J. Membrane Biol. 176, 133–141 (2000)
DOI: 10.1007/s00232001082

The Journal of
Membrane
Biology

Cysteine Residues and the Structure of the Rat Renal Proximal Tubular Type II Sodium Phosphate Cotransporter (Rat NaPi IIa)

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Received: 13 December 1999/Revised: 31 March 2000

Abstract. The rat renal Na/P_i cotransporter type IIa (rat NaP_i IIa) is a 637 amino acid protein containing 12 cysteine residues. We examined the effect of different cysteine modifying methanethiosulfonate (MTS)-reagents and the disulfide bond reducing agent tris(2-carboxyethyl)phosphine (TCEP) on the transport activity of wild-type and 12 single cysteine substitution mutants of rat NaPi IIa expressed in *Xenopus laevis* oocytes. The transport activity of the wild-type protein was resistant to three membrane impermeant MTS-reagents (MTSEA, MTSET and MTSES). In contrast, membrane permeant methyl methanethiosulfonate (MMTS) and TCEP inhibited the transport activity of both the wild-type, as well as all the single mutant proteins. This indicated the existence of more than one functionally important cysteine residue, not accessible extracellularly, and at least 2 disulfide bridges. To identify the disulfide bridges, three double mutants lacking 2 of the 3 cysteine residues predicted to be extracellular in different combinations were examined. This led to the identification of one disulfide bridge between C306 and C334; reconsideration of the topological model predictions suggested a second disulfide bridge between C225 and C520. Evaluation of a fourth double mutant indicated that at least one of two disulfide bridges (C306 and C334; C225 and C520) has to be formed to allow the surface expression of a functional cotransporter. A revised secondary structure is proposed which includes two partially repeated motifs that are connected by disulfide bridges formed between cysteine pairs C306-C334 and C225-C520.

Key words: Phosphate transport — NaPi — Cysteine residues — TCEP — MTS-reagents — Disulfide bonds

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Introduction

Renal proximal tubular reabsorption of inorganic phosphate is mediated by a sodium/phosphate (NaP_i) cotransport system. Human and rat type IIa Na/P_i cotransporters have been identified using expression cloning strategies; homology based screening procedures led to the identification of this transporter in different species including mice (Magagnin et al., 1993; Murer & Biber, 1997; Murer et al., 1998). Type IIa Na/P_i-cotransporters were found to be located in the brush border membrane of mammalian proximal tubules (Custer et al., 1994; Murer et al., 1996). Based on gene-inactivation experiments in mice, it has been concluded that brush border membrane Na^+-dependent P_i transport is mainly (*ca.* 70%) related to the activity of the type IIa Na/P_i-cotransporter (Beck et al., 1998).

The rat isoform (NaPi-2; rat NaPi IIa) is a 637 amino acid glycoprotein, with a molecular mass of 90–100 kDa (Magagnin et al., 1993; Murer et al., 1996; Murer & Biber, 1997). based on our current topology model, of the 12 cysteine residues, 3 are predicted to be extracellular, 4 to be intracellular and 5 are within transmembrane domains (Magagnin et al., 1993; Lambert et al., 1999). Rat brush border membrane vesicle Na/P_i-cotransport, mediated mostly by the type IIa Na/P_i cotransporter, is inhibited by Hg^{2+} and other heavy metal ions as well as by membrane permeant but not impermeant SH-group modifying reagents. This suggests an involvement of functionally important SH-groups not accessible from the outside (Pratt & Pedersen, 1989; Loghman-Adham, 1992). Furthermore, after treatment of brush border membranes with dithiothreitol (DTT) or other reducing agents, the rat type IIa Na/P_i cotransporter dissociates into two fragments of about 40 kDa and 45 kDa (Biber et al., 1996; Boyer et al., 1996; Xiao et al., 1997). It has been suggested that disulfide bond(s) connect the two parts of the molecular (Biber et al., 1996).

Moreover, in studies on rat brush border membranes it was observed that the appearance of the transporter specific fragments correlates with a loss in Na^+-dependent P_i-transport function (Xiao et al., 1997).

Phosphate transport mediated by the human type IIa Na/P_i cotransporter (NaPi-3) expressed in *Xenopus* oocytes is inhibited by Hg^{2+} and other heavy metal ions, which suggests an interaction of these ions with free sulfhydryl groups. Furthermore, it has been shown that only membrane-permeable cysteine modifying reagents are able to inhibit NaP_i-3 mediated Na/P_i-cotransport (Wagner et al., 1996). Therefore extracellular cysteine residues are either not accessible to the reagents or are not located in functionally important regions of the protein. For the flounder isoform of the type II Na/P_i-cotransporter (NaPi-5), injection of cRNAs corresponding either to the N-terminal or C-terminal part of the transporter (separated in the second, large extracellular loop) in *Xenopus* oocytes resulted in increased Na/P_i-cotransport activity only if both RNAs were coinjected. Although this indicates that a cleavage within this part of the transporter is still compatible with transport function (Kohl et al., 1998), the above-mentioned studies on brush border membranes would further suggest that association of the two parts by one or more disulfide bridges is necessary for function.

The aims of the present study were to identify cysteine residues that are critical for transport function and to test for their involvement in the formation of putative disulfide bridges. We characterized the effects of various membrane permeant and impermeant cysteine modifying methanethiosulfonate (MTS) reagents on the transport function ($^{32}P_i$ uptake) of wild type (WT) and 12 single cysteine mutants of rat NaPi IIa expressed in *Xenopus* oocytes. The data indicated that the inhibition observed after treatment with membrane permeant cysteine modifying reagent requires the interaction with more then one residue, not accessible from the outside. Incubation with the disulfide bridge reducing reagent tris(2-carboxyethyl)phosphine (TCEP), led to a dose-dependent loss of transport function in the wild-type (WT) transporter as well as in all single cysteine substitutions, which suggested the involvement of more then one disulfide bridge in the structure/function of the type IIa Na/P_i cotransporter. Reconsideration of secondary structure predictions and construction of 4 different double cysteine substitution mutants suggested the existence of most likely 2 disulfide bridges and their location at C306/C334 and C225/C520, respectively. The formation of at least one of these bridges is required for the proper expression of a functional transporter.

Materials and Methods

Materials

Oligonucleotide primers were obtained from Microsynth (Balgach, CH), the mutagenesis kit was obtained from Strategene (Switzerland), the different MTS reagents from Toronto research chemicals (TRC, Canada) and TCEP from Pierce (Rockford, IL). Other reagents were obtained from Fluka (Buchs, Switzerland). All constructs were cloned in pSport1 (Gibco BRL).

Mutations/constructs

We substituted 12 cysteine residues by serine residues (for numbering and position *see also* Fig. 8). Mutations were introduced following the Stratagene Quickchange Site-Directed Mutagenesis Kit manual. Briefly, 10 ng of the plasmid containing the rat NaPi IIa cDNA were amplified with 2.5 U of Pfuturbo® DNA polymerase in the presence of 250 nM of primers. PCR amplification was performed as 20 cycles at 95°C (30 sec), 55°C (1 min) and 68°C (12 min). Then 10 U of Dpn I were added directly to the amplification reaction and the sample was incubated for 1 hr at 37°C to digest the parental, methylated DNA. XL1-blue supercompetent cells were transformed with 1 μl reaction mixture and plated onto LB-ampicillin-methicillin plates. Constructs were verified by sequencing.

Xenopus laevis Oocyte Expression and Transport Assay

The procedures for oocyte preparation and cRNA injection, as well as the $^{32}P_i$-uptake assay have been described in detail elsewhere (Werner et al., 1990). briefly, in vitro synthesis and capping of cRNAs were done by incubating the rat NaPi IIa constructs, previously linearized by Not I digestion, in the presence of 40 U of T7 RNA polymerase (Promega) and Cap Analog (NEB). Oocytes were injected with either 50 nl of water or 50 nl of water containing 5 ng of cRNA. $^{32}P_i$-uptake was measured 3 days after injection (Werner et al., 1990).

MTS Incubation

Oocytes were incubated for 5 min at room temperature in Barth's solution having the following composition (in mM) KCl (1), $MgSO_4$ (0.82), $CaCl_2$ (0.41), $Ca(NO_3)_2$ (0.33), $NaHCO_3$ (2.4), NaCl (88) and Hepes (10), adjusted to pH 7.4. MTS reagent stocks were prepared each day at 100 mM in anhydrous DMSO and diluted to the desired concentration immediately before use. Concentrations of DMSO did not exceed 1%. Control experiments showed that 1% DMSO had no effect on transporter function.

TCEP Incubation

Oocytes were incubated with Barth's solution containing the indicated TCEP concentrations for 30 min at room temperature. For all of the above treatments, prior to uptake measurement, the oocytes were washed extensively (3×) with Barth's solution.

Immunoblot and Streptavidine Precipitation of Oocytes Homogenates

Yolk-free homogenates were prepared 3 days after injection (H_2O or cRNA). Pools of 5 oocytes were lysed together with 100 μl of homogenization buffer [1% Elugent (Calbiochem) in 100 mM NaCl, 20 mM Tris/HCl, pH 7.6], by pipetting the oocytes up and down (Turk et al., 1996). To pellet the yolk proteins, samples were centrifuged at 16,000 × *g* for 3 min at room temperature. Biotin-streptavidin precipitation was performed as described previously (Hayes et al., 1994): briefly, 90 μl of the homogenate were incubated for 2 hr at 4°C with

20 μl of immobilized streptavidin. After washing, the proteins were eluted with 20 μl of 2× loading buffer (4% SDS, 2 mM EDTA, 20% glycerol, 0.19 M less Tris/HCl pH 6.8, 2 mg/ml bromphenol blue) and treated as for the Western blot. For Immunoblot, 10 μl of supernatants in 2× loading buffer were separated on a SDS-PAGE gel with the indicated percentage and separated proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Kassel, Germany). The membrane was then processed according to standard procedures (Sambrook et al., 1989) using rabbit polyclonal antibodies raised either against an NH_2- or COOH-terminal synthetic peptide of the rat NaPi IIa cotransporter. The specificity of the antibodies was demonstrated previously (Custer et al., 1994). Immunoreactive proteins were detached with a chemiluminiscence system (Pierce).

All experiments have been repeated at least 3 times with different batches of oocytes; data obtained in a representative experiments are included. For uptake studies the mean values ± SE of 8 oocytes are given.

Results and Discussion

Effect of Cysteine Modifying and Reducing Agents on Wild-type Rat NaPi IIa Function

Four MTS-reagents were used to investigate the effects of modification of sulfhydryl groups on rat NaPi IIa function: Methyl methanethiosulfonate (MMTS) is uncharged and membrane permeant, whereas the positively charged 2-aminoethyl methanethiosulfonate, hydrobromide (MTSEA) and [2-(trimethylammonium)ethyl] methanethiosulfonate bromide (MTSET) and the negatively charged sodium (2-sulfonatoethyl)methanethiosulfonate (MTSES) are nominally membrane impermeant (Smith, Maggio & Kenyon, 1975; Akabas et al., 1992; Stauffer and Karlin, 1994; Holmgren et al., 1996). We found that at 1 mM none of the three charged reagents had a significant effect on the wild-type (WT) protein function (Fig. 1*A*); even when preincubated at concentrations up to 10 mM no inhibition could be observed (*data not shown*). In contrast, when oocytes expressing the WT were preincubated with MMTS, transport activity was decreased by 70% with the maximal effect at 1 mM (Fig. 1*B*). The lack of an effect from any of the charged reagents suggested that modified cysteines at or near functionally important sites were not accessible extracellularly.

As a further confirmation of this conclusion, we incubated oocytes with biotin-labeled MTSEA (MTSEA-biotin). We were unable to streptavidin-precipitate any WT protein after incubation with MTSEA-biotin. In contrast, a mutant with a novel cysteine residue in the predicted 3rd extracellular loop of the protein (A456C, Fig. 8) could be readily precipitated after exposure to 100 μM MTSEA biotin (Fig. 2*A* and *B*). Two possible explanations can be offered for the inaccessibility of cysteine residues in the wild type protein to impermeant alkylating reagents: (i) extracellular cysteine residues are involved in disulfide bridges (*see below*); (ii) extracellular cysteine residues are 'buried' in the membrane or other structures. In view of the above control experiment with the cysteine insertion mutant we favor the first explanation.

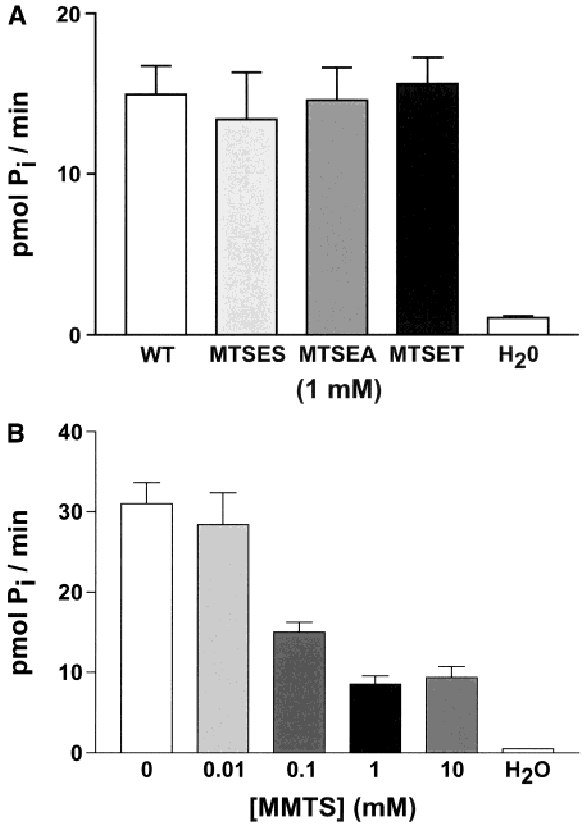

Fig. 1. (*A*) Effect of incubation in 1 mM of different charged MTS-reagents on rat NaPi IIa transport: Oocytes expressing rat NaPi IIa were incubated in 1 mM or 10 mM MTSES, MTSEA or MTSET for 5 min. After washing, the cells were incubated during 20 min at 25°C in $^{32}P_i$. After extensive washing, single oocytes were transferred to vials and the $^{32}P_i$ uptake was measured. The bars represent the mean ± SE of 8 oocytes of a representative experiment. (*B*) Dose-dependent inhibition of rat NaPi IIa transport by MMTS: Oocytes expressing rat NaPi IIa were incubated in the indicated concentrations of MMTS for 5 min. After washing, the cells were incubated during 20 min at 25°C in $^{32}P_i$. After extensive washing, single oocytes were transferred to vials and the $^{32}P_i$ uptake was measured. The bars represent the mean ± SE of 8 oocytes from a representative experiment. H_2O indicates water injected oocytes.

To test for the presence of functionally important disulfide (S-S) bridges, we incubated oocytes that expressed the WT protein with the reducing agent tris(2-carboxyethyl)phosphine (TCEP) and then tested for transport function as above. TCEP was chosen because other reagents (EtSH, DTT) must be used at very high concentrations (EtSH: 4%, DTT: 0.5 M) to reduce the S-S bridges of rat NaPi IIa when studied in isolated brush border membrane vesicles (Xiao et al., 1997). When expressed in oocytes, we found that NaPi IIa mediated P_i-transport was inhibited by TCEP with a maximal effect (80% inhibition) at 10 mM (Fig. 3). This result in-

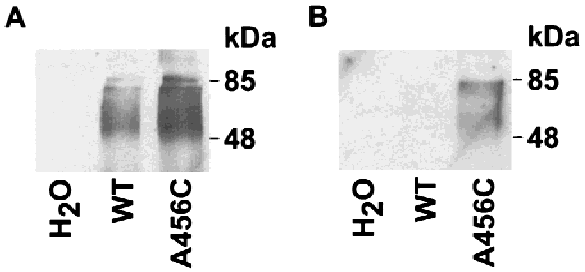

Fig. 2. Western blot and streptavidine precipitation of oocytes expressing WT or A456C protein: Five oocytes injected with water, WT or A456C cRNA were lysed in 100 μl lysis buffer after incubation for 5 min in 100 μM MTSEA-biotin. (*A*) 10 μl of the lysates were separated on a 9% SDS-gel and, after blotting, immunoreactive proteins were visualized by incubation with an antibody against the rat NaPi IIa NH_2-terminus. (*B*) 90 μl of each lysate were incubated with streptavidine beads. After washing, bound proteins were eluted with loading buffer (*see* Materials and Methods) and the elute was then treated as for *A*.

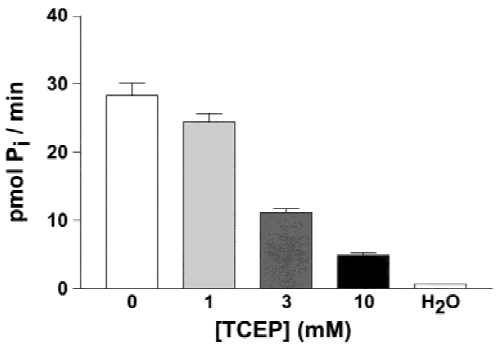

Fig. 3. Dose-dependent inhibition of rat NaPi IIa transport by TCEP: Oocytes expressing rat NaPi IIa were incubated in the indicated concentrations TCEP for 30 min. After washing, the cells were incubated during 20 min at 25°C in $^{32}P_i$ uptake was measured. The bars represent the mean ± SE of 8 oocytes from a representative experiment.

dicated, that one or more functionally important S-S bridges had been cleaved. The preceding paper describes the effects of disulfide cleavage in NaPi type IIa and type IIb in details (Lambert et al., 2000).

Single Cysteine-Serine Substitution Mutations Under Cysteine Modifying and Reducing Conditions

To ascertain if one of the 12 native cysteine residues were essential for transport function and would thus explain above inhibition by MMTS (Fig. 1*B*), we constructed the corresponding cysteine-serine single substitution mutations. The cRNAs of all these constructs were injected in *Xenopus* oocytes, the expression level was determined by Western blot and transport activity was quantitated as before.

Western blots (using an N-terminus specific antibody) of the single cysteine mutant proteins showed a broad band between approximately 60 and 75 kDa (Fig. 4). We assumed that this corresponded to the maximally glycosylated protein located at the oocyte surface that was responsible for measured transport activity (Hayes et al., 1994). The lower molecular weight bands were presumed to be either incompletely glycosylated or even nonglycosylated transporter proteins not yet expressed at the oocyte surface, which did not contribute to transport activity (Hayes et al., 1994). In the expression pattern of the WT protein, the fully glycosylated form was dominant, whereas the amount and pattern of expression varied between the different mutants. The mutants C62S, C474S and C597S were expressed less than the WT, but the expression pattern was similar to the WT, i.e., the 75 kDa band was dominant (Fig. 4). In mutants C306S and C334S the lower 60 kDa band was more pronounced, which suggested that lower amounts of fully glycosylated protein were present. In addition, a low molecular weight band of about 32 kDa was also detected; its identity will be discussed below.

All single cysteine replacement mutants still retained significant transport activity, which indicated that no single cysteine residue itself is critical for transport function (Table 1). It is difficult to compare the amount of protein expression (Fig. 4) to the amount of transport function (Table 1), since the former does not discriminate between membrane-bound protein and nonfunctioning protein in submembrane compartments. Nevertheless, some correlations were readily apparent. First, parallel to their low expression, the mutants C62S and C597S showed much lower transport activity than the WT, whereas this correlation was less obvious for C474S. Second, mutants C306S and C334S also showed less uptake than the WT, although high amounts of protein were expressed. Here, differences in the amount of fully and partially or nonglycosylated protein might explain this observation. Other mutants transported phosphate at a comparable or even higher level than the WT.

We also preincubated each of the single mutants with MMTS and tested for transport activity. We found no significant differences between the MMTS effect on the WT and the 12 mutant transporters (Table 1). This result indicated that no single cysteine residue itself was responsible for the MMTS-induced effects; i.e., there must be multiple intracellular or membrane-associated free cysteine residues that are accessible by MMTS and which are most likely located in or near functionally important regions.

Finally, we preincubated oocytes expressing the mutant constructs with TCEP, and we observed a decrease in uptake that was similar to the WT under the same conditions (Table 1). This result suggested the existence of more than one functionally important S-S bridge in the rat NaPi IIa protein, since for any one mutant at least one S-S bridge would not be present.

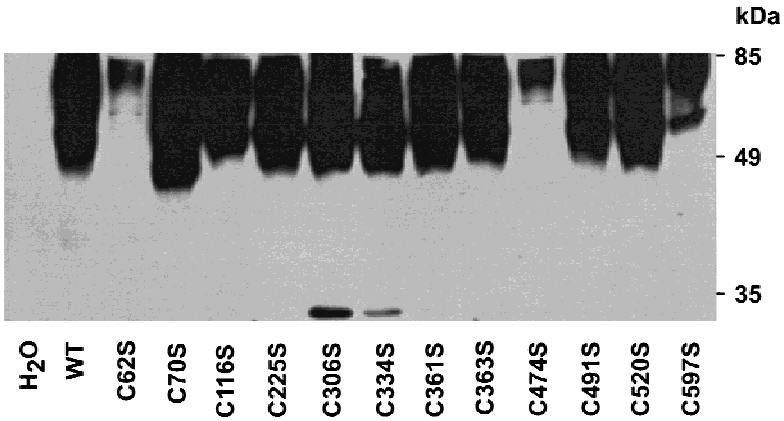

Fig. 4. Expression of the cysteine to serine mutants in *Xenopus laevis* oocytes. Oocytes were injected with WT or mutant rat NaPi IIa cRNAs. Yolk-free homogenates of 5 oocytes were separated in a 9% SDS-PAGE gel and analyzed by immunoblotting with a rabbit anti-rat NaPi IIa polyclonal antibody.

Table 1. Effect of cysteine modifying reagent MMTS, and reducing agent TCEP on WT and mutant transport

	% Wild-type activity	% Inhibition MMTS (1 mM)	% Inhibition TCEP (10 mM)
H_2O	4 ± 0.5727	0	0
WT	100 ± 18.87	49	75
C62S	21 ± 2.092	41	86
C70S	150 ± 10.46	37	83
C116S	112 ± 13.43	65	85
C225S	53 ± 8.586	41	63
C306S	35 ± 7.831	53	67
C334S	30 ± 4.611	33	75
C361S	103 ± 14.60	48	84
C363S	36 ± 12.26	60	45
C474S	67 ± 7.277	53	78
C491S	109 ± 9.972	49	72
C520S	113 ± 21.02	48	72
C597S	35 ± 4.315	52	70

The first column gives the transport rate of all the mutants in percent of the wild-type activity (±SE). The other columns give the relative inhibition of all the mutants after incubation with 1 mM MMTS (5 min), or 10 mM TCEP (30 min). The values are always based on the mean phosphate uptake rate of 8 oocytes in a representative experiment. Inhibitions were tested for significance using an unpaired *t*-test, all *P* values were below $P = 0.05$ indicating that the inhibitions were significant.

Double Cysteine-Serine Substitution Mutants and Identification of a S-S Bridge

To identify the cysteine pairs involved in forming these S-S bridges, we made serine substitutions of the three cysteine residues (C225, C306 and C334) that are predicted to be extracellular according to our previous model (Magagnin et al., 1993; Lambert et al., 1999). We constructed 3 double mutants, lacking the indicated cysteine residues in each combination (C225S/C306S; C225S/C334S; C306S/C334S). These double mutants were tested for expression (Fig. 5*A*) and transport activity (Fig. 5*B*). Two of the mutants (C225S/C306S and C225S/C334S) were only expressed in a form that was most likely partially or nonglycosylated and these showed essentially no transport activity. In contrast, mutant C306S/C334S showed an expression pattern similar to the WT and transport activity was like the single mutants C306S and C334S (Fig. 5*A* and *B*; Fig. 4; Table 1). Also in the Western blot made using the anti N-terminal antibody, a smaller band at about 32 kDa was present with these mutants (*data not shown, see below*).

To characterize this low molecular weight band, we performed Western blots of mutants C306S, C334S, C306S/C334S and the WT using higher percentage gels (12%), (*see* Fig. 6). The blots were developed using either anti-N-terminal or anti-C-terminal antibodies (Fig. 6*A* and *B*). In oocytes injected with WT or mutant cRNA, the protein pattern obtained with an N-terminal antibody (Fig. 6*A*) corresponded to those already shown in Figs. 4 and 5*A*, with major staining in the 75 to 60 kDa region and small molecular weight band(s) for the mutants at around 32 kDa (arrow, Fig. 6*A*). Figure 6*B* shows the same blot developed with a C-terminal antibody. This antibody appeared to be less specific than the N-terminal antibody because a distinct staining pattern was also observed in H_2O-injected oocytes. In WT and mutant cRNA injected oocytes, the transporter-specific band was observed in the 75 to 60 kDa range. For the 3 mutants, the C-terminal antibody recognized a peptide around 26 kDa (arrow, Fig. 6*B*), which was not visible in the WT transporter expressing oocytes. These findings would be consistent with the C- and N-terminal parts of the type IIa Na/P_i cotransporter being represented by two low molecular weight bands (~26 and ~32 kDa). If an S-S bridge is prevented between C306 and C334, the protein is apparently unstable, a proteolytic cleavage could occur and the corresponding protein fragments would then become visible (Fig. 6*A* and *B*). Since the molecular weights of the two fragments add up to approximately 60 kDa (corresponding to the partially or nonglycosylated transporter), we concluded that in the oocyte system this cleavage might occur prior to 'maturation' of the transporter, i.e., most likely prior to reaching the surface.

The two low molecular weight bands most likely also correspond to those bands observed in Western blots

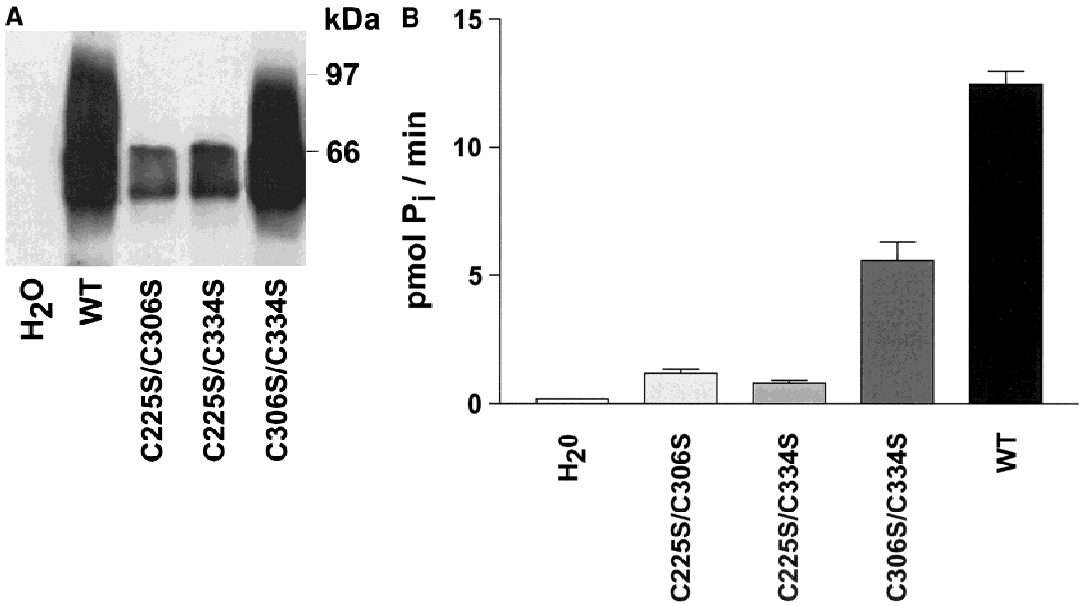

Fig. 5. (*A*) Expression of the double cysteine to serine mutants in *Xenopus laevis* oocytes. Pools of 5 oocytes expressing either the WT or the indicated double mutants were homogenized, the proteins were separated in a 9% SDS-PAGE gel and analyzed by immunoblotting with a rabbit anti-rat NaPi IIa polyclonal antibody. (*B*) Phosphate transport activity of the double mutants: Radioactive P_i uptake of oocytes was measured as described in Materials and Methods. The bars represent the mean ± SE of 8 oocytes of a representative experiment.

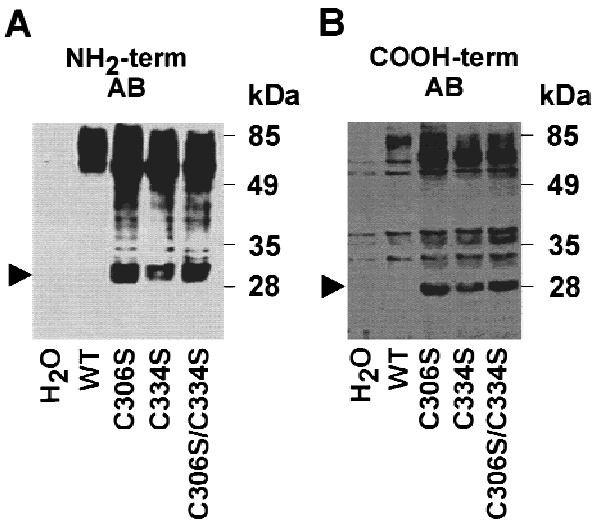

Fig. 6. Immunoblots of oocytes injected either with water, wild type cRNA or cRNA from the indicated mutants. Pools of 5 oocytes were homogenized and the proteins were separated in a 12% SDS-PAGE gel in the presence of DTT. The blots were developed with antibodies against the N-terminus (*A*) or against the C-terminus of rat NaPi IIa (*B*).

with brush border vesicles performed under reducing conditions (Biber et al., 1996; Boyer et al., 1996; Paquin et al., 1999). The identification of a cleavage site after amino acid 323, between cysteine 306 and 334 supports this interpretation (Paquin et al., 1999). Thus, in the brush border membrane a significant portion of transporter molecule might be present in a proteolytically cleaved form and held together by an S-S bridge. Alternatively cleavage might occur after reduction of the S-S bridge.

The above conclusions and experimental findings strongly support the existence of an S-S bridge between cysteines 306 and 334. Nevertheless, the sensitivity of the single mutants C306 and C334 (Table 1) as well as the double mutant C306S/C334S (*data not shown*) to TCEP pointed to the existence of yet another S-S bridge in NaPi IIa.

The behavior of the single and double mutants gave us some indication on the pair of cysteines involved. If S-S bridge C306-C334 cannot form (mutants C306S, C334S and C306S/C334S), the transporter was still active, TCEP-sensitive and partly expressed in the mature form. However, without S-S bridge C306-C334, and if C225 is also not present (mutants C225S/C306S and C225S/C334S), the protein was only expressed in the immature 60 kDa form and hardly any activity could be measured. In contrast, removal of C225 alone (C225S) resulted in an active transporter. This dependency on C225 suggested that there could be a functional relationship between the S-S bridge and C225. Since the removal of C225 was not consistent with the removal of S-S bridge C306-C334, one possible explanation would be that C225 was involved in a second S-S bridge with another cysteine residue. In this case, the TCEP effect on the C306S/C334S double mutant (*data not shown*), as well as the inactivity of the double mutants C225S/

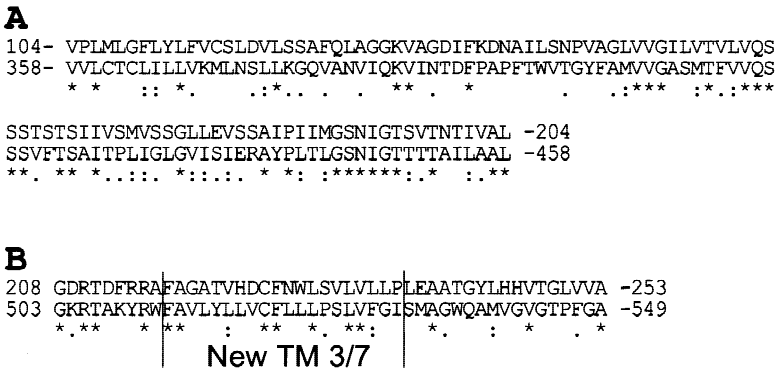

Fig. 7. Similarities between the NH_2- and COOH-terminal parts of rat NaPi IIa: Amino acids 104–204 and 358–458 (*A*) as well as amino acids 208–253 and 503–548 (*B*) are compared. Identical amino acids are marked with an asterisk, amino acids showing high similarity are marked with two points and amino acids showing low similarity are marked with one point.

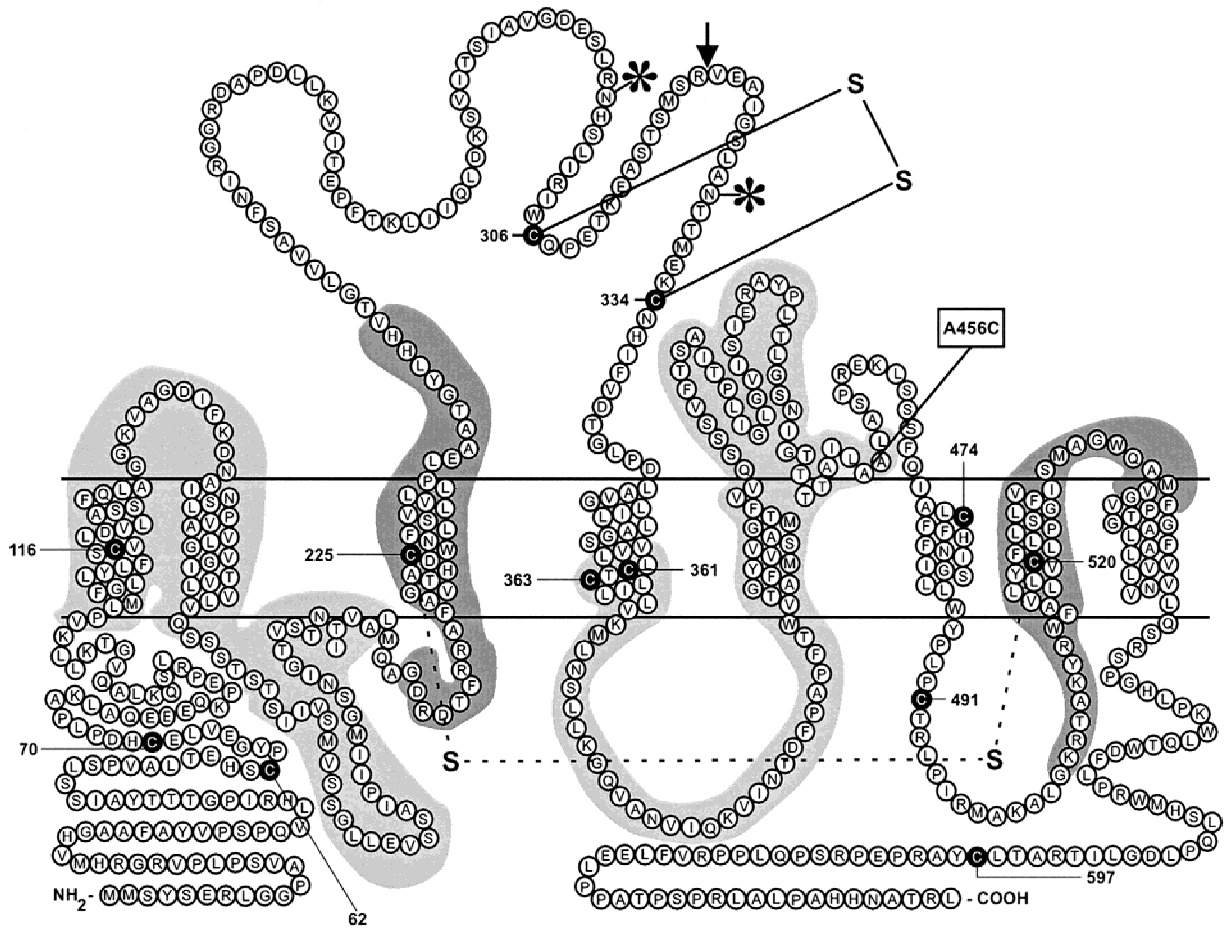

Fig. 8. Topology model of rat NaPi IIa based on our new studies: Rat NaPi IIa is a 637 amino acid (AA) protein with potentially 8 transmembrane domains (Magagnin et al., 1993). The asterisks represent two utilized glycosylation sites in the second extracellular loop (Hayes et al., 1994). The N- and the C-termini are located in the cytoplasm (Lambert et al., 1999), the 12 cysteine residues are marked black and are numbered according to the AA sequence. The arrow indicates a potential proteolytic cleavage site, which was identified in studies on brush border membrane vesicles (Paquin et al., 1999) and which separates the N- and C-terminal parts of the molecule. These are connected by one S-S bridge within extracellular loop 3 and a second, hypothesized bridge (dashed line) between cysteine residues in predicted transmembrane domain (TM) 3 and TM-7. Regions in the N-terminal and C-terminal halves which show strong sequence similarity are indicated by light gray and dark gray shadows, respectively. The two dark gray regions might be structurally associated by means of the second S-S bridge. In this model, previous TM-3 (Lambert et al., 1999) is now predicted to form a hydrophobic, membrane associated region within the intracellular loop 1, which is repeated in an homologous region in the extracellular loop (EL 3). Amino acids 216–236 formerly predicted to be part of EL-2 (Lambert et al., 1999) are now suggested to form TM-3, which is again repeated in a homologous region AA 511–531 suggested to form TM-7.

Table 2. Summary of the results obtained by the double mutants C225S/C306S, C225S/C334S, C306S/C334S and C306S/C520S

C225	C306	C334	C520	Function
X	X	✓	✓	No
X	✓	X	✓	No
✓	X	X	✓	Yes
✓	X	✓	X	No

A ✓ indicates that the cysteine residue is still present, an "X" indicates that it has been substituted by a serine residue. 'Yes' indicates at least 30% of remaining function and 'no' less than 10% of remaining function compared to the expression of the wild-type transporter.

C306S and C225S/C334S (Fig. 5*A* and *B*), would be explained by the removal of both S-S bridges.

A Revised Topology for NaPi IIa Incorporates Two S-S Bridges

To identify the possible partner cysteine of C225, we examined more closely the amino acid sequences flanking the cysteine residues. We found sequence similarities between amino acids (AA) 208-253 (containing cysteine 225) in the N-terminal part of the molecule and AA 503-549 (containing cysteine 520) in the C-terminal part (dark gray shading, Figs. 7*B*, 8). Comparing other parts of the N- and C-terminal parts with each other, again sequence similarities were found between AA 104-204 (N-terminal part) and AA 358-458 (C-terminal part) (light gray shading, Figs. 7*A*, 8). In the N-terminal part of the transporter, these sequences are separated by only 3 amino acids, whereas in the C-terminal part, there is a large stretch of 43 amino acids that separate the two repetitive units (Fig. 8).

Based on the above considerations, we have revised the current topology model (Lambert et al., 1999) for the type IIa Na/P_i-cotransporter by assuming that similar amino acid stretches might have a corresponding transmembrane orientation (Fig. 8). The similarities between the stretches AA 169-190 (formerly TM-3) and AA 423-444 (predicted EL-3), suggested that the stretch AA 169-190 is located intracellularly: this means that AA 216-236 would now form TM-3. Indeed, the stretch AA 216-236 shows a strong identity with stretch AA 511-531, which we previously predicted to form TM-7 (Fig. 7).

Our revised topology of the type II Na/P_i-cotransporter protein results in a scheme with two similar motifs. This predicted topology is also compatible with our recent studies on epitope accessibility (Lambert et al., 1999), in which we confirmed the extracellular location of AA 128 and 306, in the predicted first and second extracellular loop, respectively, as well as the intracellular location of both N- and C-termini. Based on above considerations, C520 could be a candidate to form an S-S bridge with C225, thereby connecting the two repeat motifs of the molecule.

To test this hypothesis, we constructed a mutant (C306S/C520S) in which we replaced C306 and C520 by serines. This mutant was expressed at low levels (*data not shown*) and was functionally impaired (*see* Table 2). This finding supported our hypothesis that a second S-S bridge is formed between C225 and C520. Moreover, these observations can account for the TCEP sensitivity in our mutation experiments. In the C306S, C334S and C306S/C334S mutants the TCEP sensitivity can be attributed to the cleavage of the suggested S-S bridge between C225 and C520. Likewise, in the C225S and C520S mutants, TCEP sensitivity is explained by cleavage of the bridge between C306 and C334. Thus, at least one of the S-S bridges is required for protein maturation and function (Table 2).

Conclusions

According to our revised topology for the rat type IIa Na/P_i cotransporter (Fig. 8), of the 12 native cysteine residues, only two are predicted to be extracellular (C306 and C334) and they form an S-S bridge. Four cysteine residues are predicted to be intracellular, whereas the remaining six are predicted to be in transmembrane segments. Two of the cysteine residues (C225 and C520) that are predicted in transmembrane segments may form a second S-S bridge. The effect of the membrane permeant SH-modifying reagent (MMTS) is explained by an interaction with the intracellular or membrane associated cysteine residues and the resistance to membrane impermeant SH-modifying reagents is attributed to the inaccessibility of the extracellular cysteines, since they are paired in the external S-S bridge. The proposed S-S bridges appear to link the C- and N-terminal parts of the molecule, which contain similar amino acid stretches. Finally, at least one S-S bridge is necessary to maintain the protein configuration and its associated function.

This work was supported by Grant 31-46523.96 to H.M. from the Swiss National Science Foundation. We thank Christian Gasser for professional art work.

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