Identification of Cysteine Residues in Human Cationic Amino Acid Transporter hCAT-2A That Are Targets for Inhibition by N-Ethylmaleimide

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**Background:** The mechanism of N-ethylmaleimide (NEM)-mediated inhibition of cationic amino acid transporters (CATs) was unknown.

**Results:** Cys-33 (cytoplasmic N terminus) and Cys-273 (transmembrane domain VI) in human CAT-2A are the targets of NEM inhibition.

**Conclusion:** The cytoplasmic N terminus and transmembrane domain VI are critically involved in transporter function.

**Significance:** Our results help to understand how these important amino acid transporters are regulated, probably also in vivo.

In most cells, cationic amino acids such as L-arginine, L-lysine, and L-ornithine are transported by cationic (CAT) and y⁺LAT amino acid transporters. In human erythrocytes, the cysteine-modifying agent N-ethylmaleimide (NEM) has been shown to inhibit system y⁺ (most likely CAT-1), but not system y⁺LAT (Devés, R., Angelo, S., and Chávez, P. (1993) *J. Physiol. 468*, 753–766). We thus wondered if sensitivity to NEM distinguishes generally all CAT and y⁺LAT isoforms. Transport assays in *Xenopus laevis* oocytes established that indeed all human CATs (including the low affinity hCAT-2A), but neither y⁺LAT isoform, are inhibited by NEM. hCAT-2A inhibition was not due to reduced transporter expression in the plasma membrane, indicating that NEM reduces the intrinsic transporter activity. Individual mutation of each of the seven cysteine residues conserved in all CAT isoforms did not lead to NEM insensitivity of hCAT-2A. However, a cysteine-less mutant was no longer inhibited by NEM, suggesting that inhibition occurs through modification of more than one cysteine in hCAT-2A. Indeed, also the double mutant C33A/C273A was insensitive to NEM inhibition, whereas reintroduction of a cysteine at either position 33 or 273 in the cysteine-less mutant led to NEM sensitivity. We thus identified Cys-33 and Cys-273 in hCAT-2A as the targets of NEM inhibition. In addition, all proteins with Cys-33 mutations showed a pronounced reduction in transport activity, suggesting that, surprisingly, this residue, located in the cytoplasmic N terminus, is important for transporter function.

Most cell types express at least two different transport systems for cationic amino acids (CAAs) such as L-arginine, L-lysine, and L-ornithine. System y⁺ was first described in Ehrlich cells as system Ly⁺ (to refer to the positively charged substrate lysine) and was later renamed system y⁺ when it became evident that other CAAs share the same transport system (1, 2). It works independently of the presence of Na⁺ and H⁺ ions, exhibits a relatively high affinity for CAAs, and works preferentially as an exchanger of CAAs, evidenced by its strong stimulatory effect. The second system, named y⁺LAT, was first described in erythrocytes (3). It works as an obligatory exchanger of CAAs and neutral amino acids, with transport of CAAs being Na⁺-independent (like system y⁺). However, transport of neutral amino acids by system y⁺LAT occurs in a Na⁺-dependent manner.

Working with human erythrocytes, Devés et al. (4) demonstrated that system y⁺ is inhibited by short-term treatment with the thiol-modifying agent N-ethylmaleimide (NEM; 0.2 mM, 10 min). In contrast, system y⁺LAT, also present in these cells, is insensitive to NEM. Later, molecular identification of amino acid transporters revealed that there are several isoforms of SLC7 (solute carrier family 7) (see Fig. 1A) that mediate either system y⁺ (and y⁺-like) or y⁺LAT activity. Whether sensitivity to NEM generally distinguishes all system y⁺ (and y⁺-like) from system y⁺LAT isoforms had not been elucidated so far.

Most members of the SLC7 branch of cationic amino acid transporters (CATs) exhibit transport properties similar to system y⁺. All CAT proteins transport exclusively CAAs in a Na⁺-independent manner. They are glycosylated and predicted to have 14 transmembrane domains (TMDs) (5, 6). The transport properties of CAT-1 (SLC7A1) conform most with the transport system described in Ehrlich cells (7). CAT-2B (high affinity SLC7A2) and CAT-3 (SLC7A3) are less dependent on substrate at the trans-side of the membrane, and both CAT-2 splice variants (A and B) exhibit diminished activity at low pH (7–10). CAT-2B and CAT-3 can thus be seen as transporters resembling system y⁺ and are referred to here as y⁺-like. Finally, CAT-2A is not at all trans-stimulated and has a low substrate affinity (11, 12). It is highly expressed in the liver, the only organ found so far to lack system y⁺ activity. Hence, although clearly a member of the CAT family, CAT-2A does not qualify as a system y⁺ transporter.

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3 The abbreviations used are: CAA, cationic amino acid; NEM, N-ethylmaleimide; CAT, cationic amino acid transporter; TMD, transmembrane domain; lCHAT, light chain of heteromeric amino acid transporter; y⁺LAT, system y⁺ L amino acid transporter; hCAT-2A, human CAT-2A; ANOVA, analysis of variance; CL, cysteine-less.
The second branch of the SLC7 family consists of the so-called light chains of heteromeric amino acid transporters (lcHATs), which are not glycosylated and are predicted to have 12 TMDs (13). In contrast to the CATs, lcHATs need to associate with glycoproteins to localize to the plasma membrane. When coexpressed with the glycoprotein 4F2hc, the lcHATs and y\(^+-\)LAT2 (SLC7A6) mediate system y\(^+-\)L activity (14, 15).

NEM is frequently used to inhibit enzymes with cysteine residues in their active center. In a nucleophilic attack, it forms a reaction is specific in a pH range of 6.5–7.5. At lower pH, NEM may also react with histidine (16). Our previous work with human transporters expressed in Xenopus laevis oocytes al ready demonstrated that NEM (at concentrations of 0.2 and 0.5 mM) inhibits CAT-1 but leaves y\(^+\)LAT1 (SLC7A7) and y\(^+\)LAT2/4F2hc unaffected (17).

The aim of the present study was to determine (i) whether CATs and y\(^+\)LATs can generally be distinguished by their reactivity with NEM and (ii) which cysteine residues within CAT proteins are responsible for sensitivity to NEM.

### EXPERIMENTAL PROCEDURES

**Site-directed Mutagenesis**—This was performed using the QuikChange mutagenesis kit (Stratagene). The sequence of each oligonucleotide pair used is presented in Table 1.

**Expression of RNA in X. laevis Oocytes**—The plasmids were linearized and, in the case of 3’-overhangs (restriction with ScaI), blunted using the Klenow fragment of *Escherichia coli* DNA polymerase I. cRNA was prepared by *in vitro* transcription from the SP6 promoter (Ambion mMESSAGE mMACHINE *in vitro* transcription kit, AMS Biotechnology (Europe)Ltd., Cambridgeshire, United Kingdom). 18 ng of cRNA in 36 nl of water were injected in each *X. laevis* oocyte (Dumont stages V and VI). Non-injected oocytes were used as controls. If not indicated otherwise, all experiments were performed 2 days after cRNA injection.

**Inhibition Experiments with NEM**—The oocytes were first rinsed three times with ice-cold uptake solution (100 mM NaCl, 2 mM KCl, 1 mM MgCl\(_2\), 1 mM CaCl\(_2\), 5 mM HEPES, and 5 mM Tris, pH 6.8) and then transferred to the same solution with the indicated NEM concentration (Sigma-Aldrich) and incubated for 10 min at 20 °C. The same amount of uptake solution supplemented with L-[\(^3\)H]arginine (ICN Biomedicals GmbH, Eschwege, Germany) was added at a final concentration (if not indicated otherwise) of 1 mM (10 μCi/ml). After incubation for 15 min at 20 °C, oocytes were washed five times with ice-cold uptake solution and solubilized individually in 2% SDS. The radioactivity of the lysates was determined in a liquid scintillation counter.

**Determination of Apparent K\(_m\) and V\(_{max}\) Values**—Oocytes were washed three times with ice-cold uptake solution (pH 6.8) and then equilibrated in the same solution supplemented with 0.1–10 mM unlabeled L-arginine at 18 °C. The oocytes were transferred to the same solution containing, in addition, L-[\(^3\)H]arginine (10 μCi/ml). After a 15-min incubation at 20 °C, oocytes were washed and processed as described above.

**Cell Lysates and Biotinylation of Cell Surface Proteins**—All steps were performed at 4 °C. 12 oocytes each were rinsed three times with PBS\(_{mod}\) (0.1 mM NaCl, 2 mM KCl, 1.76 mM KH\(_2\)PO\(_4\), and 10.1 mM Na\(_2\)HPO\(_4\)) and then incubated for 30 min with membrane-impermeable sulfosuccinimidobiotin (1 mg/ml in PBS\(_{mod}\); EZ-Link\(\text{TM}\), sulfosuccinimidyl 2-(biotinamido)ethyl-1,3-dithiopropionate, Thermo Fisher Scientific). The biotinylation reaction was stopped by incubating oocytes in PBS\(_{mod}\) containing 50 mM NH\(_4\)Cl for 10 min and rinsing four times with PBS\(_{mod}\) containing, in addition, 0.1 mM Ca\(_2\) and 1 mM MgCl\(_2\). After lysis in 200 μl of radioimmune precipitation assay buffer (1% deoxycholate, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 2 mM MgCl\(_2\), and 10 mM Tris-HCl, pH 7.2) containing protease inhibitors (Complete mini EDTA-free protease inhibitor tablets, Roche Applied Science) an aliquot of each whole oocyte lysate was mixed directly with an equal volume of 2× sample buffer (125 mM Tris base, 20% (v/v) glycerol, 5% SDS, 0.001% (v/v) bromphenol blue, 8 M urea, and 2% mercaptoethanol) and incubated for 10 min at 37 °C. The remaining lysate was incubated overnight with avidin-coated Sepharose beads (immobilized NeutrAvidin\(\text{TM}\), Thermo Fisher Scientific) to separate biotinylated surface proteins. The beads were then washed three times with washing buffer I (50 mM Tris, pH 8, 0.5 mM NaCl, 1 mM EDTA, pH 8, 0.5% Triton X-100, and 0.1% SDS) and once with washing buffer II (50 mM Tris, pH 8, 1 mM EDTA, pH 8,
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FIGURE 1. NEM inhibits transport activity of hCATs, but not y⁺ LATs. A, the phylogenetic tree shows the known members of the SLC7 family of amino acid transporters (AT). CATs are glycosylated, predicted to have 14 TMDs, and mediate exclusively the transport of CAAs. A4 and A14 are glycosylated orphan proteins also predicted to have 14 TMDs. iCATs are not glycosylated and are predicted to have 12 TMDs. To traffic to the plasma membrane, they need to associate with a glycoprotein (depending on the isoform: 4F2hc, rBAT, or a still unidentified protein). B, X. laevis oocytes overexpressing the indicated y⁺ (hCAT-1) and y⁺-like (hCAT-2B or hCAT-3) transporters, hCAT-2A, or y⁻ transporters (y⁻ LAT1) or y⁻ LAT2/4F2hc were preincubated in buffer with or without 1 mM NEM for 10 min, followed by incubation in buffer containing L-[³H]arginine (1 mM for hCAT-2A and 100 μM for all others) for an additional 15 min. After intensive washing, the radioactivity per oocyte was determined, and the values of non-overexpressing oocytes were subtracted. Values are expressed as a percentage of the mean of the respective control (not treated with NEM). Bars represent means ± S.E. (n = 8 from at least two different batches of oocytes). Statistical analysis between NEM-treated and untreated oocytes expressing the same transporter was performed using a t test. ***; p < 0.001; no labeling corresponds to p > 0.05.

RESULTS

hCATs, but Not y⁺ LATs, Are Inhibited by NEM—To examine whether all validated hCAT proteins are inhibited by NEM and the two y⁻ isoforms are resistant to the SH group-modifying agent, we analyzed arginine transport by the individual transporters expressed in oocytes from X. laevis in the presence and absence of 1 mM NEM (Fig. 1B). For transport studies, 1 mM arginine was used in experiments with low affinity hCAT-2A, 0.1 mM arginine was used in experiments with all other transporters. We found that NEM inhibited not only the molecular representative of system y⁺ (hCAT-1) but also the system y⁺-like transporters hCAT-2B and hCAT-3 and the non-system y⁻ transporter hCAT-2A. In each case, a t test yielded p < 0.001 when comparing values from NEM-treated oocytes and untreated controls. NEM inhibition of the CAT proteins was quite pronounced, leaving only 16% (hCAT-1), 1% (hCAT-2A), 7% (hCAT-2B), and 16% (hCAT-3) residual activity compared with untreated oocytes expressing the same transporter. In contrast, neither molecular representative of system y⁻L, y⁻LAT1 or y⁻LAT2 (each time coexpressed with 4F2hc).

Western Blotting—Lysates were separated by 10% SDS-PAGE and then blotted onto nitrocellulose membranes (Protran 83, Whatman). Staining for human CAT-2A (hCAT-2A) proteins was achieved by sequential incubations in Blotto (50 mM Tris, pH 8, 2 mM CaCl₂, 0.01% Antifoam A (Sigma-Aldrich), 0.05% Tween 20, and 5% nonfat dry milk) supplemented with 5% goat serum for 2 h at room temperature to block unsppecific binding sites; three times in antibody dilution buffer (1% BSA and 0.1% Tween 20 in PBS, pH 7.4); in a 1:100 dilution of rabbit anti-hCAT-2 antibody (18) in antibody dilution buffer overnight at 4 °C; three times in Blotto for 10 min at room temperature; in a 1:10,000 dilution of peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Sigma-Aldrich) and peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:5000; Sigma-Aldrich). Signal intensity was quantified using Chemiluminescent ECL, GE Healthcare) were then immediately exposed to Lightning Plus ECL, PerkinElmer Life Sciences) for 1 min. Chemiluminescence films (Amersham Biosciences Hyperfilm™ ECL, GE Healthcare) were then immediately exposed to the membranes. Signal intensity was quantified using ChemiDoc® (Bio-Rad). For standardization, membranes were stained with anti-β-tubulin monoclonal antibody (1:1000; Sigma-Aldrich) and peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:5000; Sigma-Aldrich).

Homology Modeling—hCAT-2A was previously predicted to exhibit 14 TMDs (5, 6) using TMHMM (19) and HMMTOP (20). Comparative models were built from structures detected by PSI-BLAST (21) and/or HHSEARCH (22), revealing that hCAT-2A is related to several bacterial transporters characterized by a “5 + 5 inverted repeat fold” formed by their first 10 TMDs, i.e. Adic (Protein Data Bank ID 3L1L (23) and 3OB6 (24)), ApcT (ID 3GIA (25)), and LeuT (ID 2A65 (26)). A three-dimensional model of the first predicted 10 TMDs of hCAT-2A was generated from crystallographic atomic coordinates of Adic as the template using the MODELLER 9.11 module (27) included in the UCSF Chimera package (developed by the Resource for Biocomputing, Visualization, and Informatics, University of California, San Francisco) (28).

Statistical Analysis—Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by the Bonferroni post hoc test. If only two groups were compared, the one-way analysis of variance (ANOVA), followed by the Bonferroni post hoc test. If only two groups were compared, the one-way analysis of variance (ANOVA), followed by the Bonferroni post hoc test. If only two groups were compared, the one-way analysis of variance (ANOVA), followed by the Bonferroni post hoc test. If only two groups were compared, the one-way analysis of variance (ANOVA), followed by the Bonferroni post hoc test.
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exhibited a significant inhibition by 1 mM NEM. A t test yielded p values of 0.27 (γ⁺LAT1) and 0.83 (γ⁺LAT2).

NEM Does Not Influence the Subcellular Distribution of hCAT-2A—To test whether the NEM-mediated reduction of CAT-mediated transport is conveyed by internalization of transport proteins, we examined cryosections of hCAT-2A-EGFP-expressing oocytes exposed for 10 min immediately before freezing to 1 mM NEM or to buffer only. By far the largest part of the fluorescent signal was found in the oocyte plasma membrane under both conditions (Fig. 2A), indicating that NEM treatment causes no change in the localization of the transporter. To verify this result, cell surface proteins of intact hCAT-2A-expressing oocytes preincubated with 1 mM NEM for 10 min or untreated were biotinylated. Subsequent isolation of the biotinylated proteins and analysis by Western blotting revealed no change in the cell surface localization of hCAT-2A upon NEM treatment (Fig. 2B). This indicates that NEM inhibits the intrinsic hCAT activity.

Identification of Cysteine Residues in hCAT-2A That Mediate NEM Inhibition—We next wondered if NEM causes hCAT inhibition by directly modifying sulfhydryl groups in cysteine residues of the transporters. To answer this question, we focused on hCAT-2A because it has a relatively small number of cysteine residues among the hCATs. Cys-33, Cys-85, Cys-264, Cys-273, Cys-299, Cys-347, and Cys-427 were found to meet this condition. Of these, only Cys-85 is also found at corresponding positions in γ⁺LAT1 and γ⁺LAT2, which are not inhibited by NEM. All seven cysteine residues in hCAT-2A were substituted individually with alanine by site-directed mutagenesis. Of the resulting seven single mutants, four exhibited transport activity comparable to WT in uptake studies using 1 mM L-[³H]arginine (Fig. 3). One mutant had a higher activity and two mutants had lower activities compared with WT. Transport measurements at different arginine concentrations revealed that, compared with WT, the C33A and C264A mutants exhibited a reduced and an increased V₅₅₀ value, respectively (1.7 ± 0.3 (C33A), 10.7 ± 0.3 (C264A), and 6.9 ± 0.7 (WT) nmol/oocyte/h; one-way ANOVA, followed by the Bonferroni post hoc test, p < 0.001 in each case). Neither total expression nor cell surface expression of the C33A mutant was different compared with WT (Fig. 3B). The apparent Kₚₚ of hCAT-2A-C33A was even lower than that of WT (1.3 ± 0.1 versus 3.0 ± 0.2 mm, p < 0.05), whereas the apparent Kₚₚ of hCAT-2A-C264A (2.6 ± 0.6 mm) was not significantly altered.

Surprisingly, all single cysteine mutants were still inhibited by NEM (Fig. 3A). To determine whether NEM affects any of the cysteine residues in hCAT-2A, we constructed a transporter devoid of cysteine residues (cysteine-less (CL), hCAT-2A-CL) by mutating all 13 cysteine residues within hCAT-2A to alanine. This mutant had only ~8% transport activity compared with WT after 2 days of expression in X. laevis oocytes (data not shown). Upon examining the total and cell surface expression of hCAT-2A-CL, it became evident that both were much lower for hCAT-2A-CL than for WT, whereas the ratio of total to surface protein was comparable to that of WT (Fig. 4A). Because of low protein expression and transport activity, the expression time was extended from 2 to 4 days, resulting in a higher amount of protein detected in Western blot analysis (Fig. 4B) and an ~25% transport activity compared with WT. Inhibition experiments revealed that the cysteine-less mutant was not inhibited at any NEM concentration used (0.2, 0.5, or 1 mm) (Fig. 4C). Even a t test comparing oocytes treated with 1 mM NEM and untreated controls yielded a p value of 0.25.

The resistance of the cysteine-less hCAT-2A protein to inhibition by NEM strongly suggested that cysteine residues within hCAT-2A were conveying the NEM inhibition of WT. As individual mutation of each of the conserved cysteine residues was not sufficient to abolish NEM inhibition (Fig. 3A), it seemed likely that more than one cysteine residue may mediate the NEM effect. We therefore had a closer look at the results in Fig. 3A and noticed that, in contrast to WT and all other mutants, (i) the C33A and C85A mutants exhibited no tendency for inhibition by NEM (Fig. 3B), and (ii) the C85A and C273A mutants were not completely inhibited by NEM at 1 mM. We therefore created double mutants, each carrying alanine mutations at two of these three cysteine residues. In fact, the C33A/C273A mutant was not inhibited at any NEM...
concentration used (Fig. 5). A t test comparing oocytes treated with 1 mM NEM and controls yielded a p value of 0.43. In contrast, the double mutants C33A/C85A and C85A/C273A were still significantly inhibited by NEM. These data strongly suggest that NEM inhibition occurs at Cys-33 and Cys-273. In fact, a mutant in which all cysteine residues except Cys-33 and Cys-273 were mutated to alanine (hCAT-2A-CL(C33/273)) was inhibited by NEM (Fig. 6A). In addition, it exhibited a much higher transport activity compared with hCAT-2A-CL. Because the two single cysteine mutants hCAT-2A-C33A and hCAT-2A-C273A were (almost) as sensitive to NEM as WT, we concluded that modification of one of these two cysteine residues by NEM would be sufficient to inhibit hCAT-2A. Indeed, mutants in which all cysteine residues except Cys-33 or Cys-273 were mutated to alanine, hCAT-2A-CL(C33) and hCAT-2A-CL(C273), were still inhibited by NEM (Fig. 6B and C). In addition, transport of hCAT-2A-CL(C33) was about three times higher than that of hCAT-2A-CL (data not shown). This was not due to increased total or cell surface expression compared with hCAT-2A-CL. In contrast to hCAT-2A-CL(C33), transport of hCAT-2A-CL(C273) was comparable to hCAT-2A-CL: even after 4 days of expres-

FIGURE 3. hCAT-2A mutants carrying single Cys-to-Ala mutations are still inhibited by NEM. A, X. laevis oocytes expressing the indicated hCAT-2A mutants with a single Cys-to-Ala mutation at the indicated positions were preincubated in buffer without (white bars) or with 0.2 mM (light gray bars), 0.5 mM (dark gray bars), or 1 mM (black bars) NEM for 10 min. L-[3H]arginine uptake buffer was then added (resulting in a final concentration of 1 mM arginine), followed by incubation for another 15 min. Arginine uptake was calculated from radioactivity taken up in each oocyte. Values of non-overexpressing oocytes were subtracted. Bars represent means ± S.E. (n = 13 from at least two different batches of oocytes). For statistical analysis, oocytes treated with 0.2, 0.5, and 1 mM NEM were compared with untreated oocytes using ANOVA with the Bonferroni post hoc test. ***, p ≤ 0.001; **, p ≤ 0.01; *, p ≤ 0.05; no labeling corresponds to p > 0.05. B, Western blotting of total and cell surface proteins from oocytes expressing WT hCAT-2A or the single mutant C33A was performed 2 days after injection of transporter cRNA as described in the legend to Fig. 2. They were probed with an anti-hCAT-2 antibody (upper panel) and subsequently with an anti-tubulin antibody (lower panel) to control loading (left lanes) and successful withdrawal of intracellular proteins (right lanes). Non-injected oocytes (ni) were used as controls.

FIGURE 4. A cysteine-less hCAT-2A mutant is not inhibited by NEM. A, representative Western blot of total and cell surface proteins from oocytes expressing WT hCAT-2A or hCAT-2A-CL 2 days after injection of transporter cRNA into X. laevis oocytes, probed with an anti-hCAT-2 antibody (upper panel) and subsequently with an anti-tubulin antibody (lower panel) to control for loading (left lanes) and successful withdrawal of intracellular proteins (right lanes). Non-injected oocytes were used as controls (cont.). Western blotting was performed as described in the legend to Fig. 2. Dotted lines indicate where lanes not shown here were cut out of the blot. B, total expression of WT hCAT-2A and hCAT-2A-CL 2–4 days (d) after injection of transporter cRNA (upper panel) and of endogenous tubulin (lower panel). Non-overexpressing oocytes served as controls. C, L-[3H]arginine uptake by WT hCAT-2A and hCAT-2A-CL in the absence of NEM (white bars) or in the presence of 0.2 mM (light gray bars), 0.5 mM (dark gray bars), or 1 mM (black bars) NEM was measured as described in the legend to Fig. 3, except that transporter expression in X. laevis oocytes was for 4 days. Bars represent means ± S.E. (n = 16 from at least three different batches of oocytes). For statistical analysis, oocytes treated with 0.2, 0.5, and 1 mM NEM were compared with untreated oocytes using ANOVA, followed by the Bonferroni post hoc test. ***, p = 0.001; no labeling corresponds to p > 0.05.
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FIGURE 5. The hCAT-2A-C33A/C273A mutant is not inhibited by NEM. Shown is the L-pipecoline uptake by the hCAT-2A-C33A/C85A, hCAT-2A-C33A/C273A, and hCAT-2A-C85A/C273A double mutants treated buffer without (white bars) or with 0.2 mM (light gray bars), 0.5 mM (dark gray bars), or 1 mM (black bars) NEM for 10 min. The experiments were performed as described in the legend toFig. 3. Bars represent means ± S.E. (n = 29 from at least four different batches of oocytes). For statistical analysis, oocytes treated with 0.2, 0.5, and 1 mM NEM were compared with untreated oocytes using ANOVA, followed by the Bonferroni post hoc test. ***p < 0.001; *, p < 0.05; no labeling corresponds to p > 0.05.

DISCUSSION

NEM Inhibits CATs, but Not y+LATs—Our study has established that all CAT proteins, but neither of the y+LAT isoforms, are inhibited by the cysteine-modifying compound NEM. Hence, NEM can be used to generally distinguish transport of CAAs by CATs and y+LATs. They belong to two different branches of the SLC7 family and are often coexpressed in mammalian cells. However, NEM does not distinguish between transporters with system y+ (CAT-1), y+–like (CAT-2B and CAT-3), and non-y+ (CAT-2A) activity within the CAT branch. In line with previous results (4), NEM inhibition occurred rapidly. A closer analysis of hCAT-2A showed that inhibition was not due to protein degradation or a change in plasma membrane localization of the transporter. Hence, NEM most likely affects the intrinsic activity of the CATs.

NEM Inhibition Occurs at Residues 33 and 273 in hCAT-2A—Mutational analysis of hCAT-2A further demonstrated that NEM inhibits CAT activity directly via interaction with Cys-33 and Cys-273, conserved in CATs, but not y+LATs. The presence of one of these residues is sufficient to confer NEM inhibition as evidenced by the observations that (i) simultaneous mutation of both residues, but not of either one alone, conferred NEM resistance to hCAT-2A, and (ii) hCAT-2A mutants in which all cysteine residues except Cys-33 and/or Cys-273 were mutated to alanine were sensitive to NEM. This strongly suggests that NEM inhibition of CATs is mediated by alkylation of either Cys-33 and Cys-273 in hCAT-2A.

Neither Cys-33 nor Cys-273 of hCAT-2A-CL(C273) was only 11% of WT.

Importance of Cys-33, but Not Cys-273, for hCAT-2A Activity—In line with an important function of Cys-33 for hCAT-2A activity, it seems unlikely that NEM inhibition through Cys-33 is simply due to steric hindrance, but rather that alkylation by NEM leads to loss of Cys-33 function in hCAT-2A (see below).

Importance of Cys-33, but Not Cys-273, for hCAT-2A Activity—In line with an important function of Cys-33 for hCAT-2A activity, it seems unlikely that NEM inhibition through Cys-33 is simply due to steric hindrance, but rather that alkylation by NEM leads to loss of Cys-33 function in hCAT-2A. However, as Cys-31 is not conserved among CATs, this motif does not seem to play a role in either CAT function or inhibition by NEM. If modification of Cys-31 has no impact on hCAT-2A activity, it seems unlikely that NEM inhibition through Cys-33 is simply due to steric hindrance, but rather that alkylation by NEM leads to loss of Cys-33 function in hCAT-2A (see below).
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The presence of a cysteine residue at positions 33 and 273 in hCAT-2A is sufficient for inhibition by NEM. Shown is the L-[3H]arginine uptake by WT hCAT-2A and mutants lacking all cysteine residues except for Cys-33 and Cys-273 in mutant hCAT-2A-CL(C33+273) (A), Cys-33 in mutant hCAT-2A-CL(C33) (B), and Cys-273 in mutant hCAT-2A-CL(C273) (C) treated without (white bars) or with 0.2 mM (light gray bars), 0.5 mM (dark gray bars), or 1 mM (black bars) NEM for 10 min. The experiments were performed as described in the legend Fig. 3, except that the expression time was extended to 4 days for hCAT-2A-CL(C273) because transport was too low after 2 days of expression. Bars represent means ± S.E. (n = 15 from at least three different batches of oocytes). For statistical analysis, oocytes treated with 0.2, 0.5, and 1 mM NEM were compared with untreated oocytes using ANOVA, followed by the Bonferroni post hoc test. ***, p < 0.001; *, p < 0.05; no labeling corresponds to p > 0.05.

Cys-33 and Cys-273 are located at the inward facing membrane in a three-dimensional model of hCAT-2A. The three-dimensional model of the first predicted 10 TMDs of hCAT-2A show the localization of the putative substrate-docking site (with arginine shown in green) as derived from the Protein Data Bank coordinate files of AdiC. The positioning of hCAT-2A in the membrane (symbolized by a red and blue plane, at the outside and inside of the membrane, respectively) was obtained via the PPM server (41). Cys-33 and Cys-273, found to be crucial for the sensitivity of hCAT-2A to NEM, are marked in red. Other cysteine residues conserved in all hCATs (positions 85, 264, 299, 347, and 427) are depicted orange. Of these, only Cys-85 is also conserved in y’LATs.

The cysteine-less mutant exhibited significant (although low) transport activity, which was resistant to NEM inhibition. The activity was even lower than that of the single mutant C33A. However, this seemed to be due mainly to poor expression of the protein, most likely because of diminished protein stability (and not diminished translation). The low activity and stability of the cysteine-less mutant are the result of the different effects of each individual Cys mutation. Among the conserved cysteine residues, only the mutation of Cys-427 to alanine also reduced hCAT-2A transport activity, but to a lesser extent than that of Cys-33. In contrast, the hCAT-2A-C264A mutant had an even higher transport activity than WT. The effects of mutating the non-conserved cysteine residues were not investigated for each individual residue. However, the hCAT-2A-C4A/C13A/C31A mutant, in which all cysteine residues in the N terminus with the exception of Cys-33 were mutated, had a transport activity comparable to WT (data not shown), suggesting that these residues have no special function in the transporter. This excludes an essential interaction of Cys-33 with any of these residues (including the CXC motif mentioned above) and further speaks for a specific function of Cys-33.

Cys-33 seems to have an important function in CAT-mediated transport. This is surprising given that it is predicted to localize to the cytoplasmic N terminus and thus is unlikely to participate in substrate binding or transition. However, the crystal structures of related proteins available so far do not allow any structural predictions in the N-terminal region of hCATs. We can therefore not exclude that Cys-33 might be part of the transition pore.

Interestingly, NEM inhibition of selenium export by the anion exchanger AE1 seems also to be mediated by cysteine residues within the cytoplasmic N terminus of the transporter (34). The specific function of these N-terminal cysteine residues needs to be elucidated. As mentioned above, Cys-33 is not conserved in any of the lCHATs. Given that these proteins all function as amino acid transporters, with some of them also recognizing CAAs as their substrate, it is puzzling to predict the specific role Cys-33 may have in CAT function.

A similar situation is seen in mitochondrial phosphate transporters: whereas the mammalian isoform contains a Cys residue essential for transporter function and inhibition by NEM, the yeast isoform does not (32). However, the amino acid residue at the corresponding position in the yeast transporter (Thr) is also essential for high transport capacity (35). It is thus tempt-
ing to speculate that the glutamic acid residue found in both y\(^{-1}\)LAT isoforms and three other hCATTs at the position corresponding to Cys-33 in hCAT-2A is also essential for transporter function.

*Could a Modification of Either Cys-33 or Cys-273 Also Play a Role in the Regulation of hCAT Activity in Living Cells?*—As for now, we can only speculate if the target residues for NEM inhibition are also subject to modification and thus inhibition by physiological processes. A number of modifications may occur at cysteine residues in proteins, some of them being regulated in a redox-sensitive manner (36). These include nitrosylation (\(-\text{SNO}\)), hydroxylation (\(-\text{SOH}\)), disulfide formation (\(-\text{S-S}\)), conjugation of glutathione (\(-\text{S-glutathione}\)), and S-palmitoylation. The latter is unique among protein acetylation reactions in that it is reversible. S-Palmitoylation of membrane proteins has been found to influence protein stability, conformation of TMDs, association with specific membrane domains, and interactions with other proteins and other post-translational modifications (37). If palmitoylation at either Cys-33 or Cys-273 were necessary for proper CAT function, the NEM effect could be explained by interfering with this modification. However, as NEM inhibition occurred very rapidly, one would have to postulate a very fast cycle of S-palmitoylation and depalmitoylation of the transporter. The reduced stability of hCAT-2A-CL could, however, be explained by a lack of S-palmitoylation at any of the Cys residues not investigated individually.

A recent report demonstrated inhibition of arginine transport in cardiac myocytes upon stimulation of NO synthesis (38). The authors concluded that “the effect of NO is likely to involve S-nitrosylation of cysteine-thiol side chains in these cationic amino acid transporters” and suggested that “putative Cys residues that react with NEM might be also the targets for NO-mediated S-nitrosylation.” As for S-palmitoylation, there is no consensus sequence deducible from the primary sequences of proteins with cysteine residues known to undergo S-nitrosylation (39). Instead, an acid-base motif in the tertiary structure has been proposed to be engaged in protein-protein interaction facilitating trans-nitrosylation. S-Nitrosylation may also compete with S-palmitoylation and thus has often opposing effects to the latter (40). Future experiments are necessary to show if CAT proteins may be S-nitrosylated or S-palmitoylated and if this occurs at the same Cys residues as alkylation by NEM.

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