Biochem. J. (2004) **380**, 283–287 (Printed in Great Britain)

Cysteine residues in the organic anion transporter mOAT1

Kunihiko TANAKA, Fanfan ZHOU, Kogo KUZE and Guofeng YOU[1]

Department of Pharmaceutical Science, Rutgers, The State University of New Jersey, 160 Frelinghuysen Road, Piscataway, NJ 08854, U.S.A.

Mouse organic anion transporter 1 (mOAT1) belongs to a family of organic anion transporters, which play critical roles in the body disposition of clinically important drugs, including anti-HIV therapeutics, anti-tumour drugs, antibiotics, anti-hypertensives and anti-inflammatories. mOAT1-mediated transport of organic anion PAH (*p*-aminohippurate) in HeLa cells was inhibited by the cysteine-modifying reagent PCMBS (*p*-chloromercuribenzenesulphonate). Therefore the role of cysteine residues in the function of mOAT1 was examined by site-directed mutagenesis. All 13 cysteine residues in mOAT1 were replaced by alanine, singly or in combination. Single replacement of these residues had no significant effect on mOAT1-mediated PAH transport, indicating that no individual cysteine residue is necessary for function. Multiple replacements at a C-terminal region (C335/379/427/434A; $Cys^{335/379/427/434} \rightarrow Ala$) resulted in a substantial decrease in transport activity. A simultaneous replacement of all 13 cysteine residues (C-less) led to a complete loss of transport function. The decreased or lack of transport activity of the mutants C335/379/427/434A and C-less was due to the impaired trafficking of the mutant transporters to the cell surface. These results suggest that although cysteine residues are not required for function in mOAT1, their presence appears to be important for the targeting of the transporter to the plasma membrane. We also showed that, although all cysteine mutants of mOAT1 were sensitive to the inhibition by PCMBS, C49A was less sensitive than the wild-type mOAT1, suggesting that the modification of Cys^{49} may play a role in the inhibition of mOAT1 by PCMBS.

Key words: *p*-aminohippurate (PAH), cysteine residue, HeLa cell, mutagenesis, organic anion transporter (OAT).

INTRODUCTION

Renal elimination of anionic drugs, xenobiotics and toxins is necessary for the survival of mammalian species. This process is mediated by vectorial transport from blood into the urine through the co-operative function of specific transporters in the basolateral and apical membranes of the proximal tubule epithelium [1]. The first step of this process is the extraction of organic anions from the peritubular blood plasma into proximal tubule cells through, in part, the OAT (organic anion transport) pathway. As a result, the OAT pathway is one of the major sites in the body for drug clearance/detoxification, but it is also the site for drug–drug interaction and drug-induced nephrotoxicity. To maximize therapeutic efficacy and minimize toxicity, the transporter structure–function relationships must be defined.

We have cloned the cDNA of a member of the family of OATs from mouse kidney (mOAT1) [2,3]. The functional characterization revealed that OATs interact with and transport a wide variety of clinically important anionic drugs [1], including β-lactam antibiotics, diuretics, non-steroidal anti-inflammatory drugs, anti-HIV therapeutics, anti-tumour drugs and inhibitors for angiotensin-converting enzyme. Computer modelling, based on hydropathy analysis, predicted that these proteins have 12 putative membrane-spanning segments and multiple consensus sites for glycosylation and phosphorylation. Indeed, we have shown that glycosylation is necessary for the targeting of mOAT1 onto the plasma membrane [3], and phosphorylation of mOAT1 resulted in a down-regulation of its function [4].

A common structural feature of all the OATs cloned to date is the presence of four cysteine residues in the first extracellular loop and several at the various other sites. In several previous studies with rabbit renal basal-lateral membrane vesicles [5], and isolated perfused snake renal tubules [6], it was reported that OAT system was sensitive to the inhibition by cysteine-modifying reagents, such as PCMBS (*p*-chloromercuribenzenesulphonate). These results indicate that OAT system contains cysteine residues, which are essential for the transport activity. Based on these previous observations, and the conservation of cysteine residues among all of the cloned OATs, we predicted that the cysteine residues in mOAT1 might play important roles for its function. The present work was undertaken to examine this hypothesis using chemical modification and site-directed mutagenesis approaches in conjunction with functional assay.

EXPERIMENTAL

Materials

PCMBS was purchased from Toronto Research Chemicals (Ontario, Canada). ^{14}C-Labelled PAH (*p*-aminohippurate) was from NEN Life Science Products (Hercules, CA, U.S.A.). NHS-SS-biotin [succinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate] and streptavidin–agarose beads were purchased from Pierce (Rockford, IL, U.S.A.). All other reagents were purchased from Sigma (St. Louis, MO, U.S.A.).

Site-directed mutagenesis

Mutant transporters were generated by site-directed mutagenesis of cysteine to alanine of mOAT1–Myc. mOAT1–Myc contains a 10-amino-acid c-Myc tag at the C-terminus of mOAT1. Previous studies from our laboratory showed that the mOAT–Myc protein retained the functional properties of the native (unmodified) structure [3,4]. The mutant sequences were confirmed by the dideoxy-chain termination method.

Abbreviations used: NHS-SS-biotin, succinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate; OAT, organic anion transporter; mOAT1, mouse OAT1; PAH, *p*-aminohippurate; PCMBS, *p*-chloromercuribenzenesulphonate; WT, wild-type; C335A etc., $Cys^{335} \rightarrow Ala$ replacement etc.

[1] To whom correspondence should be addressed (e-mail gyou@cop.rutgers.edu).

© 2004 Biochemical Society

Expression in HeLa cells

HeLa cells were grown at 37 °C and 5 % CO_2/95 % air in MEM (Invitrogen, San Diego, CA, U.S.A.) supplemented with 10 % fetal bovine serum. Confluent HeLa cells were infected with recombinant vTF-7 vaccinia virus and then transfected with DNA plasmid using Lipofectamine™ 2000 reagent (Invitrogen) following the established protocol [7]. Transfected cells were incubated for 14–20 h at 37 °C and then used for transport assays and cell surface biotinylation.

Treatment with PCMBS

HeLa cell monolayers were washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 and 1.4 mM KH_2PO_4, pH 7.3) containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS/CM). Monolayers were then incubated with PCMBS (for 10 min) at the stated concentrations at 23 °C, and then washed four times prior to isotopic transport measurements.

Transport measurements

Uptake solution was added to each well. The uptake solution consisted of PBS/CM and [^{14}C]PAH. At the times indicated in the Figure legends, the uptake was stopped by aspirating off the uptake solution and rapidly washing the well with ice-cold PBS. The cells were then solubilized in 0.2 M NaOH, neutralized in 0.2 M HCl, and aliquots were taken for liquid scintillation counting. Uptake count was standardized by the amount of protein in each well. The results are expressed as the means ± S.E.M. ($n = 3$).

Cell surface biotinylation

Cell surface expression levels of mOAT–Myc were examined using the membrane-impermeant biotinylation reagent, NHS-SS-biotin (Pierce). The transporters were expressed in HeLa cells in 6-well plates using vaccinia T7/Lipofectamine™ 2000 as described above. After 20 h, the medium was removed and the cells were washed twice with 3 ml of ice-cold PBS/CM (pH 8.0). The plates were kept on ice and all solutions were kept ice cold for the remainder of the experiment. Each well of cells was incubated with 1 ml of NHS-SS-biotin (1 mg/ml in PBS/CM) in two successive 20 min incubations on ice with very gentle shaking. The reagent was freshly prepared for each incubation. After biotinylation, each well was briefly rinsed with 3 ml of PBS/CM containing 100 mM glycine, then incubated with the same solution for 20 min on ice, to ensure complete quenching of the unreacted NHS-SS-biotin. The cells were then dissolved on ice for 1 h in 400 μl of lysis buffer [10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1 % SDS, 1 % Triton X-100 and protease inhibitors (200 μg/ml PMSF and 3 μg/ml leupeptin), pH 7.4]. The unlysed cells were removed by centrifugation at 15 700 ***g*** at 4 °C. Streptavidin–agarose beads (50 μl) were then added to the supernatant to isolate cell membrane protein. mOAT1 was detected in the pool of surface proteins by PAGE and immunoblotting using an anti-Myc antibody.

Electrophoresis and immunoblotting

Protein samples (with equal amount) were resolved by SDS/PAGE (7.5 % minigel) and electroblotted on to PVDF membranes. The blots were blocked for 1 h with 5 % non-fat dried milk in PBS/0.05 % Tween, washed and incubated for 1 h at 23 °C with monoclonal anti-Myc antibody (1:1000). The membranes were washed, and then incubated with goat anti-mouse IgG conjugated to horseradish peroxidase (1:20 000), and signals were detected by SuperSignal West Dura Extended Duration Substrate kit (Pierce).

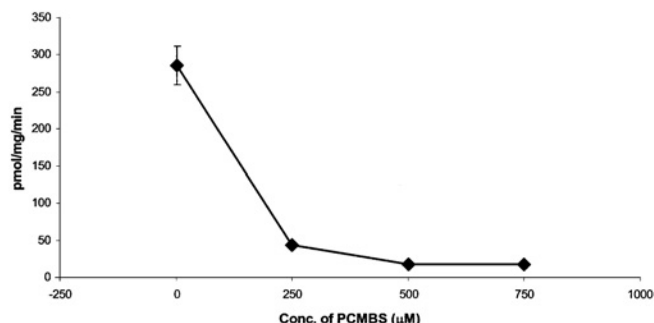

Figure 1 Dose–response curve of PCMBS inhibition

HeLa cells expressing mOAT1 were preincubated with concentrations of PCMBS of up to 0.75 mM for 10 min. The solutions were washed away and uptake of 20 μM PAH was measured over a 10 min time period. The results are expressed as the means ± S.E.M. ($n = 3$).

Immunofluorescence of transfected cells

After transfection (20 h), HeLa cells were washed three times in PBS, fixed for 15 min at 23 °C in 4 % paraformaldehyde in PBS, and rewashed in PBS. The fixed cells were then permeabilized with 0.1 % Triton X-100 for 10 min. The cells were incubated for 30 min at 23 °C in PBS containing 5 % goat serum and then incubated for 1 h in the same medium containing anti-Myc antibody (1:100) at 23 °C. The cells were washed, and bound primary antibodies were detected by reaction with FITC-coupled goat anti-mouse IgG (1:100; Chemicon) for 1 h. Cells were thoroughly washed, and the coverslips were mounted in GEL/MOUNT™ (Biomeda, Foster City, CA, U.S.A.). Samples were visualized by fluorescence microscopy.

Statistics

To test the significance of differences between data sets, Student's *t* test was performed.

RESULTS

Effects of PCMBS on mOAT1 function

Previous studies with rabbit renal basal-lateral membrane vesicles and isolated perfused snake renal tubules [5,6] indicated that the OAT system contains functionally important cysteine residues that are sensitive to the inhibition by cysteine-modifying reagents, such as PCMBS. The cloned mOAT1 expressed in HeLa cells is also sensitive to the inhibition by PCMBS. As shown in Figure 1, pretreatment of mOAT1-expressing cells with PCMBS led to a concentration-dependent decrease in mOAT1-mediated transport of PAH, a protypical substrate for OAT. About 85 % inhibition was reached with 0.25 mM PCMBS. This result is consistent with previous observations [5,6].

Cysteine-to-alanine mutations in mOAT1

To determine whether cysteine residues are involved in the transport of PAH by mOAT1, site-directed mutagenesis was used to change all 13 cysteine residues to alanine, singly or in combination. The secondary structure model of mOAT1, indicating the positions of the 13 cysteine residues, is shown in Figure 2. The nomenclature for cysteine mutants of mOAT1 containing multiple mutations is shown in Table 1.

© 2004 Biochemical Society

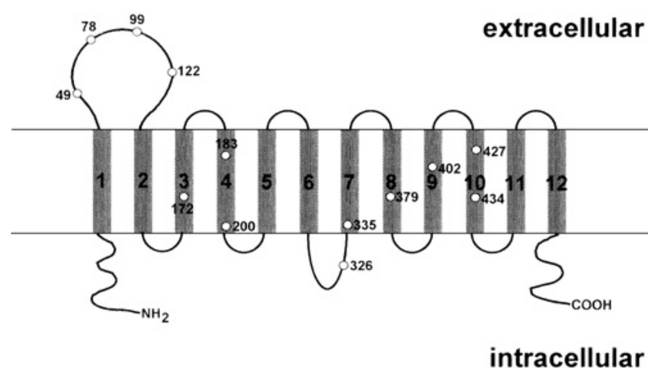

Figure 2 Secondary structure model of mOAT1

The transmembrane domains are numbered from 1–12. The positions of 13 cysteine residues are shown as open circles and numbered.

Table 1 Nomenclature of mOAT1 mutants containing multiple mutations

Name	Characteristics
A	Contains N-terminal mutations (C49/78/99/122A)
B	Contains mid-portion mutations (C172/183/326/402A)
C	Contains mid-portion mutations (C172/200/402A)
D	Contains C-terminal mutations (C335/379/427/434A)
E	All 13 cysteine residues mutated to alanine

Analysis of the effect of single replacement of cysteine residues

PAH transport was measured in HeLa cells transfected with cDNAs encoding WT (wild-type) mOAT1 and its cysteine mutants with single replacement. As shown in Figure 3, none of the single replacements had any significant effect on transport function. All the mutants were expressed at the plasma membrane, as demonstrated by cell-surface-biotinylation experiments (Table 2). The cell-surface expression levels of the mutants seemed to correlate with the transport activities observed in Figure 3.

Table 2 Quantification of cell surface expression of cysteine mutants

HeLa cells, expressing WT mOAT1 and its mutants, were labelled with NHS-SS-biotin. The labelled proteins were separated by SDS/PAGE (7.5 % gel) and followed by Western blot analysis with anti-Myc antibody. The quantification of the cell surface expression levels of cysteine mutants was obtained by densitometry analysis.

Mutant	Cell surface expression of protein (% of WT)
WT mOAT1	100.0
C49A	38.1
C78A	77.6
C99A	54.1
C122A	42.9
C172A	79.2
C183A	44.5
C200A	68.2
C326A	106.3
C335A	115.8
C379A	73.3
C402A	91.0
C427A	111.9
C434A	30.0

Analysis of the effect of multiple replacements of cysteine residues

PAH transport was then measured in HeLa cells transfected with cDNAs for WT mOAT1 and its cysteine mutants with multiple replacements. Mutants with multiple replacements (Figure 4) at the N-terminus (mutant A) and the mid-portion of mOAT1 (mutants B and C) showed transport activities comparable with that of WT mOAT1. In contrast, mutants with multiple replacements at the C-terminus (mutant D) showed a substantial (80 %) decrease in transport activity. Mutants with all 13 cysteines replaced (mutant E, C-less) showed a complete loss of transport function. Cell-surface-biotinylation experiments showed that the little or lack of transport activity of mutants D and E was correlated with the little or lack of plasma membrane expression, as compared with that of WT mOAT1 (Figure 5), although the total cellular protein of these mutants were similar to that of WT (results not shown). These results suggest that mutants D and E may have lost the ability to traffic to the plasma membrane.

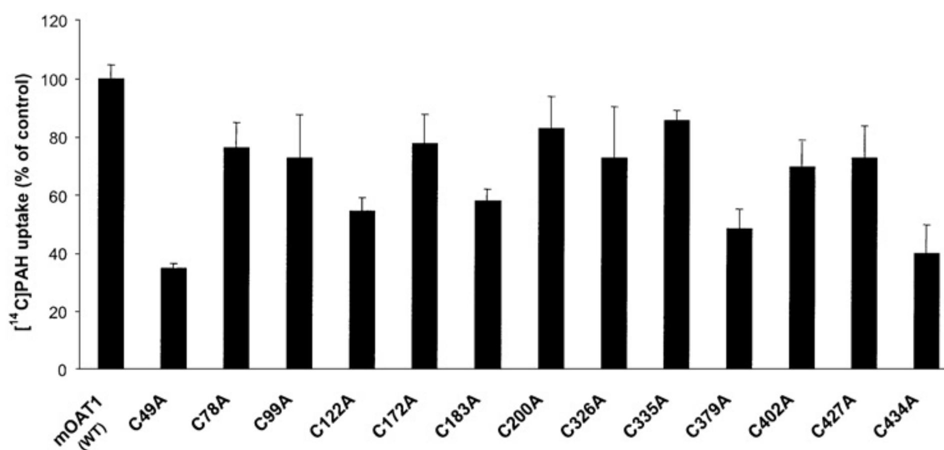

Figure 3 Effect of single replacement of cysteines on mOAT1 function

Cells were transfected with cDNAs for WT mOAT1 and the cysteine mutants. After transfection (24 h), [^{14}C]PAH (20 μM) uptake was measured. The results are presented as percentage of the control and are expressed as the means ± S.E.M. ($n = 3$).

© 2004 Biochemical Society

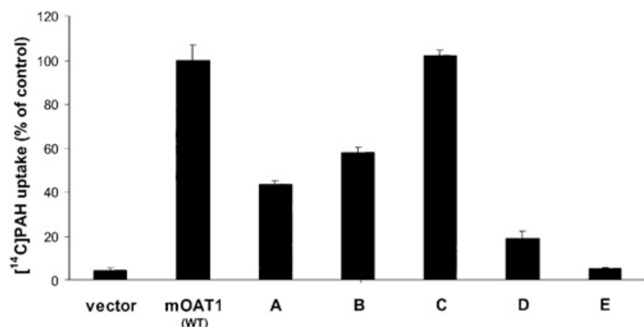

Figure 4 Effect of multiple replacement of cysteines on mOAT1 function

Cells were transfected with cDNAs for WT mOAT1, its cysteine mutants (for an explanation of the mutants A, B, C, D and E, see Table 1) and vector alone (control). After transfection (24 h), [^{14}C]PAH (20 μM) uptake was measured. The results are presented as percentage of the control and are expressed as the means $\pm$ S.E.M.

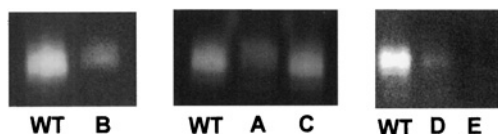

Figure 5 Effect of multiple replacements of cysteines on mOAT1 expression

HeLa cells, expressing WT mOAT1 and the mutants, were labelled with NHS-SS-biotin. The labelled proteins were separated by SDS/PAGE (7.5 % gel), and then Western blot analysis was carried out with the anti-Myc antibody. For an explanation of the mutants A, B, C, D and E, see Table 1.

Immunofluorescence analysis of mutants D and E

Further evidence of this difficulty of mutants D and E to be transported to the plasma membrane was obtained by immunofluorescence studies (Figure 6). Whereas the plasma membrane was clearly labelled (shown as green fluorescence) in cells transfected with WT mOAT1 (WT), fluorescence remained mainly in the intracellular compartment for mutants D and E. Phase-contrast images showed that cells were fully attached to the culture dishes under all conditions. Considering all these data, it can be concluded that multiple cysteine residues are necessary for proper targeting of mOAT1 to the plasma membrane.

Effect of PCMBS on cysteine mutants of mOAT1

The sensitivity of the cysteine mutants to inhibition by PCMBS was tested. As shown in Table 3, PAH transport in all the mutant transporters and WT mOAT1 was inhibited after treatment with 1 mM PCMBS. However, Cys^{49} was less sensitive to PCMBS inhibition than the WT mOAT1. Therefore, Cys^{49} probably contributes to the binding of PCMBS.

DISCUSSION

Inhibition of OAT activity by cysteine-modifying reagents has been observed in both rabbit renal basal-lateral membrane vesicles and isolated perfused snake renal tubules [5,6]. These studies led to the hypothesis that critical cysteines in OAT system are involved in its function. Due to the isolation of the cDNA for mOAT1 [2,3], the involvement of cysteine residue in transport process could be tested directly. All 13 cysteines in mOAT1 were replaced by alanine, singly or in combination. The results of the present study showed that none of the single replacements had any

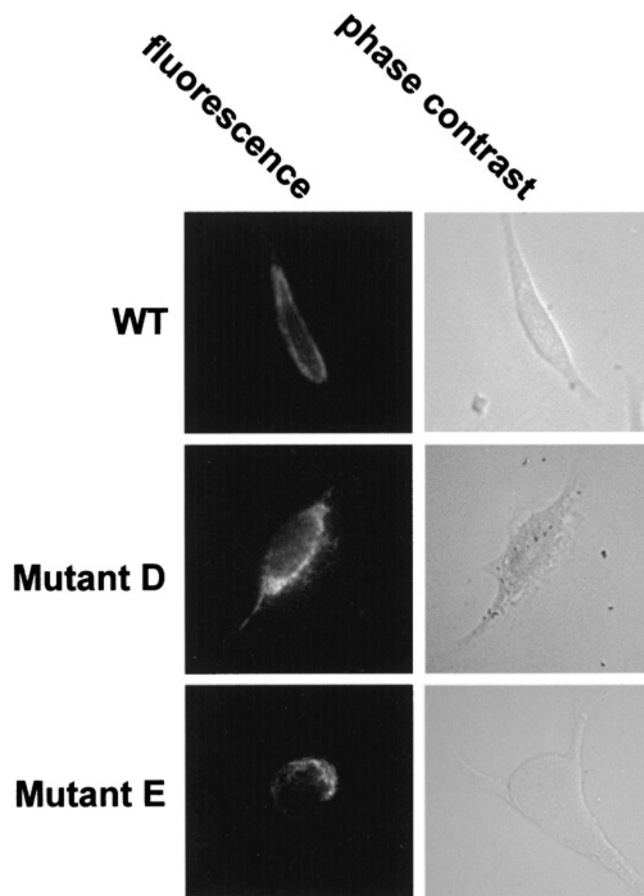

Figure 6 Immunofluorescence study of cellular localization of mutants D and E

WT mOAT1, mutants D and E (see Table 1) were expressed in HeLa cells. The cells were then stained with anti-Myc antibody and FITC-coupled goat anti-mouse IgG. Specific immunostaining appears as bright fluorescence. Phase-contrast images showed that cells were fully attached to the culture dishes under all conditions.

Table 3 Inhibition of uptake of PAH by 1 mM PCMBS

Transport of PAH was measured after preincubation with or without (control condition) 1 mM PCMBS. Uptake activity remaining after PCMBS treatment is expressed as a percentage of the uptake measured in controls. The results are expressed as the means $\pm$ S.E.M. ($n = 3$). The group that is significantly different from mOAT1 ($P < 0.05$) is indicated (*).

Mutant	Uptake remaining after PCMBS treatment (% of control)
WT mOAT1	12.9 $\pm$ 1.4
C49A	29.5 $\pm$ 5.8*
C78A	13.6 $\pm$ 3.8
C99A	12.9 $\pm$ 5.7
C122A	15.9 $\pm$ 3.6
C172A	13.0 $\pm$ 4.1
C183A	15.7 $\pm$ 4.3
C200A	14.0 $\pm$ 3.9
C326A	12.9 $\pm$ 6.7
C335A	9.4 $\pm$ 1.0
C379A	11.0 $\pm$ 2.6
C427A	10.3 $\pm$ 3.7
C434A	18.4 $\pm$ 9.3

© 2004 Biochemical Society

effect on PAH transport, suggesting that an individual cysteine in mOAT1 is not required directly for function.

The effect of multiple mutations at the various regions of mOAT1 on transport function was also examined. Replacement of cysteines at the N-terminus or mid-portion of mOAT1 had little effect on transport. In contrast, replacement of cysteines at the C-terminus (C335/379/427/434A; $Cys^{335/379/427/434} \rightarrow Ala$) resulted in a dramatic decrease in PAH transport, which is correlated with a dramatic decrease in cell surface expression of the transporter. Immunofluorescence studies demonstrated that most of the mutant C335/379/427/434A remained in the intracellular compartment, indicating that multiple cysteines at the C-terminal region may play a synergistic role in the targeting of the transporter to the plasma membrane. The targeting process was totally impaired when all of the 13 cysteines in mOAT1 were simultaneously replaced (C-less), again demonstrated by cell surface biotinylation and immunofluorescence studies. Substitution of all cysteines may alter the shape or configuration of the protein leading to the impaired trafficking of the transporter to the cell membrane. The involvement of cysteine residues in cell surface targeting has been previously reported for other transporters, such as serotonin transporter [8] and Na^+/dicarboxylate co-transporter [9].

We also showed in the present study that, although all the cysteine mutants of mOAT1 were sensitive to inhibition by PCMBS, C49A was less sensitive (70 % inhibition) than the WT mOAT1 (87 % inhibition), suggesting that the modification of Cys^{49} may contribute to the inhibition of mOAT1 function by PCMBS. Since in the present study only one of the cysteine residues has been removed, we cannot rule out the possibility that a number of cysteine residues may be involved in the full inhibition of mOAT1 function by PCMBS.

In conclusion, we have demonstrated that (i) none of the individual cysteine residue in mOAT1 is required for function, (ii) multiple cysteines may play a synergistic role for the targeting of the transporter to the cell surface, and (iii) the regulation of the transport function by cysteine modification occurs, at least in part, through direct modification of Cys^{49}.

This work was supported by grants (to G. Y.) from the J. T. Tai & Co. Foundation through the American Heart Association (New York City) (97-NIDA-019), and from the National Institutes of Health (R01-DK 60034-01). We thank Dr Gary Rudnick (Department of Pharmacology, Yale University School of Medicine, New Haven, CT, U.S.A.) and Dr Ana M. Pajor (Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, TX, U.S.A.) for their helpful suggestions during the course of this study.

REFERENCES

1 You, G. (2002) Structure, function, and regulation of renal organic anion transporters. Med. Res. Rev. **22**, 602–616
2 Lopez-Nieto, C. E., You, G., Bush, K. T., Barros, E. J. G., Beier, D. R. and Nigam, S. K. (1997) Molecular cloning and characterization of NKT, a gene product related to the organic cation transporter family that is almost exclusively expressed in the kidney. J. Biol. Chem. **272**, 6471–6478
3 Kuze, K., Graves, P., Leahy, A., Wilson, P., Stuhlmann, H. and You, G. (1999) Heterologous expression and functional characterization of a mouse renal organic anion transporter in mammalian cells. J. Biol. Chem. **274**, 1519–1524
4 You, G., Kuze, K., Kohanski, R. A., Amsler, K. and Henderson, S. (2000) Regulation of mOAT mediated organic anion transport by okadaic acid and protein kinase C in LLC-PK_1 cells. J. Biol. Chem. **275**, 10278–10284
5 Tse, S. S., Bildstein, C. L., Liu, D. and Mamelok, R. D. (1983) Effects of divalent cations and sulfhydryl reagents on the *p*-aminohippurate (PAH) transporter of renal basal-lateral membranes. J. Pharmacol. Exp. Ther. **226**, 19–26
6 Dantzler, W. H. and Bentley, S. K. (1983) Effects of sulfhydryl reagent, *p*-chloromercuribenzoate, on *p*-aminohippurate transport by isolated, perfused snake renal tubules. Ren. Physiol. **6**, 209–217
7 Stephan, M. M., Chen, M. A., Penado, K. M. and Rudnick, G. (1997) An extracellular loop region of the serotonin transporter may be involved in the translocation mechanism. Biochemistry **36**, 1322–1328
8 Chen, J. G., Liu-Chen, S. and Rudnick, G. (1997) External cysteine residues in the serotonin transporter. Biochemistry **36**, 1479–1486
9 Pajor, A. M., Krajewski, S. J., Sun, N. and Gangula, R. (1999) Cysteine residues in the Na^+/dicarboxylate co-transporter, NaDC-1. Biochem. J. **344**, 205–209

Received 12 November 2003/18 February 2004; accepted 23 February 2004
Published as BJ Immediate Publication 23 February 2004, DOI 10.1042/BJ20031724

© 2004 Biochemical Society