulpho-NHS-LC-biotin, as described in the Experimental section. Each blot contains an internal control of wild-type NaDC-1, and 'Un' refers to control, uninjected oocytes. The blots were incubated with anti-NaDC-1 antibody at a 1:10000 dilution, followed by horseradish peroxidase-linked anti-(rabbit IgG). The two protein bands represent differently glycosylated forms of NaDC-1 expressed in oocytes [6].

ments (Figure 3). The inactive C498S mutant was not present at the plasma membrane, which correlated with the lack of activity (Figure 3). Therefore, the results indicate that individual cysteine residues are not required for the function of NaDC-1.

The sensitivity of the single Cys mutants to inhibition by pCMBS was tested (Table 4). Almost all of the Cys mutants of NaDC-1 were sensitive to inhibition by pCMBS. Although C227S and C476S were significantly less sensitive than the others, the C227S mutant was more sensitive to pCMBS inhibition than the C476S mutant. Removal of Cys-476 resulted in only 20% inhibition of succinate transport by 2.5 mM pCMBS (Table 4) and no inhibition by 1 mM (results not shown). Removal of Cys-227 resulted in about 50% inhibition at 2.5 mM pCMBS (Table 4), and about 20% inhibition at 1 mM (results not shown). These results suggest that Cys-227 and Cys-476 are the inhibitory binding sites for pCMBS in NaDC-1.

Table 4 Inhibition of uptake by 2.5 mM pCMBS

Transport of succinate was measured after preincubation with 2.5 mM pCMBS in sodium buffer or sodium buffer alone (control condition). Uptake activity remaining after pCMBS treatment is expressed as a percentage of the uptake measured in controls. The results shown are means $\pm$ S.E.M. The *n* refers to the number of experiments using oocytes from different donor frogs. The two groups that are significantly different from NaDC-1 ($P < 0.01$) are indicated (*).

Mutant	Uptake remaining (% of control)	<i>n</i>
NaDC-1	17.8 $\pm$ 2.2	17
C4S	15.4 $\pm$ 2.7	4
C38S	9.9 $\pm$ 2.3	3
C50S	24.3 $\pm$ 2.7	4
C65S	14.8 $\pm$ 4.2	4
C225S	16.8 $\pm$ 3.2	3
C227S	58.5 $\pm$ 0.5*	3
C467S	11.0 $\pm$ 7.1	4
C476S	80.0 $\pm$ 7.9*	4
C498A	18.9 $\pm$ 5.3	4
C508S	23.7 $\pm$ 4.5	4
C583S	7.0 $\pm$ 3.3	4

A cysteine-less mutant of NaDC-1 (C-less) was constructed, in which all of the cysteine residues had been mutated to serine or alanine residues. Despite the indications from the single-site mutants that individual cysteine residues are not required for the function of NaDC-1, the C-less mutant of NaDC-1 exhibited very low activity (Table 5) and was very poorly expressed at the plasma membrane (Figure 4). To determine the minimal number of cysteine residues required for NaDC-1 expression, several other mutants were made, which contained up to 5 cysteine residues. The nomenclature of the mutants is shown in Table 2 and the transport activity is shown in Table 5. There was a general increase in transport activity and amount of cell-surface expression (Figure 4) with increasing numbers of cysteine residues in NaDC-1.

The C-less and 1C(476) mutants had very low cell-surface protein expression (Table 5). These mutants also showed very low total protein expression (results not shown). Interestingly, specific cysteine residues did not appear to be required for

Table 5 Succinate-transport activity in NaDC-1 mutants containing reduced numbers of cysteines

The wild-type NaDC-1 has 11 cysteines. The nomenclature and description of these mutants is given in Table 2. Results (means $\pm$ S.E.M.) are expressed as percentage of wild-type (WT) activity measured in the same batch of oocytes. The *n* refers to the number of experiments using oocytes from different donor frogs.

Mutant	Number of Cys	Succinate uptake (% of WT)	<i>n</i>
C-less	0	1.7, 1.6	2
C-less (S50A)	0	6.4 $\pm$ 2.7	3
1C(476)	1	17.1 $\pm$ 4.5	4
3C(C4S)	3	21.5 $\pm$ 5.9	3
3C(C38S)	3	18.7 $\pm$ 2.6	3
3C(C65S)	3	21.9 $\pm$ 0.5	3
4C(Nterm)	4	38.4 $\pm$ 6.5	4
4C(Cterm)	4	57.4 $\pm$ 9.4	3
5C(Cterm)	5	76.7 $\pm$ 12.4	4
C225,227S	9	50.9 $\pm$ 2.3	4

Figure 4 Western blots of cell-surface biotinylated proteins from oocytes expressing mutants of NaDC-1 containing reduced numbers of cysteines

Each blot contains an internal control of wild-type NaDC-1 (WT). The blots were prepared and probed as described in the legend to Figure 3. The number and position of the cysteine residues in each of the mutant proteins are listed in Table 2.

Figure 5 Effect of the number of cysteine residues in NaDC-1 on the transport of succinate

The transport activities (expressed as a percentage of a wild-type control) of the NaDC-1 mutants, taken from Tables 3 and 5, were replotted as a function of the number of cysteine residues in each mutant. The line represents the fit of the data by linear regression: $y = 7.59x + 5.77$, $r^2 = 0.74$.

expression of NaDC-1. The activity of the 4C(Nterm) mutant, containing 4 cysteine residues at the amino terminus, was very similar to the transport activity of the 4C(Cterm) mutant, containing four different cysteine residues at the carboxy-terminus (Table 5).

The transport results shown in Tables 3 and 5 were replotted as a function of the number of cysteine residues in each mutant (Figure 5). There was a general increase in transport activity with

increasing numbers of cysteine residues, approx. 8% for each additional cysteine residue. The data could be fit, by linear regression, with the equation $y = 7.6x + 5.8$, $r^2 = 0.74$. The results suggest that the presence of cysteine residues, regardless of their location, or alternatively, the lowest number of mutations, is positively correlated with increased expression of NaDC-1.

DISCUSSION

The NaDC-1 is sensitive to inhibition by cysteine-specific reagents, such as pCMBS. The results of this study show that the inhibitory effects of pCMBS are mediated by binding at two cysteine residues, at positions 227 and 476. These sites are not likely to be substrate or cation binding sites because the presence of substrate or sodium did not prevent the inhibition by pCMBS. However, Cys-227 and -476 are accessible from the outside of the membrane and are likely to be functionally important residues.

Cys-476 is the most sensitive of the two inhibitory pCMBS binding sites in NaDC-1. Removal of Cys-476 in the C476S mutation results in a transporter with very low sensitivity to inhibition by pCMBS, even though the mutant transporter still contains Cys-227. According to our current secondary structure model of NaDC-1 (Figure 2 and [6]), Cys-476 is located close to the outer surface of the membrane in transmembrane domain 9. The identification of Cys-476 as a functionally important residue is interesting in light of our previous study suggesting that transmembrane domain 9 may form part of the translocation pathway of NaDC-1 [8]. The adjacent residue, Glu-475, appears to be important in both sodium and substrate binding in NaDC-1, but Glu-475 is particularly important in the topological organization of the cation binding pocket [8]. It is possible that binding of pCMBS to Cys-476 could impair the flexibility of the protein or could alter the position of other functionally important residues, such as Glu-475. Since Cys-476 is accessible to pCMBS, the residue is likely to be found in an aqueous environment or pore that can be reached from the surface of the membrane.

The second site for inhibitory-pCMBS binding in NaDC-1 is Cys-227, which is located deep within transmembrane domain 5 (Figure 2). The results indicate that Cys-227 is also located in a water-filled pore that is accessible from the outside of the cell. Although it is possible that the location of Cys-227 in our secondary structure model is incorrect, there is evidence from other transport proteins that pCMBS can bind to cysteines located deep within transmembrane domains. For example, Cys-265 in transmembrane domain 7 in the bacterial glucose-6-phosphate/phosphate antiporter (Uhp T) is accessible to pCMBS from both sides of the membrane [9]. Transmembrane domain 7 in this antiporter is thought to be part of the substrate translocation pathway, forming part of a water-filled channel that is alternately exposed to each side of the membrane during transport [10].

The cysteine residues in NaDC-1 are not required for the functional activity of the protein, since replacement of any single

cysteine residue did not affect function. Because of this, it is unlikely that NaDC-1 contains functionally-important disulphide bridges, in accordance with previous studies using renal brush border membranes [2]. A remarkable finding of this study, however, is that cysteine residues alter the targeting or stability of NaDC-1 in the plasma membrane. There was a linear relationship between the number of cysteine residues, regardless of their location, and the transport activity and expression of the NaDC-1 mutants. The results suggest that the transporter can tolerate a few cysteine to serine changes without deleterious effects, but more changes could alter the shape of the protein and thus lead to its degradation within the cell. It is also possible that the presence of a certain minimal number of cysteine residues is required for efficient targeting or processing to the plasma membrane. Interestingly, the rat serotonin transporter, SERT, also contains cysteine residues that are important for targeting. Mutagenesis of these residues, many of which are conserved and located in a large extracellular loop, results in impaired processing or trafficking of the mutant transporters [11,12].

In conclusion, we find that NaDC-1 does not require any single cysteine residue for function since all eleven cysteine residues can be replaced individually without large changes in transport activity. Two residues, at positions 227 and 476, are functionally important residues that mediate inhibition by pCMBS. The location of Cys-227 and Cys-476 in transmembrane domains 5 and 9 implicates these transmembrane domains in the translocation pathway. Remarkably, we find a relationship between the number of cysteine residues in NaDC-1 and the amount of expression of the mutant transporters in oocytes. The results suggest that although single cysteine residue replacements may be tolerated in NaDC-1, substitutions of multiple cysteine residues may alter the shape or configuration of the protein leading to alterations in targeting or stability.

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