Mutational analysis of histidine residues in the rabbit Na⁺/dicarboxylate co-transporter NaDC-1

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Succinate transport by the rabbit Na⁺/dicarboxylate co-transporter, NaDC-1, expressed in *Xenopus* oocytes was inhibited by the histidyl-selective reagent diethyl pyrocarbonate (DEPC). Therefore the role of histidine residues in the function of NaDC-1 was examined by site-directed mutagenesis. All 11 histidine residues in NaDC-1 were converted to alanine, but only mutant H106A exhibited a decrease in succinate transport. Additional mutations of NaDC-1 at position 106 showed that aspartic acid and asparagine, but not arginine, can substitute for histidine. Examination of succinate and citrate kinetics of H106A revealed a decrease in $V_{\max}$ with no change in K_m . Cell surface biotinylation experiments showed that the transport activity of all four mutants

at position 106 was correlated with the amount of cell surface expression, suggesting a role of His-106 in membrane expression rather than function. Two of the histidine mutants, H153A and H569A, exhibited insensitivity to inhibition by DEPC, indicating that these residues are involved in binding DEPC. Neither of these residues is required for transport activity; thus DEPC probably inhibits NaDC-1 function by hindrance of the mobility of the carrier. We conclude that histidine residues are not critical for transport function in NaDC-1, although His-106 might be involved in determining protein expression or stability in the membrane.

INTRODUCTION

The Na⁺/dicarboxylate co-transporters mediate the transport of Krebs cycle intermediates, such as succinate and α -oxoglutarate, across the plasma membranes of mammalian cells [1,2]. The low-affinity transporter found in the apical membrane of kidney and small intestine has a K_m for a succinate of 0.5 mM [3,4]. The reaction cycle of the Na⁺/dicarboxylate co-transporter involves the ordered binding of three Na⁺ ions followed by a divalent anion substrate, with a net transfer of one positive charge across the membrane [3]. Dicarboxylate transport is also sensitive to inhibition by Li⁺, which binds with high affinity to one of the Na⁺-binding sites [5].

To date, three cDNA species coding for Na⁺/dicarboxylate cotransporters have been isolated. The rabbit and human isoforms, called NaDC-1 and hNaDC-1 [6,7], are 78 % identical in amino acid sequence. The NaDC-1 cDNA species code for proteins of 593 residues, and predicted molecular masses of approx. 65 kDa. The *Xenopus* intestinal Na⁺/dicarboxylate co-transporter, NaDC-2, has similar substrate specificity to that of NaDC-1 but exhibits altered cation selectivity [8]. The predicted secondary structure models of the Na⁺/dicarboxylate co-transporters contain 11 transmembrane domains and an extracellular N-glycosylated C-terminus [9]. The Na⁺/sulphate co-transporter [10] is also related to the Na⁺/dicarboxylate co-transporters. There is no information on the structures of the Na⁺- or substrate-binding sites in any member of this gene family. Furthermore there is very little information in the literature about residues that might be important in these functions in other transporters.

In previous studies with rabbit renal brush border membrane vesicles, treatment with the histidyl-selective reagent diethyl pyrocarbonate (DEPC) inhibited Na⁺-dependent succinate trans-

port by up to 50 % [11]. The inhibition by DEPC could be prevented by incubation with succinate, suggesting that a histidine residue might be found at or near the substrate-binding site [11]. To investigate the role of histidine residues in the function of NaDC-1, each of them in the protein was systematically changed to alanine by site-directed mutagenesis. The results of this study suggest that histidine residues in NaDC-1 might be important for cell surface expression or insertion into the membrane rather than being involved directly in transport.

EXPERIMENTAL

Site-directed mutagenesis

Histidine residues in NaDC-1 [6] (GenBank accession number U12186) were altered by site-directed mutagenesis. The mutagenesis was done by the oligonucleotide-directed method of Kunkel [12] with reagents from the Muta-gene plasmid kit from Bio-Rad, in accordance with the manufacturer's directions. Single-stranded DNA was rescued by using helper phage K409 and precipitated with poly(ethylene glycol). The sequences of the oligonucleotides used in the mutagenesis procedure, all of which code for the anti-sense strand, are listed in Table 1. Mutants were verified by sequencing. The site-directed mutants are designated by using the single letter amino acid followed by the number indicating the position of the residue in wild-type NaDC-1. The second letter after the sequence number indicates the amino acid replacement at that position. The double mutant, H153,569A was made by subcloning two single mutants.

cRNA transcription *in vitro*

The wild-type and mutant NaDC-1 cDNA species in pSPORT

Abbreviations used: DEPC, diethyl pyrocarbonate; Endo H, Endo- β -acetylglucosaminidase H; NaDC-1, rabbit renal Na⁺/dicarboxylate co-transporter; hNaDC-1, human renal Na⁺/dicarboxylate co-transporter; PNGase F, peptide-N⁴-(N-acetyl- β -glucosaminyl) asparagine amidase F.

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Table 1 Oligonucleotides used for mutagenesis

The mutated codon is shown in bold.

Mutation	Name	Sequence (5'-3') of anti-sense oligonucleotides
H102A	ST28	CCA GGC TTC CAC GGC GAT GGC
H106A	ST29	GAT CCG CTT GGC CAG GTT CCA
H153A	ST30	CAC GGC GGC CGC GAT GGG CAC
H210A	ST31	GGT CAT GGC CTC CCC TGA CTC
H306A	ST43	CTG TTG CTC GGC CTC CTG CTC
H355A	ST44	GCC CCA GCC GGC GAA GAA GCC
H456A	ST45	AGG CGG CAC GGC CTG CAG TGG
H500A	ST27	CAG CGG GGC CAG GCA GAT GGC
H569A	ST32	GAA GGT GGC CAG CTG GAA CAT
H576A	ST46	GCT GTT GGA GGC GGC CCA CGA
H582A	ST47	GGC CAG GCA GGC GGT GTT GCT
H106R	ST33	GAT CCG CTT GCG CAG GTT CCA
H106D	ST34	GAT CCG CTT GTC CAG GTT CCA
H106N	ST35	GAT CCG CTT GTT CAG GTT CCA

plasmid [6,7] were used as templates for cRNA synthesis. Plasmids were linearized with *Not* I and cRNA transcription was done *in vitro* with the T7 mMessage mMachine Kit (Ambion). The cRNA species were resuspended in water to a final concentration of 0.5–1 µg/µl.

Xenopus oocytes

Female *Xenopus laevis* were obtained from Nasco. Stage V and VI oocytes were dissected and treated with collagenase as described previously [6]. 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 50 mg/l gentamicin. Culture dishes and medium were changed daily.

Transport experiments

Transport of [¹⁴C]succinate (DuPont–NEN) and [¹⁴C]citrate (Moravsek) was measured 5–7 days after oocyte injections, also as described [6]. Sodium and choline buffers (in mM) were as follows: 100 NaCl or choline chloride, 2 KCl, 1 MgCl₂, 1 CaCl₂, 10 Hepes/Tris, pH 7.5. The oocytes were rinsed briefly with choline buffer to remove Na⁺ and serum. Transport was initiated by replacement of the choline rinse with 0.4 or 0.75 ml (kinetic experiments) of the appropriate transport buffer as described in the figure legends. Transport was stopped after the appropriate time by the addition of 4 ml of ice-cold choline buffer. Extracellular radioactivity was removed by three additional washes in cold choline buffer. Individual oocytes were transferred to scintillation vials and dissolved in 0.5 ml of 10% (w/v) SDS. Scintillation cocktail 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. Calculations of kinetic constants were done by non-linear regression with the Michaelis–Menten equation, using the SIGMAPLOT program (Jandel Scientific). Statistical analysis was done with the SIGMASTAT Program (Jandel Scientific).

DEPC experiments

Xenopus oocytes were preincubated at room temperature with the appropriate concentration of DEPC (usually 2 mM) in Barth's solution prepared without Tris buffer. The DEPC-containing solution was removed with four washes of choline

buffer at room temperature, after which the transport buffer was added. Stock solutions of DEPC in ethanol (2 M) were prepared just before starting the experiment and were added to the transport buffers just before use.

Cell surface biotinylation of oocytes

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

SDS-PAGE

Tris/glycine (Laemmli) gels were used in early studies, and the gels were run as described previously with 10% (w/v) polyacrylamide [9]. However, protein transfer from Tricine gels was more efficient, and later gels were run with the Tricine system [13]. The Tricine gels contained either 6% or 8% (w/v) polyacrylamide in 1 M Tris, pH 8.45. The gels were run with a cathode buffer containing 100 mM Tris, 100 mM Tricine, pH 8.25, 1% SDS, and an anode buffer containing 100 mM Tris, pH 8.9. The sample buffer for the Tricine gels contained 50 mM Tris/HCl, pH 7.0, 1% (v/v) glycerol, 4% (w/v) SDS, 2% (w/v) 2-mercaptoethanol and 0.13 mg/ml of Coomassie Blue. It should be noted that membrane proteins migrate anomalously relative to size standards in SDS/PAGE, particularly with differences in percentage of polyacrylamide [14]. For this reason, the protein gels always contained internal standards of NaDC-1 protein.

Western blots of cell surface proteins

The transporters precipitated by the biotinylation–streptavidin precipitation procedures were identified by Western blotting as described previously [9]. Briefly, oocyte proteins were separated in polyacrylamide gels, as described above, and blotted on nitrocellulose membranes, which were blocked for a minimum of 1 h in PBS-TM [PBS containing 0.05% (v/v) Tween 20 and 0.5% (w/v) Carnation instant dried milk]. The Western blots were probed with an antibody directed against a fusion protein of NaDC-1 [9], which was raised in rabbits [15]. 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 Ig) (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 revealed by enhanced chemiluminescence with the Supersignal CL-HRP substrate system (Pierce). Molecular masses were estimated by comparison with pre-stained protein standards (Bio-Rad). The Western blots were scanned with a Microtek Scanmaker E3

scanner, and peak mass and intensity were calculated with SIGMAGEL software (Jandel Scientific).

Western blots of purified of plasma membranes

Plasma membranes were purified from *Xenopus* oocytes by the differential centrifugation method of Geering et al. [16]. Briefly, groups of 50 oocytes were homogenized in 3 ml of a buffer containing 83 mM NaCl, 1 mM MgCl₂, 10 mM Hepes/Tris, pH 7.9, and protease inhibitors (0.5 mM PMSF, 50 µg/ml leupeptin and 16 µg/ml pepstatin). The yolk proteins were removed by two successive centrifugation steps for 10 min at 1000 *g*. The supernatants were then centrifuged at 10000 *g* for 20 min to pellet the plasma membranes. The pellets from the final centrifugation step were resuspended in homogenization buffer and used for Western blotting, as described previously [15].

Deglycosylation of plasma membrane proteins

The plasma membrane proteins were deglycosylated with peptidyl-N-glycanase F (PNGase F) (New England Biolabs) as described previously [15]. Briefly, 4 µg of protein was denatured in 0.5% SDS and 1% (w/v) 2-mercaptoethanol at 100 °C for 10 min. The deglycosylation reaction was performed in 50 mM sodium phosphate buffer (pH 7.5)/1% (v/v) NP40 containing 500–750 units of PNGase F at 37 °C for 1 h. Controls were incubated in the same buffer but without enzyme. For deglycosylation of membrane proteins with endo-β-acetylglucosaminidase H (Endo H) (Boehringer Mannheim), 4 µg of plasma membrane protein was denatured in a buffer containing 2% (w/v) SDS, 5% (w/v) 2-mercaptoethanol, 10 mM Tris, pH 7.5, 100 mM NaCl and 250 µM sucrose at 100 °C for 4 min. The deglycosylation reaction was performed in a total volume of 20 µl with the addition of 100 mM citrate, 5% (v/v) Triton X-100, 0.5 mM PMSF (final concentrations) and 4 m-units of Endo H at 37 °C for 1 h. Controls were incubated in the same buffer without enzyme. The samples were analysed by Western blotting as described above.

RESULTS

Inhibition of succinate transport in NaDC-1 by DEPC

The transport of succinate by NaDC-1 expressed in *Xenopus* oocytes was decreased after incubation with the histidyl-selective reagent DEPC. In time courses, the inhibition of succinate transport by DEPC was most effective after 20 min of incubation compared with 5 and 10 min (results not shown). As shown in Figure 1, 50% inhibition of succinate transport by NaDC-1 was seen after preincubation in 0.9 mM DEPC. The maximal inhibition by DEPC was approx. 80% at 5 mM. The inhibition of succinate transport by DEPC was less marked at pH 5.5 than at pH 7.5 (results not shown), consistent with previous studies of the effects of DEPC [17]. The inhibitory effects of DEPC on succinate transport by NaDC-1 were reversed by incubation with 25 mM hydroxylamine, verifying that the effect is due to modification of histidine sites (results not shown).

A previous study with kidney brush border membrane vesicles suggested that the inhibition of succinate transport by DEPC could be protected by the presence of substrate [11]. Experiments with NaDC-1 expressed in *Xenopus* oocytes were done by co-incubating succinate and DEPC as well as pre-incubating with succinate before adding succinate and DEPC. Table 2 shows the results of experiments in which oocytes expressing NaDC-1 were preincubated with buffer alone or containing 10 mM succinate, followed by a second preincubation with DEPC alone or together

Figure 1 Inhibition of succinate transport by DEPC

Xenopus oocytes expressing NaDC-1 were preincubated for 20 min with DEPC at concentrations between 1 and 5 mM. The DEPC was washed away and transport of 100 µM succinate was measured after 15 min. Uptake is expressed as a percentage of control uptake, measured in the absence of DEPC (2054 ± 42 pmol/h per oocyte; *n* = 5).

Table 2 Presence of substrate does not prevent inhibition by DEPC

Transport of succinate in oocytes expressing NaDC-1 was measured after two preincubations. First the oocytes were incubated with or without 10 mM succinate in Barth's solution for 10 min, after which the solutions were washed away. Second, the oocytes were incubated with or without 2 mM DEPC, in the presence or absence of 10 mM succinate, also for 10 min. The second set of solutions was also washed away and transport of 100 µM [¹⁴C]succinate was measured for 10 min as described in the Experimental section. The results are means ± S.E.M. (*n* = 6 independent batches of oocytes), expressed as percentages of controls in the absence of DEPC and succinate (1921.6 ± 134 pmol/h per oocyte; *n* = 6). The percentage inhibition by DEPC represents the mean of six individual determinations. There is no statistically significant difference between the inhibition by DEPC in the presence or absence of 10 mM succinate (*P* = 0.25, Student's *t* test).

Succinate concentration (mM) in preincubation solution	Transport of succinate (% of control)		Inhibition by DEPC (%)
	Without DEPC	With DEPC	
0	100 ± 7.0	43.4 ± 4.9	56.6 ± 4.9
10	90.6 ± 6.3	48.2 ± 7.3	46.1 ± 7.1

with succinate. Transport of succinate was then measured. No protective effect of substrate was observed, as indicated by the similar inhibition by DEPC in the presence or absence of succinate. Therefore, although NaDC-1 contains inhibitory DEPC-binding sites, the binding of DEPC at these sites is not affected by the binding of substrate.

Histidine to alanine mutations in NaDC-1

Site-directed mutagenesis was used to change each of the histidine residues systematically to alanine to determine whether a histidine residue is involved in the transport of succinate by NaDC-1. The secondary structure model of NaDC-1 [9], indicating the positions of the 11 histidine residues, is shown in Figure 2. According to this model, only one of the histidine residues, at position 102, is predicted to lie within a transmembrane domain. All of the other histidine residues in NaDC-1 are located in loops between transmembrane helices or at the extracellular C-terminus.

Figure 2 Secondary structure model of NaDC-1

The positions of the 11 histidine residues in the sequence are indicated by filled circles. Y represents the N-glycosylation site at the C-terminus.

Table 3 Succinate transport by histidine to alanine mutants of NaDC-1

The transport of 100 μ M [14 C]succinate was measured during a 15 min incubation in Na⁺-containing buffer. Transport is expressed as a percentage of wild-type NaDC-1 activity measured in oocytes from the same frog. Results are presented as means $\pm$ S.E.M.; *n* is the number of experiments using oocytes from different donor frogs.

Mutation	Succinate transport (% NaDC-1 activity)	<i>n</i>
H102A	91.1 $\pm$ 17.5	5
H106A	5.2 $\pm$ 1.7	4
H153A	115.0 $\pm$ 24.2	4
H210A	125.1 $\pm$ 10.4	5
H306A	68.2 $\pm$ 8.4	3
H355A	82.3 $\pm$ 7.9	3
H456A	97.5 $\pm$ 15.8	3
H500A	101.4 $\pm$ 15.8	3
H569A	96.5 $\pm$ 28.2	4
H576A	75.9 $\pm$ 5.1	3
H582A	116.5 $\pm$ 18.1	5

Transport activity in histidine mutants

Succinate transport was measured in *Xenopus* oocytes injected with cRNA for the wild-type NaDC-1 and each of the 11 histidine-to-alanine mutants. As shown in Table 3, most mutants showed little or no change in succinate transport compared with the wild-type control. Two of the mutants, H306A and H576A, exhibited some delay in their expression, by 1–2 days (results not shown), suggesting that those residues could be involved in protein trafficking. Only one of the 11 mutants, H106A, had a very low transport activity. The succinate transport rate in mutant H106A was decreased by approx. 95% compared with the transport in wild-type NaDC-1. However, the succinate transport activity of H106A was still measurable and approx. 4-fold that in uninjected oocytes (control uninjected oocytes,

5.8 $\pm$ 0.3 pmol/h per oocyte; H106A, 24 $\pm$ 3 pmol/h per oocyte; *n* = 5).

Kinetics of succinate and citrate transport in H106A

Figure 3 shows the kinetics of succinate transport in *Xenopus* oocytes expressing mutant H106A. This mutant transporter had a *K_m* for succinate of 0.6 mM and a *V_{max}* of 1.0 nmol/h per oocyte. For comparison, the wild-type NaDC-1 had a *K_m* for succinate of 0.6 $\pm$ 0.1 mM, and a *V_{max}* of 15.4 $\pm$ 1.3 nmol/h per oocyte (*n* = 4) (results not show). Although mutant H106A had very low rate of citrate transport at pH 7.5, approximately half that of succinate, it was possible to measure citrate transport at acidic pH. The stimulation of citrate transport at acidic pH was observed previously with the wild-type NaDC-1 [18]. At pH 5.5, mutant H106A had a *K_m* for citrate of 0.9 mM and a *V_{max}* of 0.4 nmol/h per oocyte (Figure 3B). Wild-type NaDC-1 has a *K_m* for citrate of 0.3 mM and a *V_{max}* of 23 nmol/h per oocyte at pH 5.5 [18]. Therefore the predominant effect of the H106A mutation is a large decrease in *V_{max}* for both succinate and citrate, and little or no change in substrate affinity.

Mutational analysis of position 106 in NaDC-1

Additional mutations were made at position 106 in NaDC-1, replacing the histidine residue with an arginine, aspartic acid or asparagine residue. As shown in Table 4, the replacement of His-106 with arginine resulted in a non-functional protein. The transport rate in mutant H106R was identical with that in uninjected control oocytes. However, replacement of His-106 with either an aspartic acid or asparagine residue resulted in little or no loss of activity. The histidine residue at position 106 is conserved in all of the members of the family related to NaDC-1, including hNaDC-1 and the renal Na⁺/sulphate co-transporter NaSi-1 [10]. Mutagenesis of His-106 to alanine in hNaDC-1 also

Figure 3 Kinetics of succinate (A) and citrate (B) transport in mutant H106A expressed in *Xenopus* oocytes

(A) Uptakes for 30 min were measured in Na^+ -containing buffers. Uptakes in control uninjected oocytes were subtracted from those in H106A cRNA-injected oocytes. The K_m for succinate was 0.6 ± 0.1 mM; the $V_{\max}$ was 1.0 ± 0.05 nmol/h per oocyte ($n = 5$). (B). Transport conditions were the same as in (A). The K_m for citrate was 0.9 ± 0.1 mM; the $V_{\max}$ was 0.4 ± 0.02 nmol/h per oocyte ($n = 5$).

Table 4 Succinate transport by NaDC-1 mutants at position 106

The histidine residue at position 106 was mutated to an alanine, arginine, aspartic acid or asparagine residue. The transport of $100 \mu\text{M}$ [^{14}C]succinate was measured during a 15 min incubation in Na^+ -containing buffer. Transport rates are expressed as percentages of wild-type NaDC-1 activity measured in oocytes from the same frog. The results are presented as means $\pm$ S.E.M. for experiments done with oocytes from three different donor frogs. The transport activity of NaDC-1 in the three experiments was 1130.5 ± 186 pmol/h per oocyte.

Mutation	Transport (% NaDC-1 activity)
H106A	4.6 ± 2.2
H106R	0.7 ± 0.7
H106D	96.1 ± 14.2
H106N	84.1 ± 7.7

resulted in a marked decrease in transport, by 85 % (results not shown).

Surface expression of histidine mutants

The decreases in transport $V_{\max}$ seen in mutant H106A could be the consequence of a decreased amount of transporter protein in the plasma membrane or a decrease in turnover number. Figure 4 shows the results of immunoblot analysis of biotinylated cell surface proteins from *Xenopus* oocytes probed with anti-NaDC-1 antibodies. The antibodies recognized the same two proteins in biotinylated membranes from oocytes injected with cRNA for NaDC-1 and the histidine mutants. In Tris/glycine gels containing 10 % polyacrylamide there was a smaller protein of 53 kDa and a broad band of protein centred at 66 kDa, and in Tricine gels (8 % polyacrylamide) the two proteins migrated at 50 and 68 kDa. There was no immunoreactive signal in plasma membranes prepared from control, uninjected oocytes (Figure 4A). The signal intensity in the Western blots seemed to correspond very well to the transport activity observed in NaDC-1 and the

histidine mutants. Mutant H106A was barely detectable in the membranes, indicating that the observed decrease in $V_{\max}$ is due to the absence of the protein from the membrane (Figure 4A). Interestingly, mutant H106R, which exhibits even lower transport activity than H106A, was more abundant in the membrane than H106A. The mutants H106N and H106D, which exhibit wild-type activity and kinetics (results not shown), had similar amounts of protein in the plasma membrane to those of the wild-type NaDC-1. The remaining histidine to alanine mutants of NaDC-1 were found in the plasma membrane with similar abundances to the wild-type NaDC-1 (Figure 4B).

Western blots of crude plasma membranes

Western blots were also prepared from plasma membranes of oocytes expressing NaDC-1 and mutant H106A (Figure 5). The plasma membranes were prepared by a differential centrifugation procedure and almost certainly contained other membrane populations, such as endoplasmic reticulum and Golgi. The apparent mass of the two proteins recognized by anti-(NaDC-1) antibodies in non-biotinylated membranes was slightly different from that in biotinylated samples, 55 and 77 kDa [15], and also reflects the fact that the samples were run in a Tricine gel containing 6 % polyacrylamide. The abundance of mutant H106A was approx. 55 % of that of NaDC-1 (55 kDa, 55 %; 77 kDa, 57 %), indicating that mutant H106A is synthesized by the oocytes. Therefore the major effect of the H106A mutation is the altered stability of the protein in the plasma membrane, either by decreased insertion of transporters into the membrane or by increased removal from the membrane.

Deglycosylation of plasma membrane proteins

The crude plasma membranes prepared from oocytes injected with NaDC-1 or mutant H106A were treated with the deglycosylation enzymes Endo H and PNGase F. PNGase F cleaves all

Figure 4 Western blot of cell-surface biotinylated membranes prepared from *Xenopus* oocytes injected with cRNA for NaDC-1 and His-106 mutants (A) and with cRNA for NaDC-1 and the remaining histidine mutants of NaDC-1 (B)

(A) The control lane contains proteins from uninjected oocytes. The samples were separated on a Tris/glycine gel containing 10% polyacrylamide. The blot was probed with a 1:10000 dilution of primary antibody directed against NaDC-1, followed by a 1:10000 dilution of horseradish peroxidase-conjugated anti-(rabbit IgG). The positions of size standards (in kDa) are shown at the left. (B) The samples were separated on Tricine gels containing 8% polyacrylamide. The blots were probed as described for (A).

types of N-linked oligosaccharides [19] and, as seen previously [15], treatment with PNGase F resulted in the loss of the two major immunoreactive bands at 55 and 77 kDa and the appearance of a single protein at approx. 48 kDa (Figure 5). This 48 kDa protein has the same apparent molecular mass as a non-glycosylated mutant of NaDC-1 called GM2, which lacks N-glycosylation sites [9,15]. Treatment of the membranes with Endo H, which prefers high-mannose oligosaccharides [19], affected the mass of only the smaller immunoreactive protein at 55 kDa, whereas the 77 kDa protein was Endo H-resistant (Figure 5). Interestingly, the buffer used for PNGase F digestion revealed the presence of an immunoreactive protein at 106 kDa, which was present in both the NaDC-1 and H106A oocytes. After treatment with PNGase F the molecular mass of the 106 kDa protein was diminished to approx. 96 kDa. The 106 kDa protein might represent a dimer of the 55 kDa protein, which is deglycosylated to a protein of 48 kDa. The results of the deglycosylation experiments show that the two immunoreactive proteins seen routinely when NaDC-1 is expressed in heterologous systems, such as oocytes and COS-7 cells (results not shown), represent differentially glycosylated forms of the transporter. Furthermore the relative amounts of the two glycosylated

Figure 5 Western blot of plasma membranes treated with the deglycosylation enzymes Endo H or PNGase F

Plasma membranes were prepared from oocytes expressing NaDC-1, mutant H106A or a mutant called GM2 that lacked N-glycosylation sites. The membranes were incubated in the appropriate buffers (see the Experimental section) with (+) or without (–) Endo H or PNGase F. The last three lanes show untreated membranes from the non-glycosylated GM2 mutant, wild-type NaDC-1 and mutant H106A. The samples were separated on a Tricine gel containing 6% polyacrylamide. The blot was transferred to nitrocellulose and probed as described in the legend to Figure 4. The positions of size standards (in kDa) are shown at the left.

Table 5 Effect of DEPC on transport by histidine mutants of NaDC-1

The transport of 100 μ M [14 C]succinate was measured during a 15 min incubation in Na $^{+}$ -containing buffer. Transport is expressed as a percentage of wild-type NaDC-1 activity measured in oocytes from the same frog. Results are presented as means $\pm$ S.E.M. (* $P < 0.05$) except where there were only two subjects; n is the number of experiments using oocytes from different donor frogs.

Mutation	Transport remaining (% control activity)	n
NaDC-1	42.2 $\pm$ 3.6	10
H102A	44.2 $\pm$ 4.3	5
H106D	45.8, 33.9	2
H106N	21.0, 16.5*	2
H153A	72.5 $\pm$ 5.2*	7
H210A	46.6 $\pm$ 4.6	5
H306A	31.8 $\pm$ 3.4	3
H355A	38.1, 37.4	2
H456A	44.8 $\pm$ 2.5	3
H500A	28.2, 27.0	2
H569A	55.4 $\pm$ 5.4*	6
H576A	31.8 $\pm$ 1.3	4
H582A	39.0 $\pm$ 12.7	4
H153,569A	82.1 $\pm$ 6.3*	4

proteins in mutant H106A seem to be very similar to those of wild-type NaDC-1.

Effect of DEPC on histidine mutants of NaDC-1

The sensitivity of the histidine mutants to inhibition by DEPC was tested. As shown in Table 5, succinate transport by most of the mutant transporters and wild-type NaDC-1 was inhibited by

approx. 58% after treatment with 2 mM DEPC. One of the histidine mutants, H106N, was more sensitive than NaDC-1 to inhibition by DEPC. Two of the histidine mutants, H153A and H569A, were less sensitive to DEPC inhibition than the wild-type NaDC-1. The double mutant H153,569A was also much less sensitive to DEPC inhibition. Therefore His-153 and His-569 probably represent multiple binding sites for DEPC in NaDC-1. However, these histidine residues are not required for transport because their replacement by alanine does not decrease transport activity. The histidine mutants were also tested for possible substrate protection by 10 mM succinate, similar to the experiment described in Table 2. There was no effect of pre-incubation with succinate on the extent of inhibition by DEPC (results not shown). Therefore the removal of a DEPC-binding site does not reveal a substrate-protectable histidine residue.

DISCUSSION

Previous studies of the Na⁺/dicarboxylate co-transporter in renal brush border membranes have implicated a histidine residue in substrate binding or transport [11]. With the recent isolation of the cDNA coding for NaDC-1 [6], the involvement of a histidine residue in transport could be tested directly. Each of the 11 histidine residues in NaDC-1 was replaced by alanine by using site-directed mutagenesis. The results of this study show that none of the histidine residues in NaDC-1 is required directly for transport. The replacement of 10 of the 11 histidine residues with alanine had no effect on the transport rate, whereas mutant H106A had markedly diminished transport activity. However, cell surface biotinylation experiments showed that mutant H106A was not present at the cell surface. Therefore the His-106 residue affects the expression or stability of NaDC-1 rather than its transport activity.

Of the 11 histidine residues in NaDC-1, only two (His-106 and His-576) are absolutely conserved in all members of the family related to NaDC-1, including hNaDC-1 and the Na⁺/sulphate co-transporter NaSi-1 [10]. His-106 is also found in distantly related proteins with no known function, in *Caenorhabditis elegans* and *Saccharomyces cerevisiae* (GenBank accession numbers Z30974 and Z77395). According to our secondary-structure model for NaDC-1, His-106 lies just outside transmembrane helix 3 in an extracellular loop. On the basis of our studies, it seems that His-106 is involved in the insertion or retention of the transporter in the plasma membrane. Mutant H106A is synthesized and glycosylated in the oocytes but the cell surface expression is very low. The mechanism by which His-106 regulates protein insertion or stability is not known. The imidazole side chain is not absolutely required because the histidine residue can be replaced with an asparagine or aspartic acid residue without much change in protein abundance or functional activity. Therefore the size of the residue at this position might be important. Interestingly, His-94 of melibiose permease in *Escherichia coli* seems to be involved in regulating protein insertion into the membrane. Mel permease mutants containing Asn, Gln or Arg residues in place of His-94 exhibit decreased rates of permease synthesis and membrane insertion, but the degradation rate of the mutant proteins is the same as that of the wild-type protein [20].

Our studies with the cloned Na⁺/dicarboxylate co-transporter show that histidine residues are not directly involved in transport and that there are no substrate-protectable histidine residues in NaDC-1. In contrast, Bindslev and Wright [11], using DEPC-treated brush border membrane vesicles, showed a decrease in transport inhibition in the presence of substrate. The reason for the apparent difference in the results is not known. One possible

explanation is that the effects seen in the previous study [11] were due to contamination of the brush border membranes by basolateral membranes, which contain a high-affinity Na⁺/dicarboxylate co-transporter [21]. Alternatively, a decrease in the amount of active DEPC in solution, either by degradation or by interference of the carbethoxylation reaction by succinate (as reported in other studies [22]), would lead to the same lack of transport inhibition as true substrate protection. In any case, with the use of the cloned transporter in an overexpression system there is no evidence for substrate protection of DEPC binding in NaDC-1, and the sequential removal of histidine residues from NaDC-1 does not impair function.

The DEPC-binding sites in NaDC-1 have been identified as His-153 and His-569. Replacement of these histidine residues by alanine markedly decreased the inhibition by DEPC of succinate transport. However, there was no change in transport function in the single mutants H153A or H569A, or in the double mutant H153,569A. Therefore the DEPC-binding sites in NaDC-1 are not located in or near the substrate-binding site. It is likely that the inhibitory effect of DEPC on NaDC-1 occurs by hindrance of the mobility of the carrier rather than the removal of a functional side group.

There is limited information on residues involved in substrate and cation binding in ion-coupled transport proteins. Typically, the direct involvement of a histidine residue in transport is seen in proteins that carry protons or that sense proton concentrations, such as *lac* permease [23], the Na⁺/H⁺ exchangers [24,25] and the anion exchanger AE2 [26]. The mechanism seen in *lac* permease involves alternating interactions between His-322 and Glu-269 [27]. A second histidine residue in *lac* permease, His-205, is thought to be involved in lactose transport through hydrogen bonding by both imidazole nitrogens [23]. Transport by NaDC-1 does not involve proton movements or pH sensitivity. The transport of succinate by NaDC-1 is insensitive to changes in pH, and transport is completely dependent on Na⁺ as the co-transported cation [18]. Although citrate transport by NaDC-1 is stimulated by acidic pH, this is mediated by a change in K_m for citrate but no change in V_{max} [18]. The different effects of pH on succinate and citrate transport suggest that the preferred substrates of NaDC-1 are divalent anions. The decrease in K_m for citrate with acidification is consistent with an increase in the concentration of protonated citrate species at acidic pH.

In conclusion, none of the 11 histidine residues in NaDC-1 is directly involved in transport of succinate. All of the residues, with the exception of His-106, can be replaced without affecting transport rate. Replacement of His-106 with alanine results in a decreased abundance of the mutant proteins in the membrane, with a consequent loss of activity, suggesting that this residue is involved in the expression or stability of the transporter protein. Finally, NaDC-1 contains at least two binding sites for DEPC, at His-153 and His-569, but neither of these residues seems to be located in the substrate-binding site.

We thank Dr. Bruce Hirayama for sending us his protocol for running Tricine SDS/PAGE gels. This work was supported by National Institutes of Health grants DK 46269 and DK 02429. H.G.V. was supported by the Howard Hughes Medical Institute Undergraduate Biological Science Education Program.

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Received 3 October 1997/8 December 1997; accepted 6 January 1998