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Evidence suggesting that the minimal functional unit of a renal cystine transporter is a heterodimer and its implications in cystinuria

Review Article

S. S. Tate

Department of Biochemistry, Cornell University Medical College, New York, New York, U.S.A.

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Summary. Cystinuria, one of the most common genetic disorders, is characterized by excessive excretion of cystine and basic amino acids in urine. The low solubility of cystine results in formation of kidney stones which can eventually lead to renal failure. Three types of cystinurias have been described. All involve defects in a high-affinity transport system for cystine in the brush border membranes of kidney and intestinal epithelial cells. The molecular properties of proteins involved in epithelial cystine transport are incompletely understood. A protein (NBAT, neutral and basic amino acid transporter), initially cloned by us from rat kidney and shown to be localized in the renal and intestinal brush border membranes, has been implicated in this transport, and mutations in human NBAT gene have been found in several cystinurics, making it a prime candidate for a cystinuria gene. However, mutations in NBAT were found only in Type I cystinurics and not in Types II and III suggesting that defects in other, as yet uncharacterized, genes may also be involved. NBAT has an unusual (for an amino acid transporter) membrane topology. We proposed that the protein contains four membrane-spanning domains, a model disputed by other investigators. We subsequently obtained experimental data consistent with a four membrane-spanning domain model. Furthermore, recently we showed that kidney and intestinal NBAT (85 kDa) is associated with another brush border membrane protein (about 50 kDa) and have proposed that the heterodimer represents the minimal functional unit of the high-affinity cystine transporter in these membranes. These findings raise the tantalizing possibilities that defects in the NBAT-associated protein might account for cystinurias in individuals with normal NBAT gene (such as the Types II and III cystinurics).

Keywords: Cystine transporter – Cystinuria – Subunits – Topology – Amino acid transport

Introduction

Cystinuria is an autosomal recessive disease in which excessive amounts of cystine and basic amino acids, Arg, Lys, and Orn, are excreted in the urine (Segal and Thier, 1995; McKusick, 1994). Cystinuria was one of the first four diseases defined by Sir Archibald Garrod in 1908 as "inborn errors of metabolism." The disease is now known to be caused by an amino acid transport defect in the brush borders of the proximal renal tubule and the intestinal epithelium (Rosenberg et al., 1965). Cystinuria is one of the most common genetic disorders with an overall prevalence of 1 in 7,000, ranging from about 1 in 2,500 in Jews of Libyan origin to 1 in 15,000 in the U.S.A. (Segal and Thier, 1995; Levy et al., 1972; Weinberger et al., 1974). The major clinical feature of cystinuria is the development of kidney stones which result from the low solubility of cystine. The consequent urinary obstuction can lead to colic, infection, and, eventually, renal failure (Dahlberg et al., 1977). Three types of classic cystinurias have been described, the classification being based on urinary excretion of amino acids in heterozygotes and intestinal absorption in homozygotes (Segal and Thier, 1995). In type I, the heterozygotes have no abnormal aminoaciduria, whreas types II and III heterozygotes exhibit cystinuria intermediate between normals and affected individuals. In type III, in contrast to types I and II, the homozygotes show near normal cystine transport across the intestinal epithelium. It has long been assumed that the three carrier types are due to allelism of the same gene, but more complex multilocus models have not been entirely excluded (Goodyear et al., 1993).

The molecular mechanisms involved in the transport of cystine across renal and intestinal brush border membranes are not completely understood. Most of the uptake of cystine into renal brush border membrane vesicles is Na^+-independent (Foreman et al., 1980; McNamara et al., 1981; McNamara et al., 1992) and seems to involve both a *high-affinity system* (K_m values in the μM range) that also accepts basic amino acids, and a *low-affinity system* which is relatively specific for cystine (McNamara et al., 1981; Segal et al., 1977). Transport of cystine in jejunal brush border membrane vesicles appears to involve only the high-affinity system that is shared with basic amino acids (Ozegovic et al., 1982). The high-affinity system appears to be the one that is deficient in cystinuric individuals (Coicadan et al., 1980; Thier et al., 1964). Studies with perfused isolated renal tubules indicate that the high-affinity transport system is located in the proximal straight tubules (Schafer and Watkins, 1984).

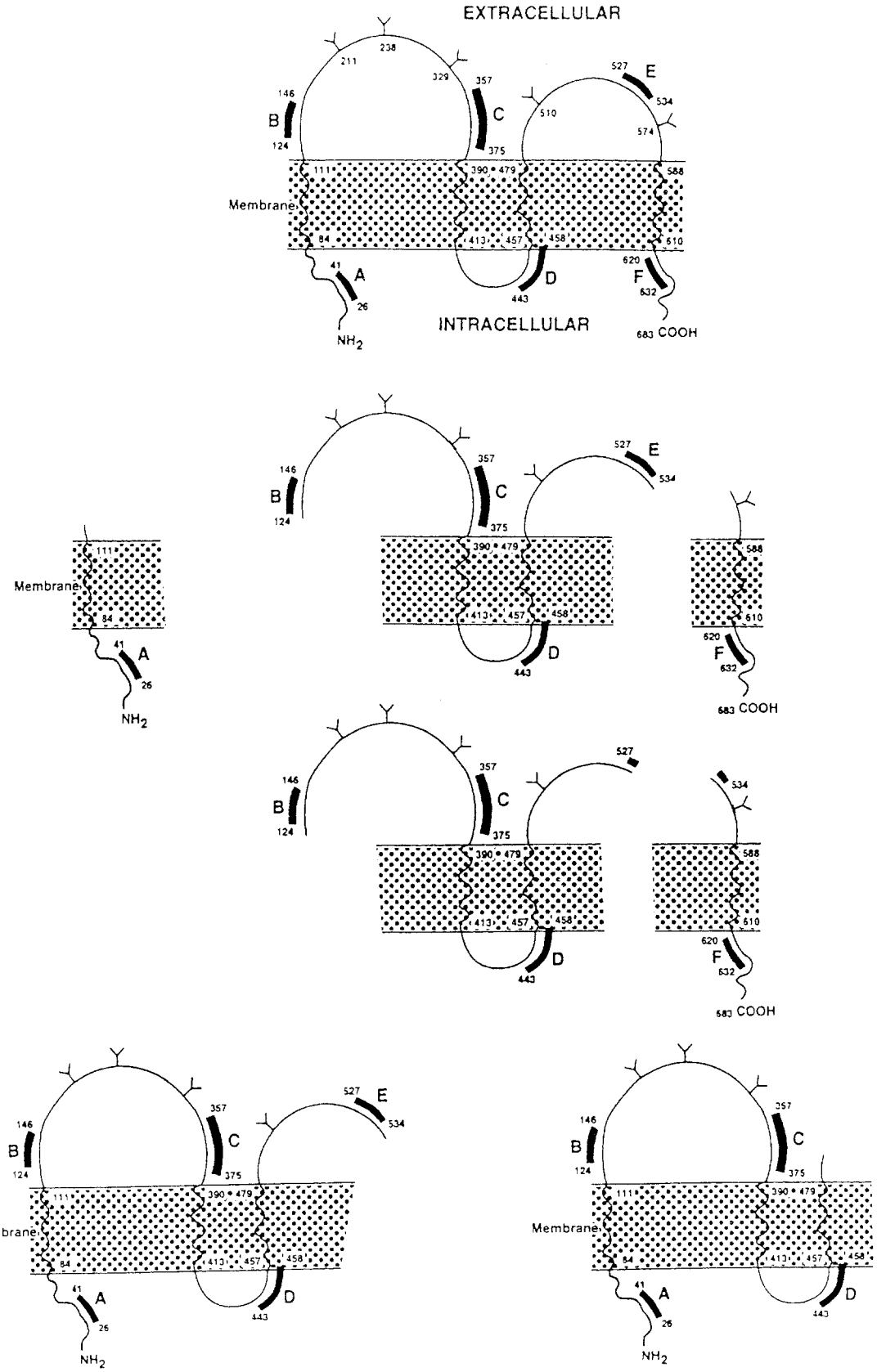
A candidate protein for renal cystine transport was first cloned by us from a rat kidney cDNA library using the *Xenopus* oocyte expression strategy (Tate et al., 1992). We initially believed it to be a variant of one of the Na^+-independent neutral amino acid transporters and hence named it NAA-Tr. This was soon followed by the cloning of an identical rat kidney protein (termed D2) (Wells and Hediger, 1992) and highly homologous proteins from rabbit (named rBAT) (Bertran et al., 1992) and human kidney (Bertran et al., 1993; Lee et al., 1993). The human clone has been denoted SLC3A1 in the

Gene Data Base. These two groups showed that expression of these proteins in oocytes leads to induction of a high affinity Na^+-independent transport system for not only some of the neutral amino acids but also for the basic amino acids and cystine. The induced transport exhibits some of the characterisics ascribed to system $b^{0,+}$, a functionally defined transport system first detected in mouse blastocysts (Van Winkle et al., 1988). (See Kilberg et al., 1993 and Bertran et al., 1994 for recent reviews on mammalian amino acid transport systems.) We confirmed these findings and introduced the term NBAT to denote these proteins (for neutral and basic amino acid transporter). In a recent review, Christensen et al. have recommended that these proteins be designated bAT (Christensen et al., 1994). However, in this review we will continue to use the term NBAT to refer to the rat, rabbit, and human proteins. This review focuses primarily on the structural organization of rat NBAT and implications of this organization in cystinuria.

Structure, function, and tissue expression of NBAT

The rat kidney NBAT/cDNA encodes a protein of 683 amino acids, with a calculated molecular weight of 78,506 and containing five potential N-glycosylation sites. The deduced sequence contains no N-terminal signal sequence that conforms to the generally accepted empirical rules for such sequences. Based on this and on our interpretation of its hydrophobicity profile (Tate et al., 1992), we proposed a topological model for this protein containing four membrane-spanning domains (MSDs) (see Fig. 1) (more on the topology of NBAT later). This model places the N- and the C-terminii of the protein in the cytoplasm (Nin-Cin) and all potential N-glycosylation sites extracellularly. About six months after the publication of our paper, Wells and Hediger (1992) reported cloning of a rat kidney protein (named by them, D_2) exhibiting 100% identity to the protein we had cloned, and Bertran et al. (1992) reported cloning of a highly homologous protein from rabbit kidney (termed rBAT). Susequently these two groups independently cloned human kidney NBAT (Bertran et al., 1993; Lee et al., 1993), the amino acid sequence of which is about 80% identical to that of the rat and rabbit NBATs.

cRNA transcribed from NBAT/cDNA (rat, rabbit, and human), when injected into oocytes, induces a high affinity, Na^+-independent transport system for neutral amino acids such as Leu, Phe, and Met (Tate et al., 1992), as well as for cationic amino acids (Arg, Lys, Orn) and cystine (Wells and Hediger, 1992; Bertran et al., 1992, 1993; Lee et al., 1993). The highest rates of uptake are seen with Leu, Phe, Met, Arg, and Lys. Cystine uptake rate is about 30 to 40% that of Leu and Arg for the rat and rabbit NBAT/cRNA-induced transport system in oocytes. In contrast, for the human NBAT/cRNA-induced transport, the cystine uptake is about 18% relative to Arg and 27% relative to Leu (Bertran et al., 1993). The transport Km's for these amino acids are in the micromolar range (20 to 100μM). As noted above, the substrate specificity and kinetics of the transport induced in oocytes by NBAT/cRNA resemble those ascribed to system designated $b^{0,+}$ (Van


EXTRACELLULAR
INTRACELLULAR
Membrane
NH2
683 COOH
A
B
C
D
E
F

Winkle et al., 1988). Recently, Busch et al. (1994) and Coady et al. (1994) independently obtained results which seem to indicate that, in oocytes, NBAT aparently catalyzes transport of neutral amino acids which is accompanied by the countertransport of basic amino acids and vice versa. These findings demonstrate the need for a more detailed investigation of the transport induced by NBAT.

The characterization and ultrastructural localization of NBAT in rat tissues became possible when we made specific rabbit antibodies (Abs) against rat NBAT. Six peptides representing sequences within the putative intra- and extracellular regions of NBAT (based on the proposed 4-MSD model for NBAT) were synthesized as antigens for the production of site-specific Abs (Mosckovitz et al., 1993, 1994). Location of these epitopes is depicted by heavy bars in Fig. 1. All six antisera specifically recognized and precipitated rat NBAT (Mosckovitz et al., 1994).

These Abs were employed to characterize NBAT protein in rat tissues and in various cellular and *in vitro* expression systems (Mosckovitz et al., 1993; Wang and Tate, 1995). In brief: *in vitro* translation (IVT) of NBAT/cRNA produced a 78kDa product, a size close to that predicted by the cDNA-deduced amino acid sequence. IVT in the presence of microsomal membranes yielded an additional approximately 89kDa species which was shown to be a coreglycosylated form. In Xenopus oocytes, rat NBAT/cRNA yields two products, approx. 89 and 87kDa, the difference in mass being apparently due to differences in the extent of glycosylation. The human NBAT/cRNA directs the synthesis of an approximately 94kDa N-glycosylated species in oocytes (Bertran et al., 1993). Transfection of COS7 cells with NBAT/cDNA led to the synthesis of a 88kDa species (presumably glycosylated), most of which was located in the membrane fraction of these cells. In rat kidney and jejunum the major NBAT band was approximately 85kDa (N-glycosylated), being enriched in the brush border membranes.

The anti-NBAT Abs were also useful in the immunocytochemical localization of NBAT in various rat tissues using light and electron microscopic immunoperoxidase labeling techniques. The most prominent localization of NBAT was in the brush border membranes of the epithelial cells lining renal proximal tubules. Somewhat less intense labeling was seen in the brush border membranes of jejunal epithelial cells (Pickel et al., 1993). Renal proximal tubular brush border membrane localization of rat NBAT was independently confirmed by Furriols et al. (1993). Note that distribution of NBAT in rat

Fig. 1. Propose membrane topology of rat kidney neutral and basic amino acid transporter (NBAT) (figure at the top) and a schematic representation of the fragments generated by limited surface proteolysis of rat kidney brush border membrane-associated NBAT. The approximate start and end points of the membrane-spanning domains are numbered. N-Glycosylation sites are numbered and each maked by a "Y". Heavy bars represent epitopes against which site-specific antibodies were made. Approximate sites of cleavage by papain were derived by estimation of the size of each fragment (following SDS/PAGE and Western analysis) and determination of the epitopes present on these fragments by Western analysis using each of the site-specific antibodies (Mosckovitz et al., 1994)

tissues parallels the tissue expression of its mRNA (Yan et al., 1992; Markovich et al., 1993).

An unexpected finding of our immunocytochemical localization studies was that, in the intestine, intense NBAT-specific immunolabeling was seen in a select population of enteroendocrine cells and enteric neurons (Pickel et al., 1993). The neuronal labeling was localized within dense core vesicles in axon terminals apposed to the basal lamina near fenestrated blood vessels. Recently, in an extension of these studies, we found intense, highly granular immunolabeling for NBAT in the chromaffin and ganglion cells of rat adrenal medulla (Nirenberg et al., 1995). Furthermore, intensely labeled varicose processes were detected in brainstem and spinal cord nuclei. Ultrastructural examination of the nuclei of the solitary tract of rats showed that NBAT was localized predominatly, as in the enteric neurons, to axon terminals. Although further work is required to characterize the anti-NBAT Ab reactive protein(s) in these regions, these findings, nevertheless, suggest additional transport and, perhaps, other functions for NBAT (or NBAT-related proteins).

NBAT is a cystinuria gene

Enrichment of NBAT in the brush border membranes of renal proximal tubule and jejunal epithelial cells is in accord with its proposed role in amino acid transport. Furthermore, hybriddepletion of renal and intestinal mRNA with NBAT anti-sense oligonucleotides results in almost complete (>90%) inhibition of cystine uptake (as well as the uptake of neutral and basic amino acids) induced in oocytes by these mRNAs (Bertran et al., 1993; Magagnin et al., 1992). These results indicate that NBAT is responsible for most, if not all, of the high-affinity cystine uptake elicited in oocytes by kidney and intestinal mRNAs making it a prime candidate for the cystinuria gene. To test this hypothesis, Calonge et al. (1994) undertook a search for cystinuria-specific mutations in NBAT. Six cystinuria-specific missense mutations in the NBAT gene, confirmed in genomic DNA, were found in 30% of the independent cystinuric chromosomes derived from Italian and Spanish cystinuric patients. All these mutations affect well-conserved amino acid residues in human, rat, and rabbit NBATs. The most frequent mutation, M467T (i.e., substitution of Met at residue 467 by Thr in human NBAT), almost completely attenuates the expression of amino acid transport activity in oocytes. Additional mutations in the NBAT gene in cystinurics have recently been found by Pras et al. (1994b), Gasparini et al. (1995), and Miyamoto et al. (1995) (for detailed discussion of the mutational analysis of NBAT gene in cystinurics see the article by Palacin et al. in this volume). The human NBAT gene was assigned to chromosome 2 by somatic cell hybrid analysis (Lee et al., 1993), and Pras et al. (1994) independently mapped the disease susceptibility locus to chromosome 2p in a panel of 17 Israeli and American families. Using the fluorescence *in situ* hybridization FISH) technique, this gene has been localized to segment p21 on chromosome 2 (Yan et al., 1994; Zhang et al., 1994). Recently, however, using a variation of the FISH technique, Calonge et al. (1995a) assigned

the human NBAT gene to segment p16 of chromosome 2 (2p16.3). This discrepancy needs to be resolved. It should be noted, however, that 2p16.3 is the nearest band to 2p21 perhaps accounting for the differences in the interpretation of the localization data. Nevertheless, taken together, these findings provide strong evidence that NBAT is a cystinuria gene. However there are other considerations which raise the possibility that other gene products might also be involved in cystinuria and, thus, in renal cystine transport. Thus, as noted above, differences are seen in both cystine transport and excretion of amino acids in urine amongst the three types of cystinurics. In a mentally handicapped 3 year old child with cystinuria, Sharland et al. (Sharland et al., 1992) found an apparently balanced *de novo* translocation with break points at 14q22 and 20p13, indicating that other genes might also be involved in the etiology of cystinuria. None of the eight mutations found in NBAT gene by Calonge et al. (1994) and Pras et al. (1995) were found in 16 other cystinuric families screened by Pras et al. Furthermore, Calonge et al. (1995b) and Gasparini et al. (1995) recently reported that all chromosomes carrying a NBAT mutated gene belong to type I cystinurics but not to types II and III. Indeed, analysis of 70% of the NBAT coding region indicated normal NBAT sequences in types II and III cystinurics. These considerations make our recent findings, discussed below, that another protein in kidney and jejunal brush border membranes might be functionally associated with NBAT, particularly enticing, since mutations in this NBAT-associated protein might also contribute to the transport defects seen in cystinuria, perhaps in individuals with normal NBAT.

Membrane topology of NBAT

Although there is now considerable evidence that NBAT participates in a high-affinity transport system for cystine, its precise role in this transport still remains to be elucidated. The uncertainty about its role stems from the fact that the membrane structure proposed for this protein does not conform with the structures proposed for other well-characterized mammalian metabolite transporters. Thus, two contrasting topological models were proposed for NBAT based on different interpretations of the hydropathy profiles of its amino acid sequence. As noted above, we postulated a topological model containing 4 membrane-spanning domains (MSDs) for the rat kidney NBAT (Tate et al., 1992; see Fig. 1). Others proposed a strikingly different model based on a single N-terminal MSD (Wells and Hediger, 1992; Bertran et al., 1992, 1993; Lee et al., 1993), and raised the possibility that NBAT itself may not be the transporter but might, in fact, be an effector subunit of a larger transporter complex. To distinguish between the two proposed topological models, we took two independent experimental approaches, both of which depended on the availability of a battery of site-specific Abs against NBAT. In the first approach, NBAT in rat renal brush border membranes was subjected to limited surface proteolysis and the fragments thus produced were probed with each of the site-specific Abs to determine approximately where the cleavages occurred. Second method involved an immunofluorescent

labeling technique in which intact or membrane-permeabilized cells expressing NBAT were probed with Abs directed against the putative extracellular and cytoplasmic domains. Both approaches yielded results consistent with our proposed four MSD Nin-Cin topological model for NBAT (Moscovitz et al., 1994) and it is gratifying to note that Palacin et al. have used this model to depict cystinuria-specific mutations in NBAT (see Palacin et al. article in this volume).

In the first approach, NBAT in closed, right-side-out rat renal brush border membrane vesicles was subjected to limited surface proteolysis by papain. The fragments produced were separated by SDS/PAGE and probed by each of the six site-specific Abs. The data obtained were then used to predict approximately where in NBAT the cleavages occurred. A summary of these findings is schematically depicted in Fig. 1. The findings in brief were: (i) All of the fragments produced by papain remained membrane-associated. (ii) The fact that fragments containing either only the N- or the C-terminal epitope (A and F, respectively; Fig. 1) remained membrane-associated indicates that both these peptides contain membrane-anchoring domains and that the C-terminus is most likely intracellular. (iii) Since the fragments containing epitopes B, C, and D and B, C, D, and E (i.e., the epitopes between the putative first and the fourth MSDs) remained membrane-associated, we conclude that this intervening stretch between the terminal epitopes must contain at least one and most likely two MSDs (two MSDs to account for the intracellular orientation of the C-terminus of NBAT). (iv) Even longer incubation with papain (upto 1h) failed to produce cleavages in regions proposed to be intracellular in our four MSD model. Thus, the results of this approach are in accord with our proposed Nin-Cin topological model containing approximately four MSDs. More importantly, our results conclusively rule out the single N-terminal MSD model (Nin-Cout) proposed by other investigators.

Results supportive of the four MSD topological model were also obtained from the second approach in which the site-specific Abs were employed as topological probes of NBAT expressed in COS7 cells. Such cells express a glycosylated form of NBAT, almost all of which is found in the plasma membrane (Mosckovitz et al., 1993). The transfected cells were treated with each of the anti-peptide Abs either in the absence or in the presence of a detergent (Triton X-100). The detergent was added at a concentration sufficient to permeabilize the plasma membrane but not to damage the cells extensively. Cells were then washed and subsequently treated with rhodamine-conjugated goat anti-rabbit IgG. Abs directed against the NBAT sequences 124–146 (Ab124), 357–375 (Ab357), and 527–534 (Ab527) (epitopes B, C, and E, respectively; Fig. 1) were readily visualized on the surface of transfected cells in the absence of the detergent (39), indicating that the epitopes recognized by these Abs are located, as predicted by our model, on the external surface of the plasma membrane. In contrast, the Abs directed against sequences 26–41 (Ab26), 443–458 (Ab443), and 620–632 (Ab620) (epitopes A, D, and F, respectively; Fig. 1) reacted only when the cells were previously permeabilized with Triton X-100, indicating that these epitopes are

not accessible on the cell surface but are presumably localized along the cytoplasmic side of the plasma membrane. These results are in accord with those expected for a four-MSD, Nin-Cin, model for NBAT and, taken together with limited proteolysis approach, make a strong case for this topological model.

Figure 2 compares the amino acid sequences of the four proposed MSDs in rat NBAT with the corresponding sequences in the rabbit and human NBATs. Of note is the considerable sequence similarity in these regions in the three species. MSD1 in the three NBATs is composed entirely of hydrophobic residues and MSD3 is almost so. In all three species, MSD2 and MSD4 contain, in addition to apolar residues, one or two amino acids with charged side chains, a feature NBAT shares with other transporters and several ion channels. Another feature that NBAT shares with many other transport proteins is the presence of proline residues (e.g., in MSD2 and MSD3) in some of the MSDs. Brandl and Deber (1986) have noted that nearly all transport proteins contain membrane-buried proline residues and have proposed that

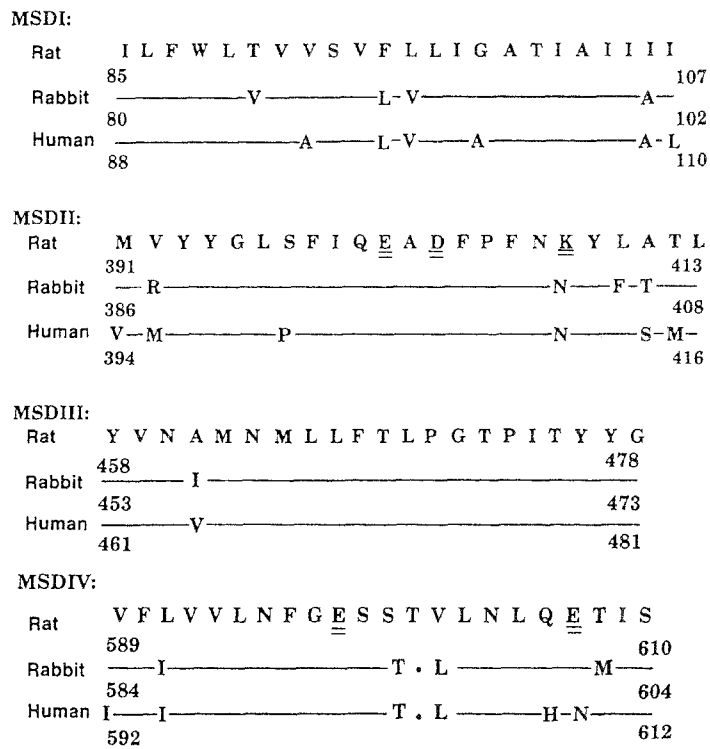


Fig. 2. Comparison of the putative membrane-spanning domains (MSDs) of the rat, rabbit, and human NBATs. For each MSD the first line refers to the rat NBAT MSDs Tate et al., 1992). Below this are the corresponding sequences in the rabbit (from Bertran et al., 1992) and human (from Bertran et al., 1993) NBATs with only the differences shown. Numbers below the sequences denote the corresponding residue within each protein. Amino acid residues with charged side chains are underlined. The filled circles in the fourth MSD of the rabbit and human NBATs are inserted to align the subsequent sequences with the corresponding sequence in the rat NBAT. Figure taken from Mosckovitz et al., 1994

such proline residues might be involved in transport channel formation. The role of membrane-buried proline residues in NBAT requires further study. The high degree of homology between the putative MSD regions in the NBATs suggests that rabbit and human NBATs most likely also assume topologies based on four MSDs. Interestingly, the most frequent mutations detected in cystinurics occurs in the conserved methionine residue (M467 in human NBAT) in the putative third MSD. It will be interesting to determine what role, if any, this region plays in ligand binding and/or in the formation of a transmembrane channel for transport.

Oligomeric organization of NBAT in brush border membranes

The four MSD topology of NBAT is in marked contrast to those of other well-characterized mammalian metabolite transporters (including the amino acid transporters cloned to date) most of which appear to contain from 8 to as many as 12 MSDs (Palacin, 1994; Kanner and Kleinberger-Doron, 1994; Baldwin, 1994; Uhl and Hartig, 1992). We, therefore considered the possibility, as did other investigators (Wells and Hediger, 1992; Bertran et al., 1992), that NBAT might in fact be a component of a multimeric transporter complex. To investigate these possibilities, the oligomeric organization of NBAT in renal and jejunal BBMs as well as in other cell systems in which NBAT is expressed was examined. The findings of these studies (Wang and Tate, 1995) are summarized below.

As noted above, when rat kidney brush border membranes were subjected to SDS/PAGE under reducing conditions (ie., when the BBMs were treated with SDS in the presence of a disulfide reducing agent such as 2-mercaptoethanol [MSH], prior to SDS/PAGE) followed by Western analysis using NBAT-specific Abs, an approximately 85 kDa immunoreactive band corresponding to the NBAT monomer was detected. We initially used chemical cross-linking of surface proteins to probe the subunit organization of NBAT in renal brush border membranes. When the membranes were treated with the cross-linking reagent, dimethylsuberimidate (DMS), followed by SDS/PAGE under reducing conditions, a cross-linked species exhibiting a molecular mass of approximately 135 kDa was observed in addition to the expected 85 kDa NBAT monomeric band. Qualitatively similar results were obtained when cross-linking with DMS was carried out on membranes which had been previously treated with a non-ionic detergent, Lubrol WX, to solubilize the membrane proteins, indicating that the components of the 135 kDa species remain associated even when the membrane structure is disrupted by a detergent. The 135 kDa cross-linked species was also produced when the brush border membranes were treated with Bis(sulfosuccinimidyl)-suberate (BS^3), a membrane-impermeant cross-linking reagent. The molecular mass of the cross-linked species (135 kDa) is significantly less than that expected for a homodimer containing two NBAT subunits (expected size, about 170 kDa). Therefore, the most likely interpretation is that NBAT is closely associated with a membrane protein about 50 kDa in size and that the

cross-linked 135kDa species represents a heterodimer containing an NBAT subunit and this 50kDa protein.

During the course of the cross-linking experiments, we noted that a similar 135kDa NBAT-containing species was also observed when the brush border membranes were subjected to SDS/PAGE under non-reducing conditions (i.e., when the membranes were treated with SDS in the absence of a reducing agent such as MSH). However, the relative amount of the 135kDa species varied from about 15 to 50% of the total NBAT-containing species when separate batches of brush border membranes were examined under non-reducing conditions. Irrespective of such variation, only the 85kDa NBAT monomer band was seen in all brush border membrane preparations under reducing conditions. These findings were disturbing since if one assumes that the heterodimer represents the functional unit of the NBAT-containing transporter, most, if not all, of the NBAT in these membranes should be found in this complex. Furthermore, the variation in the relative amount of the 135kDa species in different batches of the membranes suggested that the disulfide bonds holding this complex together are easily reduced during isolation of the brush border membranes by sulfhydryl compounds normally present in rat kidney (eg., glutathione, concentrations of which in kidney can approach 5mM). To test this possibility, we included a thiol-blocking reagent,

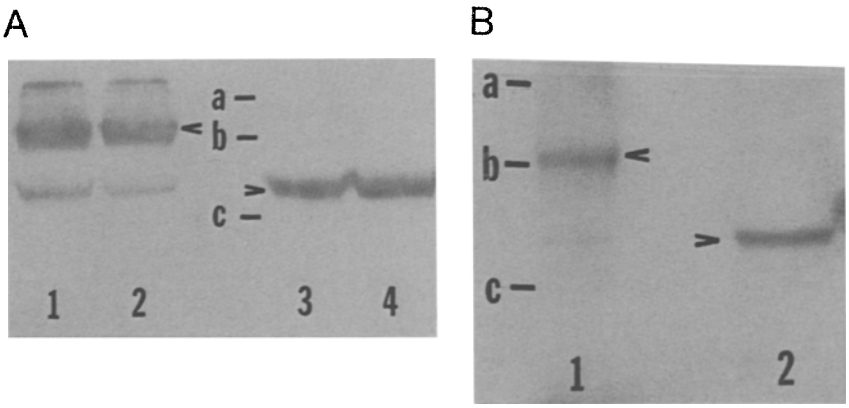


Fig. 3. Western analysis of NBAT from rat kidney and jejunal brush border membranes (BBMs). The BBMs were isolated using buffers containing N-ethylmaleimide for homogenization of the freshly obtained tissue. Aliquots of the BBM suspension were subjected to SDS/PAGE following treatment with SDS either in the absence or in the presence of 2-mercaptoethanol (MSH). The proteins were electroblotted onto a nitrocellulose membrane and detected using a mixture of all six anti-NBAT Abs. Arrowheads indicate position of the NBAT-containing 135kDa species (upper band) and the 85kDa NBAT monomer (lower band). Molecular mass standards *a*, *b*, and *c* are, respectively, 202, 133 and 71kDa. **A** Western analysis of rat kidney BBMs. Lanes *1* & *2*, SDS/PAGE under non-reducing conditions; lanes *3* & *4*, SDS/PAGE under reducing conditions. The bands at the top of lanes *1* & *2* in this gel represent aggregates (>200kDa) which remain at the upper edge of the gel and, thus, are presumably non-specific associations of NBAT subunits under the non-reducing conditions. **B** Western analysis of rat jejunal BBMs. SDS/PAGE under non-reducing (lane 1) and under reducing conditions (lane 2). Adapted from Wang and Tate, 1995

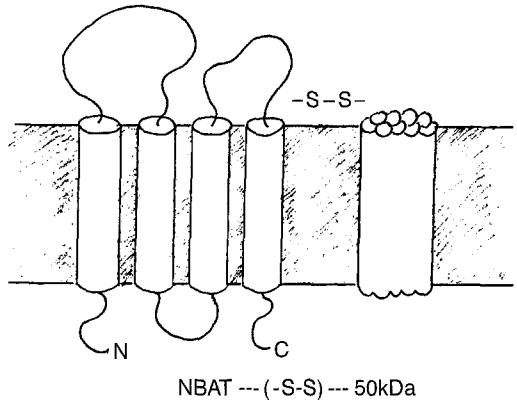


Fig. 4. Proposed heterodimeric model for the minimum functional unit of the NBAT-containing amino acid transporter. NBAT is shown on the left as a four MSD protein. The associated 50 kDa protein is depicted as an integral transmembrane protein although its structure, at present, remains uncharacterized. The two proteins in the complex are shown linked to each other by one or more disulfide bonds

N-ethylmaleimide (NEM) in the buffers used for homogenization of the kidneys. When prepared in this manner, the 135 kDa band consistently accounted for greater than 90% of the total NBAT-containing molecules in brush border membranes subjected to SDS/PAGE under non-reducing conditions (Fig. 3A, lanes 1 and 2). Only the 85 kDa NBAT monomer was seen when membranes prepared in the presence of NEM were subjected to SDS/PAGE under reducing conditions (Fig. 3A, lanes 3 and 4). Thus, these results suggest that the 135 kDa NBAT-containing complex indeed has functional relevance.

As noted above, in addition to kidney brush border membranes, jejunal brush border membranes also contain significant amounts of NBAT. We found that virtually all NBAT in the rat jejunal membranes, isolated in the presence of NEM, migrated as a 135 kDa species when subjected to SDS/PAGE under non-reducing conditions (Fig. 3B, lane 1). Under reducing conditions, only the 85 kDa NBAT monomer was seen (Fig. 3B, lane 2). Furthermore, evidence for such a heterodimeric complex containing NBAT was also obtained in rabbit kidney brush border membranes (rabbit NBAT monomer about 90 kDa and the disulfide-linked complex containing NBAT, about 145–150 kDa). The fact that NBAT in two different tissues of rat and in kidneys from two different species is associated with a smaller protein (approximately 50 kDa) provides compelling argument that this heterodimer plays an essential role in the physiological function of NBAT and, thus, probably represents the minimal functional unit of the NBAT-mediated amino acid transport. A schematic version of this heterodimer is shown in Fig. 4.

Relationship of the NBAT-containing heterodimer to transport

If the heterodimer described above is indeed the minimal functional unit of the NBAT-mediated transport, how does then one account for the fact that NBAT/cRNA alone suffices to induce system $b^{o,+}$-like transport in *Xenopus*

oocytes? To answer this question we considered the possibility that NBAT, synthesized in oocytes from injected cRNA, might recruit endogenous oocyte proteins and, thus, generate the functional transporter. We have detected small but significant amounts of such disulfide-liked oligomers (135–140kDa) in oocytes injected with NBAT/cRNA (see figure in Wang and Tate, 1995), in addition to NBAT monomers and higher aggregates (180–200kDa), the latter presumably representing disulfide-linked homodimers of NBAT subunits (probably formed as a consequence of the relatively high density of NBAT expressed in oocytes). Since NBAT/cRNA directs synthesis of only the NBAT subunits, it follows that the formation of the 135–140kDa species must involve association of NBAT with approx. 50kDa protein(s) of oocyte origin. The resultant dimers are similar in size to those seen in rat kidney and intestine and, thus, might perform similar function in oocytes (i.e., system $b^{0,+}$-like transport activity). If formation of such a heterodimer is, indeed, a prerequisite for expression of transport activity, the upper limit of transport must then depend upon the available amounts of the 50kDa protein(s) in oocytes. Such constraints probably explain data obtained earlier which showed that, whereas the amount of total NBAT synthesized in oocytes increased greater than 10-fold between 24 and 48h following the injection of NBAT/cRNA, the rate of amino acid uptake increased only about 1.5-fold (Mosckovitz et al., 1993). It should be noted that the approximately 180–200kDa species believed to be homodimers of NBAT subunits were not seen in either the rat kidney or the jejunal brush border membranes. Thus, it is unlikely that they represent functional units of NBAT-mediated transport.

We had previously found that, although NBAT is readily expressed in COS7 cells transfected with NBAT/cDNA, no significant increments in amino acid uptake ascribable to the expressed NBAT could be demonstrated (Mosckovitz et al., 1993). To resolve this anomaly, we subjected the membranes of COS7 cells, that had been transfected with NBAT/cDNA, to Western analysis under non-reducing conditions. Although intense bands corresponding to the NBAT monomers (about 87kDa) and disulfide-linked homodimers (about 200kDa) were readily detected, bands corresponding to species in the 135–140kDa region were not seen (Wang and Tate, 1995). Thus, it appears that the failure to detect increments in amino acid uptake in COS7 cells following expression of NBAT could conceivably be due to the absence of the 50kDa or related proteins in these cells. If further studies bear this out, then the COS7 cells could conceivably serve as a useful cell expression system to investigate the relative role of NBAT and the 50kDa protein(s) in amino acid transport.

Perspectives

The heterodimeric model proposed for the minimal functional unit of NBAT-mediated transport is, to our knowledge, unique for a metabolite transporter. Determination of the precise role of NBAT and the associated 50kDa protein in cystine and other amino acid transport awaits cloning and charac-

terization of the 50kDa protein and such efforts are currently underway taking advantage of the fact that the complex can be immunoprecipitated by one or more of the anti-NBAT Abs. Mutational analysis of the human gene encoding the 50kDa protein are necessary to determine its role, if any, in cystinuria, particularly in types II and III cystinurics in which no abnormalities in NBAT have thus far been detected. Another question which requires further study is whether NBAT (or, more precisely, NBAT-like proteins), detected immunologically in cell types other than the kidney and intestinal epithelial cells (for e.g., in a select population of enteroendocrine cells and enteric neurons [Pickel et al., 1993], in the chromaffin cells of adrenal medulla, and certain neurons in the brainstem and spinal cord nuclei [Nirenberg et al., 1995]), is also associated with 50kDa (or analogous proteins). If so, the function (or functions) such complexes perform in these locations needs to be elucidated. In view of the rapid advance over the past four years in our knowledge of the molecular basis of epithelial cystine transport and the molecular genetics of cystinuria, some of the unanswered questions raised here, and by other investigators in this field, will no doubt be answered in the foreseeable future.

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Author's address: S. S. Tate, Ph.D., Department of Biochemistry, Cornell University Medical College, 1300 York Avenue, New York, NY 10021, U.S.A.

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