# Interactions between the thiol-group reagent *N*-ethylmaleimide and neutral and basic amino acid transporter-related amino acid transport

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The neutral and basic amino acid transport protein (NBAT) expressed in renal and jejunal brush-border membranes is involved in amino acid and cystine absorption. NBAT mutations result in Type 1 cystinuria. A C-terminal *myc*-tagged NBAT (NBAT*myc*) retains the amino acid transport and protein–protein interaction properties of NBAT when expressed in *Xenopus* oocytes. Neutral amino acid (Ala, Phe)–cationic amino acid (Arg) heteroexchanges related to NBAT*myc* expression in oocytes are inactivated by treatment with the thiol-group reagent *N*-ethylmaleimide (NEM), although significant Arg–Arg and Ala–Ala homoexchanges persist. Inactivation of heteroexchange activity by NEM is accompanied by loss of $> 85\,\%$ of alanine and cystine uptake, with smaller ($< 50\,\%$) inhibition of arginine and phenylalanine uptake. NEM-sensitive cystine uptake and arginine–alanine heteroexchange (system $b^{0,+}$ activity) are not expressed by an NBAT truncation mutant (NBAT*myc*-*Sph*1) lacking the 13 C-terminal amino acid residues, but the mutant expresses NEM-resistant transport activity (system $y^+$L-like) equivalent to that of full-length NBAT*myc*. The deleted region of NBAT*myc*-*Sph*1 contains two cysteine residues (671/683) which may be the targets of NEM action. The synthetic amino acid 2-trifluoromethylhistidine (TFMH) stimulated alanine efflux at pH 7.5 and arginine at pH 5.5, but not vice versa, establishing the existence of distinct pathways for cationic and neutral amino acid homoexchange (TFMH is zwitterionic at pH 7.5 and cationic at pH 5.5). We suggest that NBAT expresses a combination of system $b^{0,+}$ and $y^+$L-like activities, possibly by interacting with different light-chain subunits endogenous to oocytes (as does the homologous 4F2hc protein). The C-terminus of NBAT may also have an additional, direct role in the mechanism of System $b^{0,+}$ transport (the major transport activity that is defective in Type 1 cystinuria).

Key words: cystine, cystinuria, exchanger, membrane transport, truncation mutant.

## INTRODUCTION

The neutral and basic amino acid transport protein NBAT [1,2], also known as rBAT [3] or D2 [4], is an 85 kDa glycoprotein predominantly expressed in brush-border membranes of epithelium lining the jejunum and renal proximal straight (S3) tubule [5,6]. Expression of NBAT in *Xenopus* oocytes results in a marked increase in the $Na^+$-independent transport of neutral and cationic amino acids, plus cystine [1,3,4,7,8], suggestive of a role for NBAT in amino acid and cystine absorption from the gut and kidney. Indeed, mutations of human NBAT are associated with the inherited disease Type I cystinuria [6,9], which is characterized by urinary hyper-excretion of cationic amino acids and cystine due to a defect in their reabsorption. However, membrane topology of NBAT is predicted to include at most four membrane-spanning domains [1,6,10], far fewer than for typical transporter proteins [6]. NBAT is linked by disulphide bonds to a smaller ($\sim 45$ kDa) membrane-bound protein(s) in the cell membrane [11,12], leading to the suggestion that NBAT is a regulatory subunit of a heteromeric protein complex which acts as the minimal 'functional transporter unit' for NBAT-related amino acid transport [6,11]. A similar role has been proposed for 4F2hc (a membrane glycoprotein displaying significant structural as well as functional similarity to NBAT) [6,8,13], and recent studies [14–17] have identified several members of a new family of amino acid transporter-related proteins (E16 [14], LAT1 [15], $y^+$LAT-1 [16]) which exhibit activation of amino acid transport in oocytes only when co-expressed with 4F2hc. These highly hydrophobic, permease-related proteins of $\sim 40$ kDa (light-chain) are suggested to interact with 4F2hc (heavy-chain) via disulphide bridges, involving $Cys^{109}$ in 4F2hc, to produce a heterodimeric transporter unit [14,16,17]. There is additional evidence that 4F2hc regulates the intracellular trafficking and membrane topology of these heterodimers [14,17]. The light-chain transporter subunits cloned to date have not been shown to interact with NBAT, but the available evidence is consistent with the idea that NBAT, as well as 4F2hc, functions to direct catalytic light-chain subunits to the cell surface, although an additional, active role for NBAT in the transport mechanism cannot be excluded.

Our previous studies on amino acid transport in NBAT-expressing *Xenopus* oocytes [7,18] provided direct evidence that NBAT-related amino acid uptake occurs by two prominent, functionally distinct pathways. We identified two components of arginine uptake [7,18], one which was resistant to inactivation by the thiol-group reagent *N*-ethylmaleimide (NEM) and insensitive to alanine, plus a second which was inactivated by NEM and inhibited by alanine. The NEM-sensitive transport pathway bears functional characteristics similar to those of $Na^+$-independent transport mediated by systems $b^{0,+}$ and asc [6–8], whereas the NEM-resistant pathway more closely resembles system $y^+$L [6–8,18]. NBAT-related amino acid transport involves obligatory

Abbreviations used: MBM, modified Barths medium; MTBSTFA, *N*-t-butyldimethylsilyl-*N*-methyltrifluoroacetamide; NBAT, neutral and basic amino acid transport protein; NEM, *N*-ethylmaleimide; TFMH, 2-trifluoromethylhistidine; TMA, tetramethylammonium.

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hetero- or homo-exchange of substrates [19–21], but it is not clear whether these distinct modes of exchange occur through the same or different transport pathways, although we have previously [7,18] presented evidence for separate binding sites for neutral and cationic substrates. In the present study, we have therefore sought to distinguish between the exchange properties of the major NBAT-related transport pathways and their relative importance in cystine transport.

Recent studies are suggestive of an important role for the NBAT C-terminus in functional transport expression. Removal of the terminal 175 amino acid residues (Δ511–685) of human NBAT [22] ablates expression of cystine transport, leaving residual arginine and leucine transport with system $y^+$L-like properties [6,8]. Progressive C-terminal deletions of rat NBAT [12] produced a bimodal pattern of functional expression in which transport was lost by short (25–68 amino acid) truncations, restored with a 95 amino acid truncation then again lost by longer (117–175 amino acid) truncations. We now report on the possible role of Cys residues in the extreme C-terminus (final 13 residues) of NBAT in the differential expression of NBAT-related transport activities, using *c-myc* epitope-tagged NBAT (wild-type and mutant) to facilitate studies of protein expression and protein–protein interactions.

## EXPERIMENTAL

### Materials

*Xenopus laevis* toads were purchased from the South African Xenopus Facility. Chemicals were obtained from Sigma with the exception of collagenase A (Boehringer), Ultraspec water (Ambion, Austin, TX, U.S.A.), 9E10 monoclonal antibody and 9E10-coated Sepharose beads (Santa Cruz Biotechnology) and secondary antibodies (Scottish Antibody Production Unit, Carluke, Scotland, U.K.). Radiotracers were purchased from NEN, except L-[$^{35}$S]cystine which was from Amersham. All cDNA clones for oocyte expression were ligated into pSG5 (a simian virus 40 driven expression plasmid) modified by inclusion of a sequence encoding the *c-myc* epitope EQKLISEEDL (target of the 9E10 monoclonal antibody) distal to a unique *Xho*1 site within the multiple cloning region. NBAT*myc* (Figure 1) was constructed by ligating an *EcoR*1–*Sph*1 fragment of rat NBAT cDNA [1], which includes the entire coding region except the final 13 amino acids, into modified pSG5 using a synthetic oligonucleotide linker, such that the full coding region was restored immediately 5′ and in-frame to the *myc* epitope-tag. The NBAT*myc*-*Sph*1 truncation mutant (Figure 1) was constructed by ligating the *EcoR*1–*Sph*1 NBAT fragment into pSG5 using an oligonucleotide linker which converted Cys residue 671 into Trp, followed immediately by the *myc* epitope-tag. Plasmid construction was confirmed by DNA sequencing. Oligonucleotides were synthesized by the MRC Protein Phosphorylation Unit (University of Dundee, Scotland, U.K.).

### Isolation and maintenance of *Xenopus* oocytes

Oocytes were isolated by collagenase treatment of ovarian tissue obtained from mature female *Xenopus laevis* toads (South African Xenopus Facility) using methods described previously [7]. Defolliculated, stage V–VI (prophase-arrested) oocytes were selected and maintained at 18 °C in modified Barths medium (MBM) containing (in mM): 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.82 $MgSO_4.7H_2O$, 0.66 $NaNO_3$, 0.75 $CaCl_2.2H_2O$, 5.0 Hepes, pH 7.6 with Tris base, and 10 mg/l gentamycin sulphate.

| | 666 683 |
|---|---|
| **NBAT** | -VSNRACYSSVLNILYTSC |
| **NBAT*myc*** | -VSNRACYSSVLNILYTSC-LE-*EQKLISEEDL* |
| **NBAT*myc-Sph1*** | -VSNRA-WE-*EQKLISEEDL* |

**Figure 1 Amino acid sequences of the C-terminal regions of NBAT, NBAT*myc* and NBAT*myc-Sph*1**

Numbers denote amino acid residues of NBAT protein sequence [1]. Letters in italics indicate the *myc* epitope tag.

### DNA injection procedure

Oocytes were transferred into individual wells of Tetraski plates pre-filled with MBM and centrifuged at 600 ***g*** for 8 min at 18 °C, which causes migration of the nucleus to the cell surface and facilitates nuclear injection [23]. The visible nucleus of each oocyte was injected with 2 ng of DNA in 15 nl of Ultraspec water using a pneumatic delivery system [7]. The nuclei of control oocytes were injected with either Ultraspec water, pSG5 plasmid vector or were not injected (no differences in transport properties between the three control types was observed). Injected oocytes were incubated in MBM at 18 °C for 1–6 days to allow expression of injected DNA before experimentation.

### Measurement of amino acid uptake and efflux

Amino acid transport in oocytes was measured as influx or efflux of radiolabelled amino acid tracer. Unless otherwise stated, all experiments were carried out at 22 °C on oocytes at 3–4 days post-injection using an $Na^+$-free transport buffer containing 100 mM tetramethylammonium chloride (TMACl), 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM Hepes (pH 7.5 with Tris). Radiolabelled amino acid uptake was measured as described previously [7,24]. For measurement of amino acid efflux, 50 nl (0.00185 MBq) of amino acid tracer in deionized water was micro-injected into the cytoplasm of individual oocytes. After a 15 min recovery period, each oocyte was loaded into an efflux tube (a plastic tube fitted with a nylon mesh base) which was then transferred into the first of a series of 5 ml scintillation vials (each containing 0.5 ml of efflux medium). The tubes containing oocytes were moved progressively from one vial to another along each series at 10 min intervals, the efflux medium alternating between $Na^+$-free transport buffer and buffer containing unlabelled amino acid or cystine. After each exposure to unlabelled amino acid, oocytes were washed rapidly ($< 5$ s) in 0.5 ml of transport buffer before transfer to the next vial. At the end of the efflux experiment, each oocyte was lysed in 0.2 ml of 1 % SDS and the residual radioactivity (d.p.m.) in the cell and each vial (including wash vials) was measured by liquid-scintillation counting. Tracer efflux was assessed from plots of $-\ln(C_t/C_0)$ against time, where $C_0$ and $C_t$ are the levels of radioactivity (d.p.m.) contained within the cell at the start and finish respectively of each experimental period (obtained by summation of residual oocyte radioactivity and sequential radioactivity losses to vials by tracer efflux). Preliminary results (not shown) demonstrated that the time-course of depletion of injected tracer (L-[$^3$H]alanine, L-[$^3$H]-arginine) by efflux stimulated by 5 mM external L-arginine was described by a single rate constant (over at least 90 min) for both NBAT*myc*- and water-injected oocytes, indicating that injected tracer exchanged from a single intracellular pool.

In experiments using the thiol-group reagent NEM, oocytes were pre-incubated in MBM containing 2 mM NEM for 10 or 30 min [7], rinsed in transport buffer and then uptake or efflux experiments were carried out in the absence of NEM. Experiments involving cystine were performed in the presence of 1 or 10 mM diamide [21,22]; the stability of [$^{35}$S]cystine tracer under experimental conditions was confirmed by TLC [21] with direct autoradiography (Packard Instant-Imager).

### Amino acid analysis

Amino acids were analysed by GC–MS after derivatization of the amino acids to their t-butyldimethylsilyl derivatives. Groups of three oocytes for analysis were washed in ice-cold MBM then lysed in 0.5 ml of 100 % ethanol, incubated on ice for 30 min and centrifuged at 10000 ***g*** for 3 min at 4 °C. The supernatant was dried down under vacuum, resuspended in 25 $\mu$l of pyridine and 25 $\mu$l of *N*-t-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) and incubated at 80 °C for 1 h to derivatize free amino acids. A 1 $\mu$l aliquot was then injected for analysis on an MD800 GC mass spectrometer. Samples were injected in the splitless mode, the GC programme was started at 100 °C and held isothermal for 2 min, then a ramp of 10 °C/min was applied. The column used for separation was a capillary Econocap BX3, 30m. The carrier gas was helium at an injector pressure of 28 lb/in$^2$ (1 lb/in$^2$ = 6.9 kPa). Individual amino acids were quantified by integration of the area of signal from specific mass fragments by comparison with a standard of known amino acid composition, using added norleucine as an internal standard.

### Membrane isolation, immunoprecipitation and Western blotting

Oocyte membranes were isolated using the method of Wang and Tate [11]. For immunoprecipitation studies, oocytes were preloaded with [$^{35}$S]methionine/[$^{35}$S]cysteine (*$^{35}$S-Express*; NEN Life Sciences, Hounslow, U.K.) for 3 days before membrane isolation and precipitation with 9E10 antibody attached to Sepharose beads according to manufacturers protocol. Proteins were resolved by PAGE (± 5 % 2-mercaptoethanol as a reducing agent) and electroblotted onto nitrocellulose membranes. NBAT*myc* was detected by enhanced chemiluminescence (ECL; Amersham) using 9E10 monoclonal antibody (1 $\mu$g/ml) and horseradish-peroxidase-labelled rabbit anti-mouse secondary antibody (1:300 dilution). Radiolabelled proteins on gels and membranes were detected and quantified by direct autoradiography.

### Data analysis and presentation

Results are expressed as means ± S.E.M.; $n$ = number of observations. Experimental measurements in each batch of oocytes were made on individual oocytes (9–11 for uptake experiments and 6–8 for efflux experiments, unless otherwise stated). Differences between mean values were assessed using Student's unpaired $t$-test, with significance assigned at $P < 0.05$. Line-fitting in Hanes plots was performed by the method of least-squares using commercial software (Slidewrite 4; Advanced Graphics Software).

## RESULTS

### Characterization of NBAT*myc*

NBAT*myc* was detected in membranes isolated from NBAT*myc* DNA-injected oocytes as a glycosylated protein band (~ 85 kDa) by Western blot analysis under reducing conditions (Figure 2a).

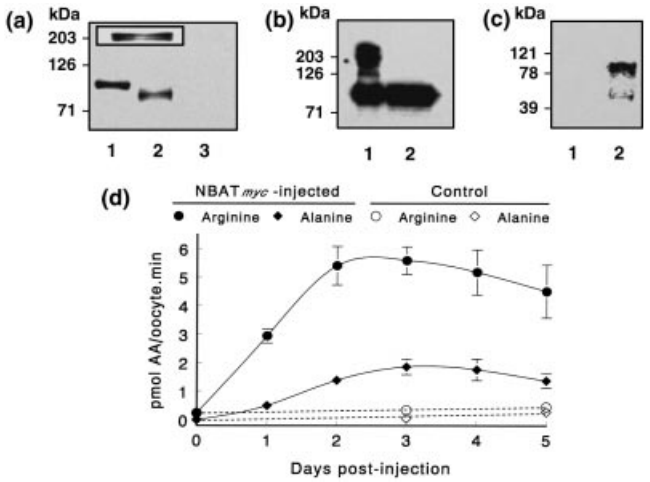


**Figure 2 Characterization of NBAT*myc* expressed in *Xenopus* oocytes**

(**a**) Western blot of cell membranes isolated from NBAT*myc* (lanes 1 and 2, before and after deglycosylation respectively) and control (lane 3) oocytes. Each lane contained 10 $\mu$g of protein probed with 9E10 anti-*myc* antibody. Deglycosylation was performed by pre-treatment of membranes with *N*-glycosidase F (0.6 units at 37 °C overnight) before electrophoresis. Inset: short ECL exposure reveals two closely apposed NBAT*myc* protein bands. (**b**) Western blot of cell membranes isolated from NBAT*myc*-injected oocytes, after electrophoresis under non-reducing (lane 1) or reducing (lane 2) conditions. Each lane contained 40 $\mu$g of protein probed with 9E10 anti-*myc* antibody. (**c**) Direct autoradiogram of polyacrylamide gel containing proteins immunoprecipitated with anti-*myc* antibody from membranes isolated from oocytes pre-loaded with [$^{35}$S]methionine/[$^{35}$S]cysteine (*$^{35}$S-Express*) for 3 days before isolation (lane 1, control oocytes; lane 2, NBAT*myc*-injected oocytes). (**d**) Time course of induction of arginine and alanine transport in oocytes after nuclear injection of NBAT*myc* expression plasmid. Transport was measured from uptake of radioactive tracer (50 $\mu$M L-[$^3$H]alanine and L-[$^3$H]arginine) in TMA transport buffer and is presented as mean uptake ± S.E.M. for 9–11 oocytes at each time-point. Similar time-courses of expression were observed for phenylalanine and cystine uptake (results not shown).

Two closely apposed NBAT*myc* protein bands could be seen at short autoradiographic exposure (see inset to Figure 2a). The protein was also detected in higher molecular-mass bands (of approx. 130, 170 and > 200 kDa) under non-reducing conditions (presumably reflecting formation of hetero- and homo-meric protein complexes through disulphide linkages; see Figure 2b). NBAT*myc* immunoprecipitated from oocyte membranes appeared to co-immunoprecipitate with protein(s) of lower molecular mass (45–50 kDa; Figure 2c). These results are consistent with previous observations on wild-type NBAT [11,12]. Uptakes of radiolabelled amino acids (L-[$^3$H]alanine, L-[$^3$H]arginine, L-[$^3$H]phenylalanine) and L-[$^{35}$S]cystine in NBAT*myc*-injected oocytes were significantly (8–25 times) greater than in water- or pSG5 plasmid-injected control oocytes, with maximum uptake values obtained by day 3 post-injection (Figure 2d; Table 1). NBAT*myc*-expressing oocytes also exhibited exchange transport (e.g. *trans*-stimulation of injected AA tracer efflux by external $\beta$-phenylalanine; Table 1). Addition of the *myc* epitope tag did not appear to affect the transport functions of NBAT significantly: we were unable to distinguish any differences in transport kinetics and inhibition profiles of arginine, phenylalanine or cystine transport induced by NBAT and NBAT*myc* in oocytes ([7,18], Table 1, Figure 2d and also G. J. Peter, A. Davies and P. M. Taylor, unpublished work).

### Effect of NEM on NBAT-related alanine and cystine transport

We have previously [7] identified an NEM-sensitive component of neutral and cationic amino acid transport which, on the basis

**Table 1 Amino acid influx and efflux (*trans*-stimulation) in NBAT*myc* DNA injected *Xenopus* oocytes**

All influx measurements made at 0.05 mM substrate. $n > 3$ preparations for influx experiments and for 8–24 individual oocytes for efflux measurements. Values in parentheses are uptakes in control (pSG5- or water-injected) oocytes. *, **, Significantly different from basal value with $P < 0.05$, 0.005 respectively.

| | Influx (pmol/min per oocyte) | | | $10^2 \times$ Efflux rate constant ($min^{-1}$) | |
|---|---|---|---|---|---|
| | Basal | + NEM† | + 5 mM β-Phe | Basal | + 1 mM β-Phe |
| Arginine | 6.28 ± 0.71 (0.33 ± 0.04) | 3.62 ± 0.45* | 2.35 ± 0.31** | 0.015 ± 0.003 | 0.81 ± 0.06** |
| Alanine | 1.62 ± 0.27 (0.15 ± 0.05) | 0.22 ± 0.04** | 0.29 ± 0.03** | 0.08 ± 0.02 | 0.72 ± 0.05** |
| Cystine | 1.50 ± 0.31 (0.06 ± 0.035) | 0.12 ± 0.02** | 0.25 ± 0.03* | ND‡ | ND‡ |
| Phenylalanine | 2.68 ± 0.54 (0.32 ± 0.14) | 1.71 ± 0.69 | 0.41 ± 0.04* | 0.07 ± 0.025 | 0.35 ± 0.02** |

† 2 mM NEM pre-treatment for 10 min.
‡ ND, not determined.

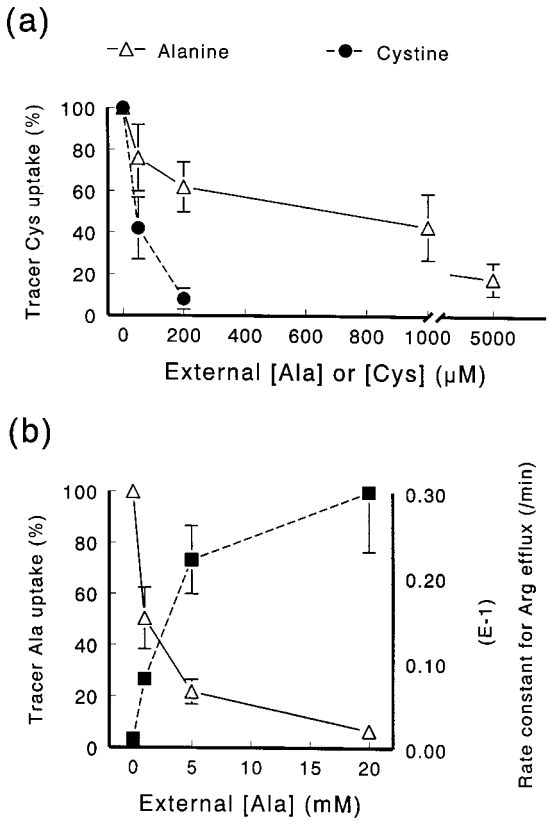


**Figure 3 Cystine and alanine transport in NBAT*myc*-expressing oocytes**

(**a**) Effect of unlabelled cystine or alanine on [$^{35}$S]cystine tracer (2 μM) uptake into NBAT*myc*-injected oocytes (after subtraction of uptake into control oocytes). Results are means ± S.E.M. for 11 oocytes. Raw tracer uptakes used to generate these inhibition profiles were also used to calculate cystine transport rates. Hanes plots of transport data were then used to derive $K_m$ for cystine uptake (45 μM) and an approximate $K_i$ for alanine inhibition of cystine uptake (900 μM). (**b**) Effect of increasing external [Ala] on [$^3$H]alanine tracer (0.1 μM) uptake (△) and rate constant for [$^3$H]arginine tracer efflux (■). Data points represent means ± S.E.M. values for 11 oocytes (uptake) or 8 oocytes (efflux) and are corrected by subtraction of corresponding value in control oocytes. The $K_m$ for alanine uptake (1000 μM) was derived as described for (**a**).

of inhibition studies, appeared to be the major pathway for NBAT-related alanine transport. Alanine is reported to be a competitive inhibitor of NBAT-related cystine transport [3,25],

**Table 2 Effects of NBAT*myc* expression and NEM pre-treatment on amino acid concentrations in *Xenopus* oocytes**

Results represent means ± S.E.M. of five separate analyses. All values refer to oocytes at 4 days post-injection. *Significantly different from control value in water-injected oocytes ($P < 0.05$).

| | Intra-oocyte amino acid concentrations (nmol/oocyte) | | | |
|---|---|---|---|---|
| | NBAT*myc*-injected | | Water-injected | |
| | Control | + NEM† | Control | + NEM† |
| Total‡ | 8.7 ± 2.5 | 7.3 ± 1.9 | 6.9 ± 2.2 | 6.2 ± 2.1 |
| Alanine | 0.92 ± 0.31 | 0.84 ± 0.28 | 0.41 ± 0.09 | 0.27 ± 0.11 |
| Leucine | 0.55 ± 0.16* | 0.42 ± 0.08 | 0.13 ± 0.04 | 0.15 ± 0.03 |
| Serine | 0.75 ± 0.16 | 0.68 ± 0.21 | 0.58 ± 0.15 | 0.60 ± 0.16 |
| Phenylalanine | 0.10 ± 0.02 | 0.08 ± 0.015 | 0.06 ± 0.01 | 0.06 ± 0.01 |
| Lysine | 0.32 ± 0.11* | 0.33 ± 0.08 | 1.05 ± 0.28 | 0.73 ± 0.25 |

† Pre-treatment with 2 mM NEM for 30 min.
‡ The sum of measured amino acid concentrations (excludes Pro, Trp, Cys); glutamate contributed 40% to this total under all conditions.

so we proceeded to investigate directly the effects of NEM on NBAT-related alanine and cystine transport and the 'system $b^{0,+}$-like' alanine–arginine heteroexchange transport component observed in NBAT-expressing oocytes [6,20]. We found that NBAT-related alanine and cystine transport was more sensitive to inactivation by NEM than was arginine transport (> 85% compared with 42% inhibition under experimental conditions Table 1). Similarly, alanine and cystine transport were most sensitive to inhibition by β-phenylalanine (Table 1). Cystine uptake was saturable with an apparent $K_m$ of 45 μM (Figure 3a). Alanine inhibited cystine uptake with a $K_i$ of the order of 900 μM (Figure 3a), a value similar to the apparent $K_m$ for alanine uptake (Figure 3b), supporting the idea that alanine and cystine share a single NBAT-related transport pathway.

NBAT expression did not have a major effect on total amino acid concentration in oocytes (Table 2), although there were significant effects on concentrations of specific amino acids, notably increases in leucine and isoleucine and decreases in lysine and arginine; representative results are shown in Table 2. NEM pre-treatment had only a minor effect on amino acid concentrations of oocytes (generalized 15% decrease; see Table 2), which did not achieve statistical significance ($P > 0.1$).

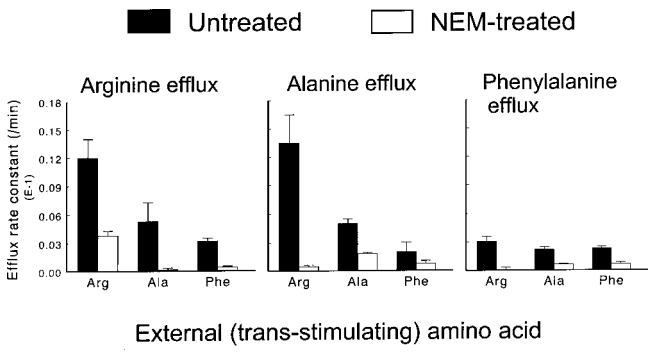


**Figure 4 Effects of NEM pre-treatment (2 mM NEM for 10 min) on the efflux of L-[$^3$H]arginine, L-[$^3$H]alanine and L-[$^3$H]phenylalanine by NBAT*myc*-injected oocytes**

*Trans*-stimulation of tracer efflux by 1 mM external unlabelled amino acid was measured in TMA transport buffer. Results are presented as the mean rate constant ($min^{-1}$) $\pm$S.E.M. for three oocyte preparations after subtraction of the appropriate value for control (pSG5-injected) oocytes.

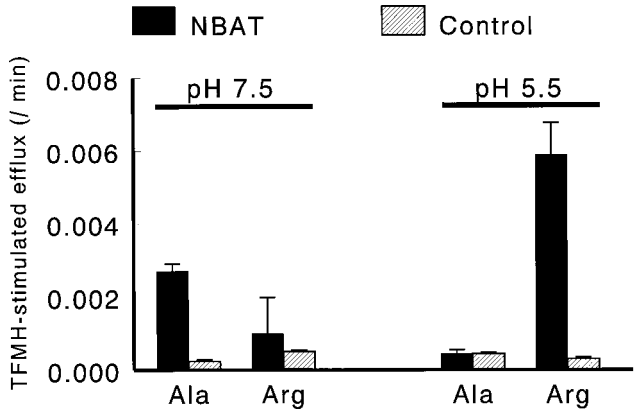


**Figure 5 Effect of external pH on *trans*-stimulation of alanine and arginine efflux by TFMH in NBAT*myc*-expressing oocytes**

L-[$^3$H]alanine and L-[$^3$H]arginine efflux from NBAT*myc* and control (pSG5 injected) oocytes was measured in TMA transport buffer in the presence of 1 mM TFMH at external pH 7.5 and 5.5. Results are presented as mean rate constants for efflux $\pm$S.E.M. of 14 oocytes from two cell batches, after subtraction of the appropriate value measured in the absence of external TFMH.

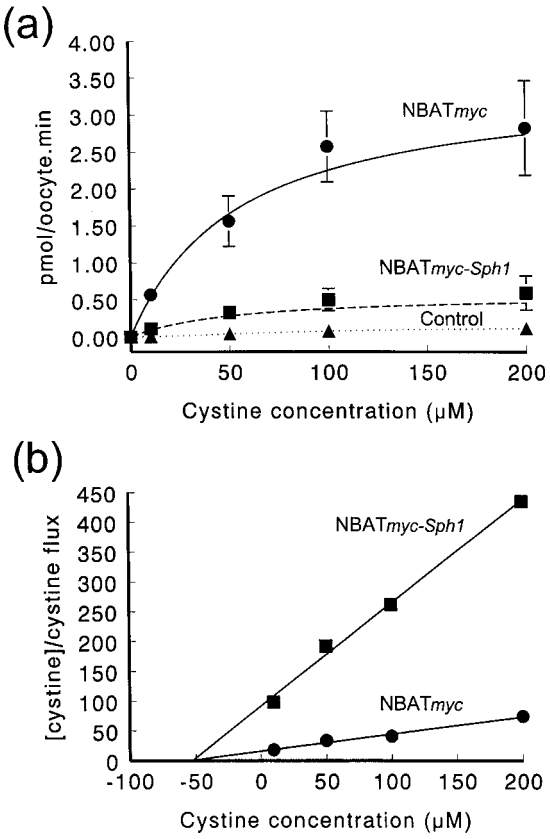


**Figure 6 NBAT-related L-[$^{35}$S]cystine uptake is reduced in the NBAT*myc-Sph*1 truncation mutant**

Results are presented as the mean uptake $\pm$S.E.M. for 9–11 oocytes from a single batch (similar results were obtained in a second experiment using a different batch of oocytes). (**a**) Total cystine uptake in NBAT*myc*, NBAT*myc-Sph*1 and control (water-injected) oocytes. The lines shown are Michaelis–Menten plots derived using $V_{max}$ and $K_m$ values obtained from (**b**) Hanes plots of transport data (here after subtraction of value in control oocytes). For NBAT*myc*, $V_{max}$ = 3.48 pmol/min per oocyte, $K_m$ = 55 $\mu$M; for NBAT*myc-Sph1*, $V_{max}$ = 0.58 pmol/min per oocyte, $K_m$ = 53 $\mu$M.

## Effect of NEM on NBAT-related exchange transport

L-Arginine, L-alanine and L-phenylalanine all displayed mutual *trans*-stimulation of tracer efflux from NBAT-injected oocytes (Figures 3b and 4), but efflux in the absence of external amino acid was not significantly different from that in control oocytes (which exhibited relatively minor *trans*-stimulation of efflux).

**Table 3 Effect of NEM on influx of amino acids and cystine (all at 10 $\mu$M) in NBAT*myc-Sph*1-injected oocytes**

$n$ = 5–7 oocyte batches for arginine and cystine flux; $n$ = 3 for phenylalanine flux. Values in parentheses represent percentage of control uptake after NEM treatment (2 mM for 30 min). *, **, Significantly different from basal value with $P < 0.05$, 0.005 respectively.

| | Influx (pmol/min per oocyte) | | | | | |
|---|---|---|---|---|---|---|
| | NBAT*myc* | | NBAT*myc-Sph*1 | | Uninjected | |
| | Control | + NEM | Control | + NEM | Control | + NEM |
| Arginine | 1.07 $\pm$ 0.09 | 0.65 $\pm$ 0.03** (61%) | 0.82 $\pm$ 0.07 | 0.58 $\pm$ 0.02* (71%) | 0.21 $\pm$ 0.07 | 0.18 $\pm$ 0.02 (86%) |
| Cystine | 0.48 $\pm$ 0.07 | 0.12 $\pm$ 0.04** (25%) | 0.10 $\pm$ 0.01 | 0.105 $\pm$ 0.03 (105%) | 0.013 $\pm$ 0.004 | 0.016 $\pm$ 0.010 (123%) |
| Phenylalanine | 0.42 $\pm$ 0.06 | 0.15 $\pm$ 0.03** (36%) | 0.28 $\pm$ 0.08 | 0.17 $\pm$ 0.04 (61%) | 0.055 $\pm$ 0.01 | 0.036 $\pm$ 0.018 (65%) |

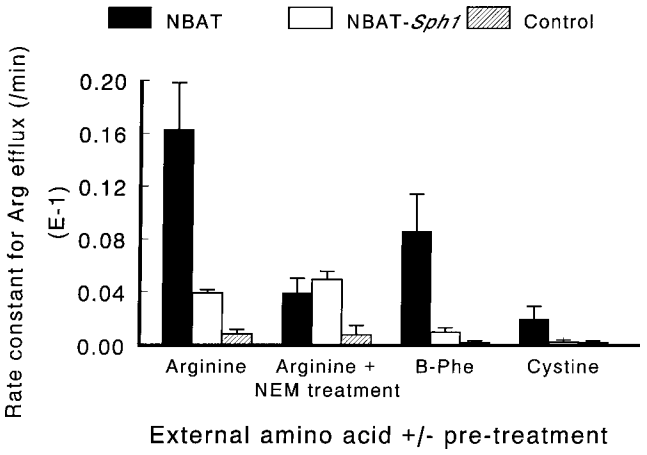


**Figure 7** ***Trans*-stimulation of L-[$^3$H]arginine efflux by external arginine, $\beta$-phenylalanine and cystine in oocytes injected with NBAT*myc* or NBAT*myc-Sph*1**

NEM pre-treatment was at 2 mM for 10 min where indicated. Efflux was measured in TMA transport buffer in the presence of 1 mM unlabelled amino acid or 0.2 mM cystine (+1 mM diamide). Uninjected oocytes were used as controls. Results are presented as the mean rate constant ($min^{-1}$) ±S.E.M. for 6–8 oocytes after subtraction of the appropriate value in the absence of external substrate. Similar results were obtained in a second experiment using a different batch of oocytes.

Arginine elicited the greatest *trans*-stimulatory effect on efflux of all three amino acids at both 1 mM (Figure 4) and 5 mM (results not shown). Arginine and phenylanine efflux were also stimulated by external cystine (0.2 mM), with rate constants of 0.002± 0.0007 $min^{-1}$ and 6±0.5 ($\times 10^{-4}$) $min^{-1}$ respectively.

NEM pretreatment (Figure 4) virtually abolished neutral–cationic amino acid hetero-exchange (Arg–Ala and Arg–Phe exchanges) and Cys–Arg exchange (results not shown), with lesser effects on homo-exchanges (Arg–Arg, Ala–Ala, Phe–Phe) and neutral amino acid heteroexchange (Ala–Phe). Efflux of L-[$^3$H]arginine from NEM-treated oocytes injected with NBAT*myc* was *trans*-stimulated by external arginine with a $K_{0.5}$ (50 $\mu$M; results not shown) of similar value to the apparent $K_m$ for the NEM-resistant component of arginine uptake [7].

### Trifluoromethylhistidine (TFMH) is a substrate for a subset of NBAT-related exchanges

The histidine analogue TFMH had differential inhibitory effects on NBAT-related transport activities related to its net charge at different pH values (effectively cationic at pH 5.5 and neutral at pH 7.5) [7]. Here we demonstrate directly that TFMH is a substrate of distinct NBAT-related exchanges at different external pH values (Figure 5). Alanine efflux from NBAT-injected oocytes was *trans*-stimulated by TFMH at pH 7.5 but not at pH 5.5, whereas TFMH stimulated arginine efflux at pH 5.5 but not 7.5 (Figure 5). Arginine and alanine tracer uptakes and Arg–Arg and Ala–Ala homo-exchanges were not affected by reduction of the external pH from 7.5 to 5.5 (results not shown). TFMH therefore appears to be a substrate for neutral–neutral and cationic–cationic amino acid exchanges but not for the neutral–cationic amino acid heteroexchange mechanism.

### Functional properties of NBAT*myc-Sph*1 mutant

The NBAT*myc-Sph*1 mutant (Δ671–683; see Figure 1) showed an 80% reduction in cystine (0.01 mM) transport relative to NBAT*myc*, with substantial retention of arginine and phenylalanine transport (Table 3). This reduction in cystine transport resulted from a decrease in $V_{max}$ (from 3.48 to 0.58 pmol/min per oocyte) without a change in transport $K_m$ (54 $\mu$M; see Figure 6). The residual cystine transport activity of NBAT*myc-Sph*1 differed markedly from that of NBAT*myc* in that it was insensitive to inactivation by NEM (Table 3). Arginine and phenylalanine transport expressed in NBAT*myc-Sph*1-injected oocytes also showed significantly reduced sensitivity to inactivation by NEM (Table 3). The NEM-resistant uptakes of all three substrates were of very similar magnitude in NBAT*myc* and NBAT*myc-Sph*1-injected oocytes, suggestive of a specific loss of NEM-sensitive transport activity in the *Sph*1 mutant (Table 3). This mutant also showed an almost complete loss of Arg–$\beta$-Phe and Arg–Cys hetero-exchange transport, but retained an Arg–Arg homo-exchange component which was NEM-resistant and of the same magnitude as the NEM-resistant Arg–Arg exchange component in NBAT*myc*-injected oocytes (Figure 7). NBAT*myc-Sph*1 protein was detected on Western blots of oocyte membranes in similar abundance to NBAT*myc* (results not shown).

## DISCUSSION

The present results extend our previous observations [7,18] that multiple amino acid transport components are induced by NBAT expression in oocytes by revealing distinct Na-independent pathways for neutral–neutral and cationic–cationic amino acid homoexhange, as well as a neutral–cationic amino acid hetero-exchange activity that is selectively blocked by NEM pre-treatment. This latter activity is the primary route for cystine and alanine transport by NBAT-related mechanisms and was selectively ablated in the rat NBAT-*Sph*1 mutant, in which the 13 C-terminal amino acids are deleted, without significant effect on expression of NEM-resistant homoexchange mechanisms. NBAT expression also generates alterations in intra-oocyte concentrations of some (but not all) amino acid substrates, presumably due to relative changes in the effective permeability of the cell membrane to these amino acids. These alterations should be taken into consideration when detailed kinetic analysis of NBAT-related transport expressed in oocytes is undertaken. NEM pre-treatment does not produce any significant, independent alteration of amino acid concentrations in oocytes, therefore its effects are not merely an indirect consequence of changes in *trans*-substrate availability for exchange.

NBAT-related transport activities appear to operate as obligatory exchange mechanisms, requiring substrate on both faces of the membrane for a transport cycle to occur. The NEM-sensitive transport pathway effects neutral–cationic amino acid exchange and has a relatively high $K_m$ for alanine (~ 1 mM) in both Na-free (Figure 3) and Na-containing [20,25] medium. These functional properties are identifiable with the 'system $b^{0,+}$-like' activity [6,8,25] that is defective in Type 1 cystinuria [9]. The transport mechanism of the system $b^{0,+}$-like activity appears to include both homoexchange (e.g. Arg–Arg, Ala–Ala) as well as heteroexchange modes, because NEM has significant inhibitory effects on both types of exchange. Cystine–neutral amino acid exchanges by this mechanism at the renal brush border are believed to form the basis of a tertiary active transport mechanism for renal cystine reabsorption [21].

TFMH is not a good substrate for the system $b^{0,+}$-like pathway but, depending upon its net charge, TFMH appears to be a substrate for separate NBAT-related homoexchange mechanisms for neutral and cationic amino acids. We have shown previously

[7] that TFMH is a potent inhibitor of NEM-resistant, NBAT-related arginine transport (which has functional properties characteristic of system $y^+L$ [7,8,18]) and now confirm that the cationic form of TFMH exchanges with arginine in NBAT-expressing oocytes. We therefore propose that system $y^+L$ is responsible for the observed NEM-resistant cationic amino acid (Arg–Arg) exchanges. Our experiments were performed in the absence of sodium and this may have largely suppressed any neutral–cationic amino acid heteroexchanges through system $y^+L$, because the affinity of this exchanger for neutral amino acids is reduced in Na-free conditions [8,16]. Nevertheless, system $y^+L$ and/or system L-like activity (possibly analogous to that produced by co-expression of SPRM1 with 4F2hc [14]) might account for the residual, NEM-resistant, neutral amino acid exchanges. In NBAT-expressing oocytes, the activity of these latter exchange mechanisms may explain the relatively high resistance to NEM of uptake of arginine and phenylalanine, which enter oocytes in exchange for internal amino acids exiting an Na-containing compartment.

There is significant amino acid homology between NBAT and 4F2hc (26 % identity, 45 % similarity; see [6] for review) and it is now evident that 4F2hc acts as a subunit of heteromeric transporter complexes [14–17], possibly involved in targetting putative catalytic light-chain subunits to the plasma membrane [14,17]. System L and $y^+L$ transport activities have recently been shown to be expressed by heterodimers of 4F2hc with different light-chain permease subunits (e.g. E16, LAT1 [14,15], $y^+$LAT-1 [16]). The functional heterogeneity in NBAT-related amino acid transport leads us to suggest that NBAT, like 4F2hc, can interact with a variety of light-chain subunits to express different transport properties, although such protein–protein interactions have not been demonstrated directly to date. Association of NBAT with different light-chain subunits endogenous to the *Xenopus* oocyte would produce the observed heterogeneity of transport properties in our experimenal system, limited by the availability of endogenous light-chain proteins for heteromer formation. Such heterogeneity might also help to explain the apparently variable stoichiometry of NBAT (rBAT) as an amino acid exchanger [20]. Confirmation of these possibilities awaits molecular identification of further members of the light-chain permease family, notably the putative system $b^{0,+}$-like light-chain.

The NEM-sensitive, system $b^{0,+}$-like mechanism transporting cystine is selectively ablated in the rat NBAT-*Sph*1 truncation mutant (Δ671–683). A cystinuria-specific mutation (L678P) has been identified within the equivalent amino acid residues (673–685) of human NBAT [6,9]. Transport activity expressed by NBAT-*Sph*1 is similar in both scope and magnitude to NEM-resistant, NBAT-related transport, which is suggestive of a crucial role for some or all of the 13 C-terminal amino acid residues of wild-type NBAT in expression of system $b^{0,+}$-like heteroexchange, but not of other NBAT-related transport activities. These observations lead us to propose that NEM specifically ablates a distinct $Na^+$-independent heteroexchange component, rather than modifying the overall functional properties of NBAT-related transport. Miyamoto et al. [22] have also noted loss of cystine transport in a (human) NBAT truncation mutant lacking C-terminal residues 511–685, with retention of some capacity for arginine and leucine transport by a system $y^+L$-like mechanism [6,8,22]. The amino acids deleted in the NBAT-*Sph*1 mutant include two cysteine residues (671 and 683) which may be the targets for NEM action; we are currently investigating this possibility using site-directed mutagenesis. These two cysteine residues are not conserved in 4F2hc, which does not express the system $b^{0,+}$-like transport component displayed by NBAT [6,8,25].

The NBAT C-terminus does not appear to be required for expression of the $y^+L$ (and putative L-like) transport activities similar to those expressed by 4F2hc in association with different light-chain subunits. A highly conserved, exofacial cysteine residue, located just distal to the first predicted trans-membrane domain of both 4F2hc and NBAT, has been proposed as a likely site for a disulphide bridge associated with heavy-chain–light-chain protein complex formation [6,12,13,17,25]. Mutation of this residue in NBAT (C111S) reduces expressed amino acid transport only when accompanied by C-terminal truncation [12]. Cysteine residues 111 and 671/683 of rat NBAT may therefore all be required for full expression of transport, but it remains unclear as to whether these residues support the same or different transport mechanisms. Surprisingly, covalent disulphide linkages between heavy-chain and light-chain subunits of NBAT and 4F2 transporters may not be absolutely essential functional requirements [12,13], possibly because heavy-chain–light-chain interactions also involve leucine-zipper motifs (e.g. residues 545–587 of NBAT) [12,17]. The suggested importance of cysteine 671/683 of NBAT may therefore result from involvement in essential intra-protein disulphide interactions, or alternatively these residues may have direct roles in substrate binding or in the production of a conformational change essential to the (system $b^{0,+}$) transport cycle. The last mentioned possibilities are most consistent with the view that C-terminal Cys residue(s) of NBAT targetted by NEM should contain free sulphydryl groups not (or perhaps only transiently) involved in -S–S- bonding. The possibility that NBAT itself has catalytic transport properties, either intrinsically or in association with other subunits of a transporter complex, should therefore not be entirely excluded at this time.

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