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The proline permease of *Aspergillus nidulans*: Functional replacement of the native cysteine residues and properties of a cysteine-less transporter

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Abstract

The major proline transporter (PrnB) of *Aspergillus nidulans* belongs to the Amino acid Polyamine Organocation (APC) transporter superfamily. Members of this family have not been subjected to systematic structure–function relationship studies. In this report, we examine the functional replacement of the three native Cys residues (Cys54, Cys352 and Cys530) of PrnB and the properties of an engineered Cys-less PrnB protein, as background for employing a Cys-scanning mutagenesis approach. We show that simultaneous replacement of Cys54 with Ala, Cys352 with Ala and Cys530 with Ser results in a functional Cys-less PrnB transporter. We also introduce the use of a biotin-acceptor domain tag to quantitate protein levels of the engineered PrnB mutants by Western blot analysis. Finally, by using the background of the Cys-less PrnB transporter, we evaluate the functional importance of amino acids Q219, K245 and F248 of PrnB, which our previous data had suggested to be involved in the mechanism of PrnB-mediated proline uptake. In the current study, we show that K245 and F248 but not Q219 are critical for PrnB-mediated proline uptake.

Keywords: Cys-less transporter; Proline permease; *Aspergillus nidulans*

1. Introduction

Amino acids and their derivatives are transported into and out of cells by a variety of transporters belonging to several distinct protein families, some of which are distantly related (Saier, 2000). The largest and best-studied amino acid transporter superfamily is the APC (Amino acid Polyamine Organocation) family, which includes members that function as solute/cation symporters and solute/solute antiporters. APC members are found in bacteria, archaea, fungi, protists, plants and animals. They vary in length from 350 to 850 residues. Most of them possess 12 putative transmembrane α-helical segments (TMSs), with both their N- and C-terminal domains oriented towards the cytoplasmic side, whereas members of some subfamilies might have 10, 11 or 14 TMSs. Fungal and bacterial APC amino acid transporters show significant sequence similarities (33–62% identity scores in binary comparisons), which may reflect a common topology and mechanism of function. Their specificities range from one to several L-amino acids and their kinetics of transport and regulation of expression may also vary significantly (Sophianopoulou and Diallinas, 1995; Saier, 2000). Hip1p, a histidine-specific transporter of *Saccharomyces cerevisiae*, member of the APC superfamily, has been also implicated in heavy metal transport (Farcasanu et al., 1998). Interestingly, recent studies have shown that homologues of amino acid transporters in yeast function as 'sensors' of amino acids rather than real transporters (Bernard and André, 2001; Forsberg and Ljungdahl, 2001). Studies addressing how yeast and *Aspergillus nidulans* amino acid transporters find their way to the plasma membrane have shown that their proper sorting into the secretory pathway is facilitated by ER

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chaperones (Martinez and Ljungdahl, 2000; Erpapazoglou et al., 2006). In humans, more than 10 APC members have been identified and characterized (Verrey et al., 2004), while 14 APC-type genes are present within the complete *Arabidopsis* genome (Su et al., 2004).

Aspergillus nidulans is able to utilize L-proline as sole nitrogen and carbon source. The activities involved in L-proline uptake and catabolism map in five genes clustered in linkage group VII (Arst and MacDonald, 1975). The *prnB*[2] gene of this cluster (Sophianopoulou and Scazzocchio, 1989) encodes an active transport system highly specific for L-proline, the PrnB transporter (Tazebay et al., 1995, 1997). The expression of the *prnB* gene has been studied in great detail and shown to be regulated by no less than six independent or partially independent mechanisms: proline induction, nitrogen metabolite repression, carbon catabolite repression, the general control system regulating amino acid pools, an independent mechanism in response to germination and an unknown conidiospore-specific mechanism (Cubero and Scazzocchio, 1994; Tazebay et al., 1995, 1997; Gonzalez et al., 1997; Cubero et al., 2000; Pantazopoulou et al., 2007). The PrnB protein belongs to the YAT (Yeast Amino acid Transporter) subfamily of the APC superfamily (Sophianopoulou and Diallinas, 1995; Saier, 2000; Wipf et al., 2002). *In silico* searches have shown that PrnB is similar (35–45% identity in amino acid sequence) to all known yeast amino acid transporters and to several putative amino acid transporters from other fungi. This is reflected to a common topology of 12 TMSs for all these transporters, including PrnB, connected with short cytoplasmic loops and both N- and C-terminal domains towards the cytoplasm. Interestingly, no close homologue of PrnB exists in any other *Aspergillus* species of known genome (G. Diallinas, V. Sophianopoulou and C. Scazzocchio, unpublished results). Its functional homologue in *S. cerevisiae*, Put4p, is specific for L-proline, but, in contrast to PrnB, it also catalyses the efficient uptake of GABA, several proline analogues, alanine and glycine (Regenberg et al., 1999). The molecular rationale of the unique specificity of PrnB is missing. In this direction, we have previously performed a mutational analysis to address structure–function relationships in PrnB (Tavoularis et al., 2003). Most isolated missense mutations affecting PrnB function were mapped in the borders of cytoplasmic loops with transmembrane domains and within TMS6. None affected PrnB specificity but specific substitutions of amino acids K245 and F248 in TMS6 and Q219 in the extracellular loop between TMS5 and TMS6 were found to significantly alter the kinetic characteristics of PrnB, without affecting its plasma membrane localization (Tavoularis et al., 2003). Despite the fact that these replacements were microscopically shown (GFP fluorescence) to have wild-type subcellular localisation, an apparent drawback of this work was the lack of a systematic methodology, such as Western blotting, to quantify PrnB protein levels in different mutants.

Cys-scanning mutagenesis combined with site-directed sulfhydryl labelling and various biochemical and spectroscopic techniques is a powerful approach to study structural and dynamic aspects of membrane protein structure and function (Frillingos et al., 1997, 1998; Kimura et al., 1998; Slotboom et al., 1999; Sahin-Toth et al., 2000; Sahin-Toth and Kaback, 2000; Unkles et al., 2005). The power of this approach in the above aspects was first demonstrated for the *Escherichia coli* lactose permease (LacY) (Frillingos et al., 1998 and references therein). Site-directed mutagenesis of the eight native Cys residues of LacY showed that only one (Cys154) is important for transport and another one (Cys148) interacts directly with the substrate, but can be replaced without loss of transport activity (Frillingos et al., 1998; Smirnova and Kaback, 2003). Apart from investigating the role of the native Cys residues *per se*, this approach yielded new transporter variants devoid of Cys residues that retained activity. Such variants are used as background for cysteine-scanning mutagenesis *in vitro*. In the case of LacY, the study of native Cys residues has permitted the production of a mutant protein (C154G) that was more stable and amenable to purification for crystallography than the wild-type protein (Abramson et al., 2003; Smirnova and Kaback, 2003).

Engineering of a functional PrnB Cys-less transporter, which can be also detected immunologically, is considered as a unique molecular tool for structure–function studies of a eukaryotic member of the APC transporter family by Cys-scanning mutagenesis. PrnB contains three native Cys residues: Cys54, Cys352 and Cys530 (Fig. 1). A significant number of studies indicate that Cys residues in membrane proteins can be replaced, in general, with small hydrophobic residues (Ala in particular) or with the more hydrophilic Ser, without affecting the functionality of the protein. In the present study, we replaced the three native Cys residues of PrnB with Ala or Ser either individually or in combination and we obtain a functional PrnB Cys-less transporter, appropriate for subsequent Cys-scanning mutagenesis in *A. nidulans*. Our study also introduced a detection/purification tag, allowing immunoblot analysis of the PrnB molecules from plasma membrane preparations of mycelia and conidiospores. As an initial application, amino acid residues K245, F248 and Q219 that were previously suggested to be important for PrnB function (Tavoularis et al., 2003), were replaced with Cys on the Cys-less PrnB background and studied for their role in PrnB-dependent proline transport.

2. Materials and methods

2.1. Media, growth conditions and A. nidulans strains

Minimal (MM), Complete (CM) media and growth conditions for *A. nidulans* have been previously

[2] *Abbreviations used:* PrnB, major proline transporter; AAS transporter, C54A/C352A/C530S PrnB; NEM, *N*-ethylmaleimide; BAD, biotin-acceptor domain.

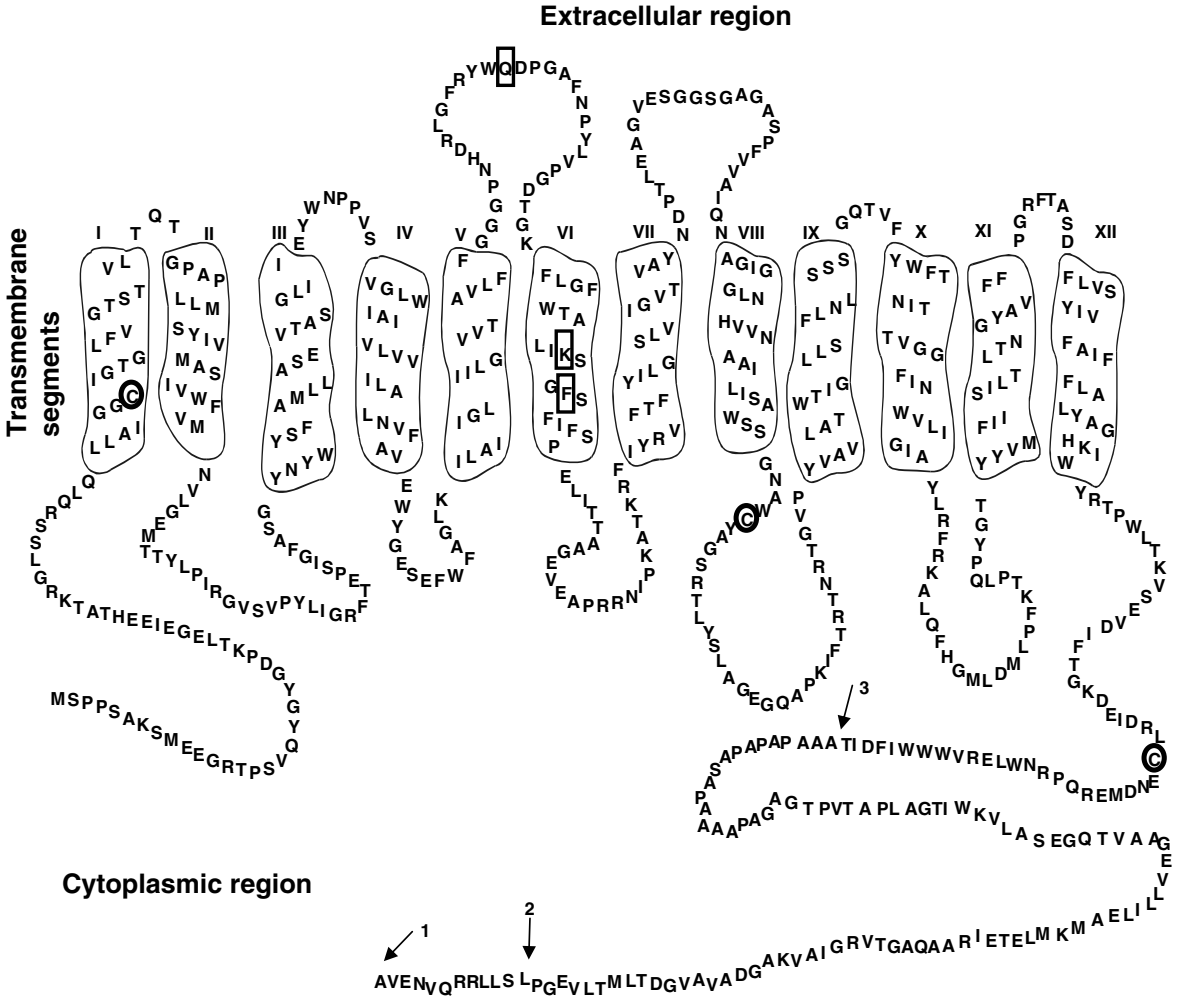

Fig. 1. Predicted secondary structure of the tagged PrnB transporter. The PrnB polypeptide chain is shown crossing the membrane 12× in a zigzag fashion with the N- and C-terminal domains retained in the cytoplasm. This model has been drawn on the basis of the hydropathy plot and the 'positive-inside' rule (Von Heijne, 1992), according to algorithm PRED-TMR (http://biophysics.bio.uoa.gr/PRED-TMR). The three native Cys residues are encircled. The C-tail tag is indicated by arrows 1 and 2 and the BAD domain by arrows 2 and 3 at the C-terminus of PrnB transporter. Amino acid residues K245, F248 and Q219 are also indicated by rectangles.

described (Cove, 1966). Supplements were added when necessary. Nitrogen sources, urea and proline were used at a final concentration of 5–10 mM. The carbon source glucose was used at final concentrations of 1% w/v. CsCl was used to a final concentration of 10 mM. The *A. nidulans* strains used have the following genotypes: *yA2 pantoB100* (*prnB*$^{+}$), *yA2 pantoB100 prnBC397* (*prn397*), *yA2 pabaA1 prnB377* (*prnB377*). *prn397* carries a deletion starting at the *Pst*I site of the *prnB* gene and extending up to the *Pst*I site of the *prnC* gene (Tavoularis et al., 2001). *prnB377* carries a deletion in the open-reading frame of the *prnB* gene that was described previously in Tazebay et al. (1995). All other strains, including *prnB* alleles isolated in this work, were constructed in strain *yA2 pantoB100 prn397*. *pantoB100* indicates auxotrophy for D-pantothenic acid. *yA2* results in yellow conidia. These markers do not affect the regulation of gene products involved in proline uptake and catabolism.

2.2. DNA manipulations and protoplast transformation

Plasmid pA-tag was constructed using plasmid pA2. This plasmid contains the *prnB* gene fused in-frame with the *gfp* open-reading frame, and part of the *prnC* gene, in a pBluescript KS (+) vector (Tavoularis et al., 2001). pA2 was restricted with *Eco*RV–*Hin*dIII and the *gfp* sequence within these restriction sites was replaced by a 338 bp PCR fragment containing the biotin-acceptor domain (BAD) sequence followed by a dodecapeptide epitope (C-tail). This PCR product was amplified from plasmid pT7-5/*melY*-BAD (Frillingos and Kaback, 2001) using primers F-BAD ($^{5'}$TTCACGGATATCACGGCTG CTGCTCCTG$^{3'}$) and R-BAD ($^{5'}$GCGCGCTTCGAATC AGCTGGTCCCAG$^{3'}$) having the restriction sites *Eco*RV and *Hin*dIII, respectively.

Plasmid isolation from *E. coli* strains and standard DNA manipulations were performed according to Sambrook et al. (1989). Polymerase Chain Reaction (PCR)

was carried out using AmpliTaq DNA polymerase (Perkin-Elmer) and the Expand High Fidelity PCR system (Roche Molecular Biochemicals, Mannheim, Germany). DNA sequencing of plasmid constructs was carried out using the ABI 310 Genetic Analyser at the Institute of Biology, NCSRD, Athens, Greece. The *prnB*-specific oligonucleotide primers used for sequencing were those described by Tavoularis et al. (2003). *Aspergillus nidulans* protoplast transformation was carried out as described by Tilburn et al. (1983). Total genomic DNA isolation from *A. nidulans* strains and Southern blot analysis were carried out as in Lockington et al. (1985). The DNA fragment used as a probe in Southern blot was a ~1.8 kb *Pst*I restriction fragment of the *prnB* gene isolated from plasmid pAN225 (Hull et al., 1989; Tavoularis et al., 2001).

2.3. Construction of targeted prnB mutations

In vitro prnB site-directed mutagenesis was performed by the method of Kunkel et al. (1987) using plasmid pA-tag. The codon changes performed and the oligonucleotides used are presented in Table 1. All the mutations were either individually or sequentially generated in plasmid pA-tag by *in vitro* site-directed mutagenesis (Ex-Site PCR Based Site-Directed Mutagenesis Kit; Stratagene, La Jolla). To construct plasmids C54A/C352S/C530S pA-tag and C54A/C352A/C530S pA-tag, the *Eco*RI–*Ppu*MI fragment of the C54A pA-tag plasmid was used to replace the corresponding segment of the C352S/C530S pA-tag and the C352A/C530S pA-tag plasmid, respectively. The whole length of the *prnB* gene was sequenced in every plasmid constructed and only the expected changes were found in each case.

The constructed plasmids were linearized using the restriction endonuclease *Xho*I, which does not cut within the *prnB, prnC* genes or the *BAD, C-tail* and the *prnB–prnC* intergenic regions of the plasmids. Linearized plasmids were introduced in the genome of *A. nidulans prn397* strain, which carries a large deletion extending from within the *prnB* open-reading frame to within the *prnC* open-reading frame, by protoplast transformation (Tilburn et al., 1983). *prnC*$^+$ transformants were isolated on proline as sole nitrogen source. Strain *prn397* lacks both PrnB and PrnC (L-Δ^1-pyrroline-5-carboxylate dehydrogenase) activities and does not grow at all on media containing proline as sole nitrogen source, since the absence of PrnC results in strong proline toxicity (Arst et al., 1981; Tavoularis et al., 2001), unlike single *prnB* null mutant strains, which show leaky growth due to a minor proline transporter (Tazebay et al., 1995). Thus, this strain allows the direct selection of *prnC*$^+$ transformants upon reintroduction of sequences containing any *prnB* allele and the missing *prnC* sequences, notwithstanding whether or not the introduced *prnB* sequences are functional. The functionality of the PrnB protein can then be assessed directly by growth on proline as a sole nitrogen source. This system leads to the targeted, single-copy integration of any *prnB-tag* chimeric gene at the resident *prnB* locus, thus avoiding complications arising from ectopic and/or multiple integrations of *prnB* copies (see Fig. 2a). Southern blot analysis of genomic DNA isolated from the transformants showed that all mutants constructed contained a single copy of *prnB* alleles integrated by homologous recombination into the *prnB* genomic locus of the recipient strain (Fig. 2a and data not shown).

2.4. Membrane protein extract preparation, avidin–Sepharose pull-down and Western blotting

Conidiospore suspensions of *A. nidulans* strains were grown in MM containing either 5 mM urea or 10 mM proline as sole nitrogen sources and the appropriate auxotrophies at 37 °C. For detection via the BAD epitope, 10 mg D-biotin/100 ml of minimal media was added. Mycelia (8 h of growth) or conidiospores (4.5 h of growth) were collected, washed with minimal media and ground in liquid nitrogen. The powder was weighed and suspended in 2 ml

Table 1
Amino acid, nucleotide changes, oligonucleotides used for construction of PrnB replacement molecules and strains isolated

Amino acid change	Codon change	Oligos used
C54A	TGT → GCT	GCTCGCCATCGGAGGAGCTATTGGCACTGGTCT
C352A	TGT → GCT	CGGTAACGCATGGGCTTACGCTGGATCGAG
C352S	TGT → TCT	CGGTAACGCATGGTCTTACGCTGGATCGAG
C530S	TGC → TCC	GAGATTGACCGTTTGTCCGAGAATGATATGGAGC

Strain	C54	C352	C530
Cysteine replacements			
prnB-C54A	A	C	C
prnB-C352S	C	S	C
prnB-C352A	C	A	C
prnB-C530S	C	C	S
prnB-C54A/C530S	A	C	S
prnB-C352S/C530S	C	S	S
prnB-C352A/C530S	C	A	S
prnB-C54A/C352A	A	A	C
prnB-C54A/C352S/C530S	A	S	S
prnB-C54A/C352A/C530S	A	A	S

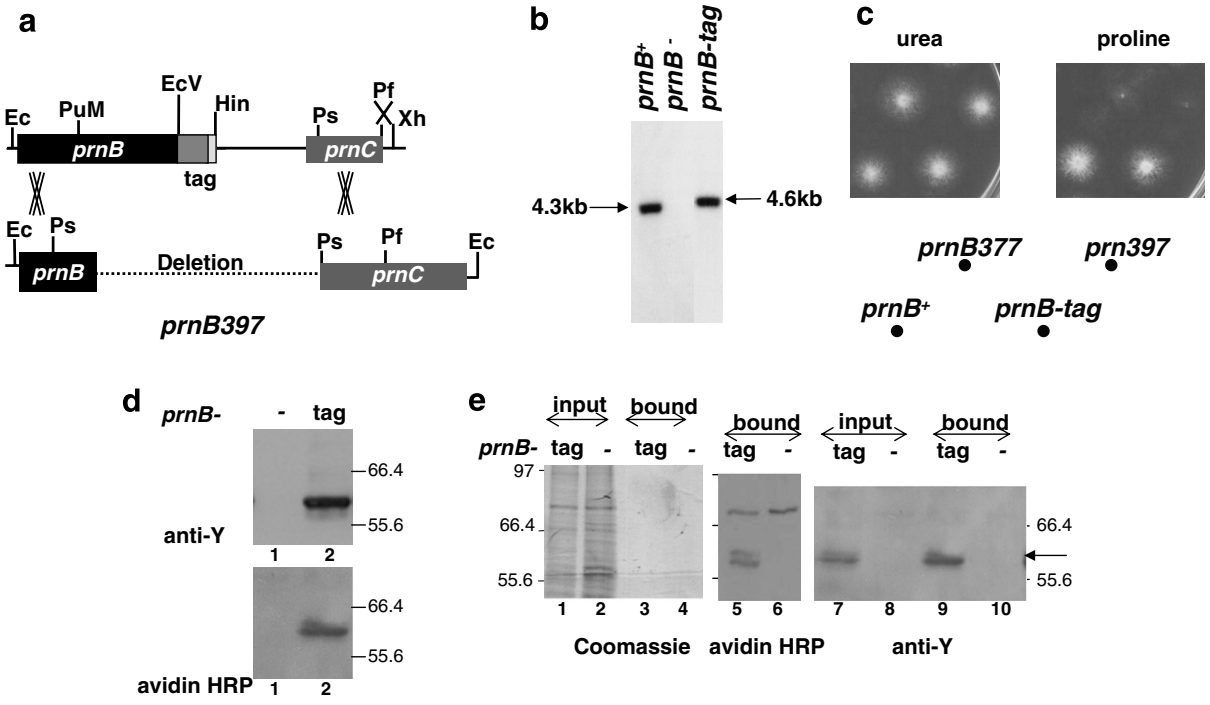

Fig. 2. Characterization of *A. nidulans* strains expressing tagged PrnB transporter. (a) Schematic presentation of the plasmid pA-tag and the structure of the *prnB–prnC* region in the recipient strain of *A. nidulans* used for transformation (*prnB397*). The positions where homologous recombination takes place are also indicated. Restriction enzyme abbreviations: Ec, *Eco*RI; Ps, *Pst*I; PuM, *Ppu*MI; EcV, *Eco*RV; Hin, *Hin*dIII; Pf, *Pfl*MI; and Xh, *Xho*I. (b) Southern blot analysis of total genomic DNA extracted from the recipient strain *prn397* (*prnB*$^-$) and isogenic strains carrying wild-type (*prnB*$^+$) and *prnB-tag* genes. Approximately 5 μg of genomic DNA from each strain were digested with *Eco*RI, transferred to nitrocellulose and hybridised with a *prnB*-specific probe. The observed size difference of ~0.3 kb between the wt and the *prnB-tag* signals verifies the presence of the engineered BAD-C-tail tag. (c) Growth of isogenic control strains *prnB*$^+$ (wt), *prnB377* (*prnB* deleted strain), *prn397* (the recipient *prnB prnC* deletion strain) and the *prnB-tag* transformed strain of *A. nidulans*, on minimal media supplemented with 5 mM urea or 10 mM proline as sole nitrogen sources at 37 °C. The strains used and their relative positions are shown. (d) Western blot analysis of PrnB-tag protein. Membrane protein extracts were prepared from mycelia of *prnB-tag* and *prnB*$^+$ strains. The PrnB-tag chimeric molecules were detected by immunoblot using (1) anti-Y antibody and (2) avidin–HRP. Molecular weight markers are indicated in kDa. (e) Binding of PrnB-tag protein on avidin–Sepharose beads. Membrane protein extracts were prepared from conidiospore suspensions of *prnB-tag* and *prnB*$^+$ strains and incubated with avidin–Sepharose beads as described in Section 2. Input membrane protein extracts as well as proteins bound on beads were analyzed by SDS–PAGE and stained with Coomassie (lanes 1–4) or immunoblotted with avidin–HRP (lanes 5 and 6) or anti-Y antibody (lanes 7–10). Lanes 1, 2, 7, 8, one-fifth of the amount of the membrane protein extract incubated with avidin beads (~100 μg of total protein); lanes 3–6, 9, 10, proteins pulled down by avidin–Sepharose beads.

cold extraction buffer (10 mM Tris, pH 7.5, 5 mM $MgCl_2$, 100 mM NaCl, 0.3 M Sorbitol, 1 mM PMSF, 1 mM DTT, Protease inhibitors cocktail (Sigma, 1:500)) per gram. These suspensions were centrifuged at 2000*g* for 10 min at 4 °C followed by centrifugation of the supernatant at 42,000 rpm (Beckman OPTIMA MAX ultracentrifuge, MLA130 rotor) for 60 min at 4 °C. The gelatinous pellet was resuspended in 100 μl equilibration buffer for pull down assays (20 mM phosphate buffer, pH 7.5, 100 mM NaCl, 0.05% NP-40, 1 mM PMSF, 1 mM DTT, Protease inhibitors cocktail (Sigma) 1:500) or in 100 μl of buffer containing 20 mM sodium phosphate buffer, pH 7.5, 250 mM Sucrose, 100 mM NaCl, 0.15% NP-40, 1:1000 protease inhibitors cocktail, 1 mM PMSF, 1 mM DTT for Western blot analyses. Protein concentration was determined using a modified Bradford assay (Bradford, 1976).

For pull down assays, membrane protein extracts, after centrifugation at 13,000 rpm (~16,000 RCF) for 5–10 min, were incubated for 60 min at 4 °C with immobilized monomeric avidin–Sepharose (Pierce), pre-equilibrated with the equilibration buffer. The resin was washed twice with 20 volumes of equilibration buffer and biotinylated proteins were eluted in SDS sample buffer (10% glycerol, 100 mM DTT, 2% SDS, 0.1% bromophenol blue), incubated at 37 °C for 20 min and analysed by electrophoresis on 7.5% polyacrylamide gel. They were transferred to nitrocellulose membrane and probed either with a site-directed polyclonal antibody against the C-tail epitope (anti-Y antibody, Babco, CA; generously provided by the laboratory of H. R. Kaback, UCLA) or with avidin–HRP (Amersham) that recognizes the *in vivo* biotinylated BAD-epitope.

2.5. Proline transport assays

[2,3,4,5-^{3}H]L-proline uptake was assayed in germinating conidia at 37 °C as previously described (Tazebay et al., 1995; Meintanis et al., 2000). Standard uptake assays for the determination of initial uptake rates were performed in *A. nidulans* MM (pH 6.5) by using 10 μM [2,3,4,5-^{3}H]L-proline (specific activity 120 Ci mmol^{-1};

Moravek Biochemicals, Brea CA, USA). Initial uptake rates were expressed in pmol of substrate incorporated per 1 min per 10^8 viable conidiospores. Radioactivity was determined in sediment and supernatant by liquid scintillation counting (Beckman Instruments). Transport measurements were repeated independently, and the reported results represent the mean values of at least three to five different experiments. The apparent Michaelis constant (K_m) and maximal velocity (V_{max}) values for [^{3}H]L-proline were determined from double reciprocal plots of the initial uptake rates against substrate concentration. Initial uptake rates were corrected by subtracting background uptake values, evident in the *prnB377* total loss-of-function mutant strain (Tazebay et al., 1995). The errors given are standard deviation of the mean value.

2.6. NEM effect and [^{14}C]-labelling of PrnB-BAD-C-tail chimeric molecules

Minimal media (50 ml) containing 10 mM proline as sole nitrogen source were inoculated with 5×10^6 conidiospores/ml. Inoculated cultures were incubated at 130 rev min^{-1} for 4.5 h at 37 °C. Conidia were filtered through Millipore filters (0.45 μM), washed and resuspended in an appropriate volume of minimal medium, pH 7.5, in order to obtain 200 μl aliquots of approximately 1×10^8 conidia. Each aliquot was equilibrated for 10 min at 25 °C. The appropriate volume of 100 mM *N*-ethylmaleimide (NEM) was then added in order to reach the final concentration needed and aliquots were incubated for different periods of time at 25 °C. For [^{14}C]-labelling of transporter molecules, conidia were incubated with 0.3 mM [^{14}C]NEM for 15 min at 25 °C. Reactions were quenched by addition of 5 mM DTT. Conidia were washed with 30 ml of minimal medium and membrane protein extracts were prepared from the conidiospore suspensions. Tagged PrnB molecules were purified via their BAD domain and analysed by Western blotting with anti-Y antibody, as described above. Nitrocellulose membranes were stripped of the antibody and exposed onto Super RX Fuji films, after enhancement of the signal with Enhanced Autoradiography kit (Thistle Scientific).

3. Results

3.1. Construction and analysis of a functional tagged PrnB transporter

Plasmid pA2, containing the whole sequence of the *prnB* gene was used to generate plasmid pA-tag, producing a tagged version of the PrnB transporter. The expressed tagged PrnB contains the biotin-acceptor domain (BAD) of the oxaloacetate decarboxylase of *Klebsiella pneumoniae* (Consler et al., 1993; Frillingos and Kaback, 1996), fused in its C-terminus with an epitope corresponding to the C-terminal dodecapeptide of *E. coli* LacY (Carrasco et al., 1984), designated as C-tail (Fig. 1). This plasmid was used to transform protoplasts of a *prnB*Δ *prnC*Δ recipient strain (*prn397*, see Section 2) that is unable to grow on proline as sole nitrogen source (Tavoularis et al., 2001). Several *prnC*$^+$ transformants obtained were purified on selected media and analysed for their ability to grow on media containing 5 mM proline as sole nitrogen source at 37 °C. All transformants grew identically to each other and similarly to the wild-type strain in these media. Southern blot analyses and detection of integration of the pA-tag sequences into the genome of the transformed strains confirmed that isolated strains, designated *prnB-tag*, were the result of single homologous recombination events on the genomic *prnB–prnC* region (Fig. 2a and b and data not shown). One transformant was thus selected for further analysis.

Growth test and kinetic analysis showed that *prnB-tag* strain expresses a functional tagged PrnB transporter. As presented in Fig. 2c, strain *prnB-tag* grows similarly to an isogenic wild-type *prnB* strain (*prnB*$^+$) on proline as sole nitrogen source and on all other nitrogen sources tested (data not shown). Growth on proline was not affected by temperature when it was kept within the physiological range for *A. nidulans* (25–37 °C, data not shown). In addition, the kinetic characteristics of proline transport were examined for strains expressing both the wild-type and the tagged-PrnB transporter. Both transporters showed similar proline uptake kinetics and calculated K_m and V_{max} values (Table 2).

As shown in Fig. 2d, the BAD-C-tail tag allowed specific detection of PrnB from membrane protein preparations of *prnB-tag* strain by immunoblotting using either an antibody against the C-tail epitope (anti-Y antibody) or avidin–HRP that recognizes the *in vivo* biotinylated BAD-epitope. In addition, the tagged PrnB transporter could be efficiently pulled down using membrane protein extracts prepared from the *prnB-tag* strain by avidin–Sepharose beads, through the biotinylated BAD domain (Fig. 2e). Although the amount of proteins bound on beads was not detectable by Coomassie or silver stain (lanes 1–4 in Fig. 2e and data not shown), the pulled down PrnB transporter could be detected by Western blotting (Fig. 2e, com-

Table 2
K_m and V_{max} values for [^{3}H]L-proline uptake in the wild-type and in strains carrying PrnB mutations

Strain	K_m (μM)	V_{max} (nmoles × min^{-1} × 10^8 viable conidiospores^{-1})
prnB$^+$	41 ± 2.0	1.5 ± 0.4
prnB-tag	48 ± 5.0	1.4 ± 0.3
prnB-C54A/C352A/C530S (AAS)	37 ± 5.0	0.8 ± 0.4
prnB-C54A	50 ± 7.0	0.8 ± 0.2
prnB-C352A	52 ± 6.0	0.9 ± 0.3
prnB-C530S	63 ± 5.0	0.5 ± 0.2
prnB-C54A/C352A	40 ± 2.0	0.9 ± 0.3
prnB-C54A/C530S	35 ± 3.0	0.8 ± 0.3
prnB-C352A/C530S	40 ± 2.0	1.0 ± 0.2

pare lanes 9 and 10 to 7 and 8). Furthermore, lanes 5 and 6 of Fig. 2e, show that PrnB was the only biotinylated protein at the area of 55–66 kDa immobilised on avidin–Sepharose beads.

3.2. Construction of a functional PrnB transporter devoid of endogenous cysteine residues

Initially, individual Cys-replacements were engineered to examine their effect on PrnB function. In this respect, Cys54 of PrnB was replaced with Ala, Cys352 with Ser or Ala and Cys530 with Ser. The rationale for Ala or Ser substitutions is discussed later. In addition, four paired-Cys-replacement PrnB (C352S/C530S, C352A/C530S, C54A/C530S, C54A/C352A) and two triple-Cys-replacement (Cys-less mutants) PrnB versions (C54A/C352S/C530S and C54A/C352A/C530S) were constructed. The constructed plasmids expressing biotin-acceptor domain C-tail-tagged mutated PrnB transporters were introduced by targeted homologous integration into the *prnB* genomic locus of *A. nidulans* using *prn397* as a recipient strain (see Section 2 and Fig. 2a). Transformants were characterized by Southern blot analysis of genomic DNA with a *prnB*-specific radiolabelled probe. Integration of plasmid sequences into the genome of the transformants was detected in all cases, confirming that the isolated strains (see Table 1) were, as expected, the result of single homologous recombination events on the genomic *prnB–prnC* region (data not shown).

All mutant strains were analysed for their ability to grow on proline or other nitrogen sources. Fig. 3b shows that mutant strains expressing PrnB with the single substitutions C54A and C352A, as well as strains expressing mutant PrnB transporters with the C54A/C352A, C54A/C530S or C352A/C530S double replacements, grow slightly less than the *prnB-tag* wild-type strain on proline as sole nitrogen source. On the other hand, mutant strains expressing PrnB transporters bearing a C352S replacement, either alone or in combination with C530S, grow as badly as a PrnB total loss-of-function strain, *prnB*377 (Fig. 3b and c). The strain expressing the Cys-less C54A/C352S/C530S PrnB transporter (ASS PrnB) also shows a PrnB loss-of-function phenotype, probably due to the C352S substitution. Mutant strains expressing PrnB with the single substitution C530S as well as those expressing the Cys-less C54A/C352A/C530S PrnB transporter (AAS PrnB), grow less well than the *prnB-tag* strain, but much better than the *prnB*377 strain, at both 37 and 25 °C (Fig. 3b and c), indicating that the AAS Cys-less PrnB version of the transporter is functional. All mutant strains showed normal growth on all other nitrogen sources tested (Fig. 3a and data not shown).

Mutant strains were also analysed for their ability to grow on proline as sole nitrogen source in the presence of mildly toxic concentration of Cs^+ (as the chloride, 10 mM) that accentuates reduced nitrogen source utilization (Arst et al., 1981). The strain expressing the Cys-less C54A/C352A/C530S PrnB transporter (AAS PrnB) grows much less than the *prnB-tag* strain but significantly better than the *prnB*377 strain, confirming that the AAS Cys-less PrnB version of the transporter is functional (data not shown).

Despite this apparent drawback of reduced function of the engineered AAS Cys-less transporter, common in many cases of Cys-less versions of transporters (Frillingos et al., 1998 and references therein), these growth tests and a subsequent kinetic analysis (see below and Table 2) establish that the Cys-less AAS PrnB transporter is active to a degree proper for functional analysis by Cys-scanning mutagenesis.

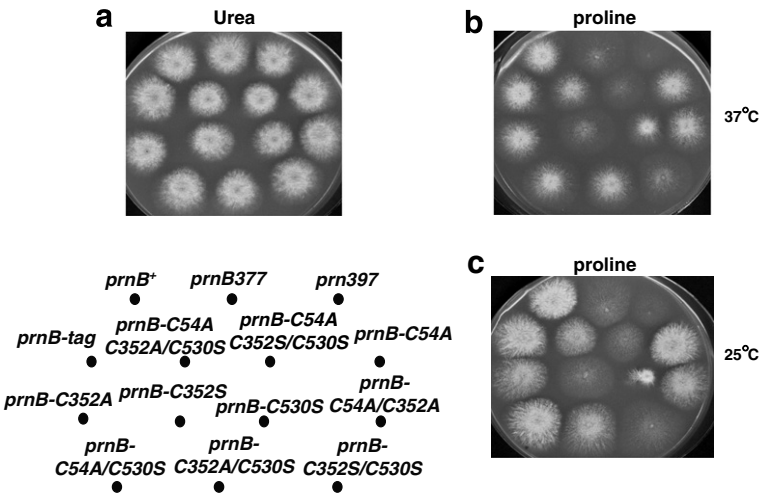

Fig. 3. Growth of *A. nidulans* mutant strains on nitrogen sources. Growth of control strains $prnB^+$ (wt), *prnB377*, *prn397* and the *prnB-tag* transformed strain of *A. nidulans* (see legend of Fig. 2) on minimal media supplemented with 5 mM urea (a) and 5 mM proline (b and c), as sole nitrogen sources at 37 °C (a and b) and 25 °C (c), respectively. The strains used and their relative positions are shown.

3.3. Immunoblot analysis of the PrnB transporters

Immunological analysis of membrane protein fractions extracted from strains expressing tagged wild-type or mutated PrnB transporter is shown in Fig. 4. Membrane protein preparations, containing approximately 20 μg of total protein per sample, were analyzed by Western blot analysis, using anti-Y antibody. The results show that the expression of the Cys-less and all tagged mutated PrnB transporters described in Table 1, including the C352S replacement mutant, is similar to that of the wild-type tagged PrnB transporter (Fig. 4 and data not shown).

3.4. Kinetic transport analysis

Kinetic analysis of proline uptake was carried out in strains expressing tagged wild-type and mutant PrnB transporters. All uptake experiments were performed at 37 °C. In accordance with growth tests, mutant strains not growing on proline as sole nitrogen source (C352S, C352S/C530S, C54A/C352S/C530S, see Fig. 3) show the same level of residual proline uptake with that found in strains carrying a deletion of the *prnB* gene (results not shown). This residual uptake is attributable to other amino acid transporter(s) that are able to incorporate proline and never exceeds 20% of the total uptake measured in wild-type strains (Arst et al., 1980; Tazebay et al., 1995). The strain expressing the PrnB-tag transporter shows the same level of proline transport as that expressing the wild-type protein (Table 2). Mutant tagged PrnB transporters with substitutions C54A, C352A, C530S, either alone or in combinations of two, retain their ability to transport proline, even though in a lesser degree compared to the *prnB-tag* wild-type strain (Table 2). The Cys-less AAS PrnB transporter retains ~55% transport activity, exhibits wild-type affinity for its substrate, and can be therefore used for the subsequent analysis of Cys-scanning mutagenesis.

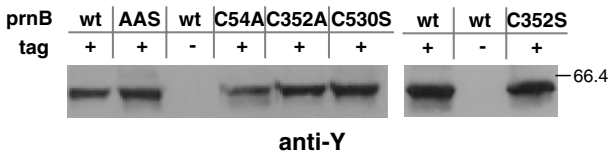

Fig. 4. Western blot analysis of the PrnB-tag protein. Approximately 20 μg of total protein from membrane fractions of mycelia derived from strains expressing untagged PrnB (wt) or PrnB-tag and mutant proteins were analyzed by SDS/7.5% PAGE followed by transfer onto nitrocellulose membrane and probing with anti-Y antibody.

3.5. Effect of NEM on the kinetic properties of PrnB, single Cys-PrnB and Cys-less PrnB

The transport activity of both the wild-type and the Cys-less PrnB-tag transporters was assayed, after incubation of cells expressing them with the SH-specific alkylating agent *N*-ethylmaleimide (NEM). Conidiospore suspensions from strains expressing the wild-type tagged PrnB protein or the AAS Cys-less tagged transporter were incubated with 0.5–4 mM NEM, at 25 °C, for time periods ranging from 5 to 30 min (Fig. 5a and data not shown). Incubation of conidia expressing wild-type tagged PrnB transporter with 0.5–2 mM NEM for up to 15 min, does not significantly affect

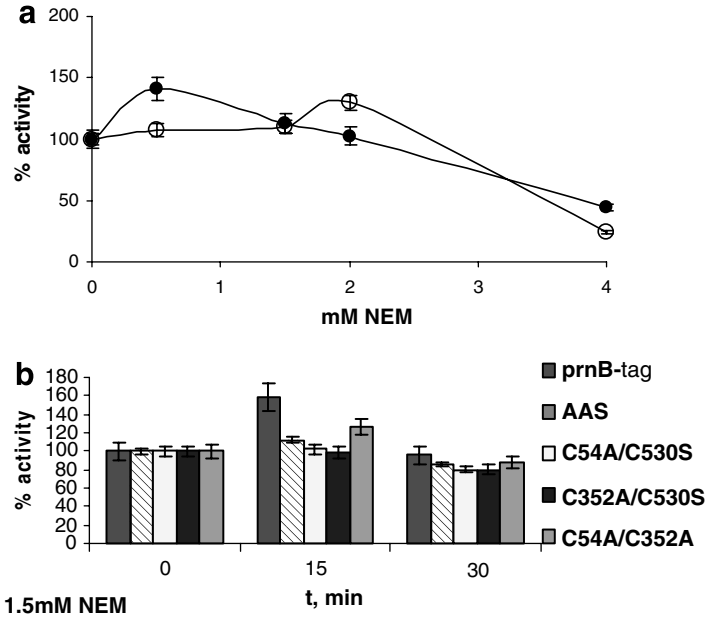

Fig. 5. Transport activity of PrnB mutants and effect of NEM on proline transport. (a) Conidia expressing wild-type tagged-transporter or the AAS Cys-less transporter were incubated with different concentrations of NEM for 15 min at 25 °C. Reactions were stopped with DTT and conidia were washed with minimal media and assayed for their ability to transport proline as described in Section 2 (○, AAS PrnB; ●, wild-type PrnB-tag transporter). (b) Conidia expressing wild-type, mutant or the AAS Cys-less PrnB transporter were incubated with 1.5 mM of NEM for 15 or 30 min at 25 °C. Reactions were stopped with DTT and conidia were washed with minimal media and assayed for their ability to transport proline as described in Section 2.

transport activity. Incubation for longer periods (>30 min) or with higher NEM concentrations (4 mM) resulted in a non-specific decrease in transport activity, evident in both the Cys-less and the wild-type tagged PrnB (Fig. 5a and data not shown). The transport activity of each single Cys-PrnB transporter (C352A/C530S, C54A/C530S and C54A/C352A PrnB) was also assayed after incubation of the cells expressing them with 1.5 mM NEM for 15 or 30 min. None of the individual native Cys residues of the PrnB transporter was found to be essential for PrnB-mediated proline uptake (Fig. 5b), since alkylation of each one separately does not affect the uptake activity of the transporter. These results suggest that conditions allowing specific sulfhydryl alkylation of the native Cys residues of wild-type PrnB have no effect on the PrnB-mediated proline transport.

3.6. [^{14}C]NEM-labelling of PrnB-tag transporter

Given that PrnB-dependent transport of proline was not affected at the presence of NEM (Fig. 5a and b) we examined whether native Cys residues of the PrnB-tag transporter were actually modified by the SH-alkylating agent. For that reason, we investigated whether [^{14}C]NEM directly reacts with this PrnB molecules. Conidiospore suspensions from strains expressing the wild-type tagged PrnB protein were incubated in minimal media supplemented with 0.3 mM [^{14}C]NEM. The tagged transporter molecules were purified by avidin–Sepharose from membrane protein extracts prepared from these strains and subjected to autoradiography and Western blotting. Results presented in Fig. 6 show that PrnB-tag transporter molecules identified by anti-Y antibody are labelled by [^{14}C]NEM, while the AAS PrnB transporter could not be detected via [^{14}C]NEM-labelling under the same experimental conditions. These data indicate that the lack of PrnB sensitivity to NEM is due to the inability of the SH-specific adduct, bonded at native Cys residues of PrnB, to influence the proline translocation mechanism rather than due to the inability of NEM to react with these residues.

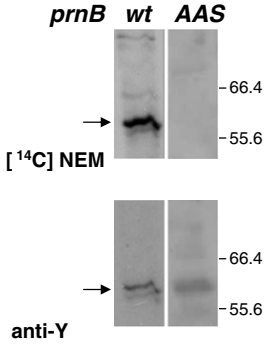

Fig. 6. [^{14}C]NEM-labelling of PrnB transporter. Conidia expressing wild-type or AAS Cys-less PrnB-tag transporter were incubated with [^{14}C]NEM and used to prepare membrane protein extracts. *In vivo* biotinylated proteins were pulled down by avidin–Sepharose beads and analyzed by SDS–PAGE and immunoblotting. PrnB molecules labelled through NEM interactions were detected by autoradiography. The exact position of PrnB protein on the blot was verified by Western blotting using anti-Y antibody.

3.7. Study of structure–function relations of the PrnB transporter by Cys-scanning mutagenesis

In order to address structure–function relations of the PrnB transporter, we replaced each one of the amino acids K245, F248 and Q219 individually with Cys. As discussed in the introduction, specific replacements of these residues affect PrnB kinetics and indicate that they might be important molecular determinants for proline binding and transport. Single Cys replacements were carried out on the plasmid expressing the Cys-less AAS PrnB transporter. The constructed plasmids were introduced by targeted

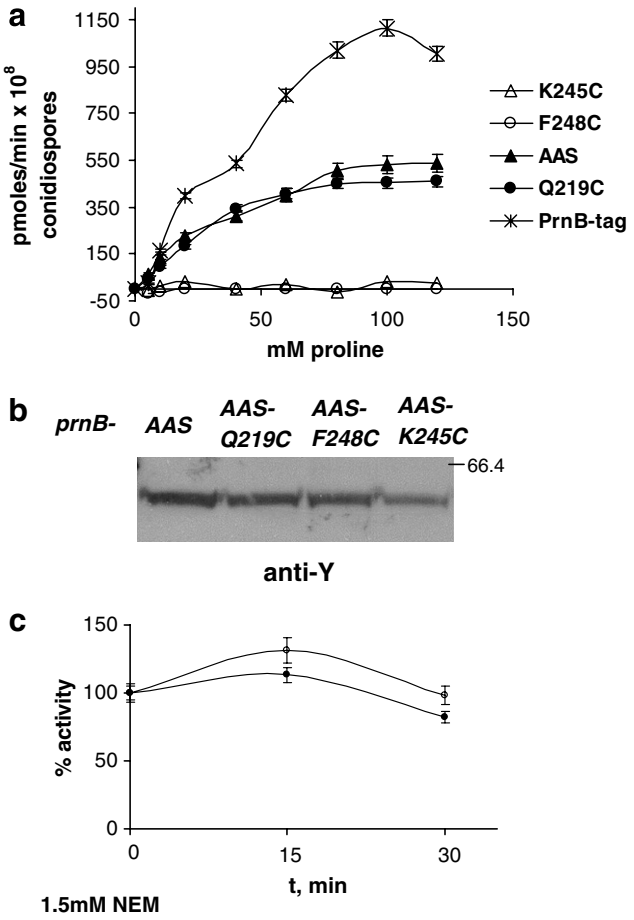

Fig. 7. Analysis of AAS PrnB-Q219C, -K245C and -F248C mutant Cys-less transporters. (a) Conidia from strains expressing either the wild-type tagged PrnB or the AAS Cys-less tagged-transporter or mutant tagged Cys-less transporter molecules were assayed for their ability to transport proline as described in Section 2. (b) Approximately 20 μg of total proteins from membrane fractions of mycelia derived from strains expressing tagged Cys-less PrnB transporter molecules, either wt or mutant, were analyzed by SDS/7.5% PAGE followed by transfer onto nitrocellulose membrane and probing with anti-Y antibody. (c) Conidia expressing AAS PrnB or AAS PrnB-Q219C transporter were incubated with 1.5 mM of NEM for 15 min at 25 °C and assayed for their ability to transport proline as described in Section 2 ○, AAS PrnB-Q219C; ●, AAS PrnB.

homologous integration into the *prnB* genomic locus of *A. nidulans* using *prn397* as a recipient strain (see Section 2). Transformants were characterized by Southern blot analysis of genomic DNA with a *prnB* specific radiolabelled probe. Integration of plasmid sequences into the genome of each transformant was detected in all cases, confirming that the isolated strains resulted from single homologous recombination events on the genomic *prnB–prnC* region (data not shown and see above).

All mutant strains were analysed for their ability to grow on proline or other non-repressing nitrogen sources. Mutant strains expressing C54A/C352A/C530S/K245C (AAS PrnB-K245C) or C54A/C352A/C530S/F248C (AAS PrnB-F248C) were found to grow on proline as sole nitrogen source (data not shown) indistinguishably from a total-loss of PrnB function strain, *prnB*377, with which they showed the same residual proline uptake activity (Fig. 7a). In both strains the level of PrnB protein expressed was similar to that of the strain expressing the wild-type AAS Cys-less transporter (Fig. 7b). In this context, strains expressing these mutant PrnB molecules could not be further assayed for their ability to transport proline after treatment with NEM. On the other hand, the mutant strain expressing C54A/C352A/C530S/Q219C PrnB tagged transporter (AAS PrnB-Q219C) grows similarly to the strain expressing the Cys-less AAS PrnB-tag transporter and both strains showed similar PrnB protein levels and kinetic characteristics (data not shown and Fig. 7a and b). Incubation of cells expressing AAS PrnB-Q219C molecules with 1.5 mM NEM for 15 or 30 min at 25 °C marginally affected the kinetic characteristics of the transporter, indicating that alkylation of Q219 does not affect the mechanism of PrnB-mediated proline transport (Fig. 7c).

4. Discussion

The ascomycete *A. nidulans* has been extensively used as a model system to study molecular genetics and regulation of eukaryotic membrane transporters, in particular of those transporting amino acids, purines and other nitrogen sources. Part of these studies refer to structure–function analyses of such transporters, albeit in a non-systematic manner (see Diallinas et al., 1998; Amillis et al., 2001; Tavoularis et al., 2003; Unkles et al., 2004, 2005; Goudela et al., 2005; Kinghorn et al., 2005). To gain systematic insight into the structure and the mechanisms of function of such transporters by applying Cys-scanning and other specific site-directed techniques in the expression system of *A. nidulans*, a number of problems have to be addressed first. These refer to (*a*) development of methods for detection, quantitation and purification of transporters from the *Aspergillus* membrane, (*b*) elaboration of protocols for sensitive and specific site-directed modification assays *in situ*, and (*c*) establishment of the appropriate functional transporter background. In this direction, we have (*a*) used a dual-utility C-terminal tag (BAD domain followed by the C-tail epitope) that allows immunodetection and avidin-affinity purification of PrnB as a functional tagged transporter, (*b*) presented a sulfhydryl-specific alkylation assay of PrnB in *Aspergillus* conidiospores, (*c*) constructed and used a Cys-less version of PrnB that is functional and appropriate for the application of Cys-scanning mutagenesis strategies. Furthermore, all PrnB mutations studied in this work were expressed from the native *prnB* promoter and in strains in which prnB alleles were integrated at the endogenous genomic *prnB* locus by homologous recombination.

Wild-type PrnB contains three native Cys residues (Fig. 1), predicted to map in the TMS1 (Cys54), the borderline region between TMS8 and cytoplasmic loop 8/9 (Cys352) and the cytoplasmic C-terminal tail (Cys530). The present study shows that none of the three Cys residues is necessary for the PrnB transport mechanism, since all are functionally replaceable with either Ala (Cys54, Cys352) or Ser (Cys530); moreover, they can all be replaced in a combinatorial manner to yield a normally expressed and functional Cys-less PrnB transporter. To optimally design the corresponding site-directed replacements on the basis of the predicted topology (Fig. 1), we introduced a more hydrophobic side chain (Ala) in place of a predicted transmembrane-region Cys (C54A), a more hydrophilic side chain (Ser) in place of a putative hydrophilic-region Cys (C530S) and/or both versions (Ala, Ser) in place of Cys352, that is mapped at the borderline between a TMS and a loop (C352A, C352S). With respect to position 352, we found that an Ala residue is well tolerated (both PrnB-C352A and PrnB-C54A/C352A/C530S are functional), while a Ser replacement leads to inactivation (C352S, C352S/C530S and C54A/C352S/C530S PrnBs are expressed but inactive). This finding implies that Cys352 in PrnB might be involved in a hydrophobic interaction that is essential for proline transport, so that the presence of a more polar residue (Ser) at this position is disruptive while the presence of a small hydrophobic residue (Ala) is well-tolerated. However, a more extensive mutagenesis at this position is necessary to address this issue in more detail.

Apart from introducing a functional PrnB Cys-less background, we have also introduced the application of a dual detection/purification tag for immunodetection on Western blots, purification of PrnB in small scale and application of the [^{14}C]NEM-labelling assay in conidiospores. This tag, consisting of a biotin-acceptor domain followed in-frame by a linear dodecapeptide epitope from LacY (Consler et al., 1993; Frillingos and Kaback, 1996, 2001; Karatza and Frillingos, 2005), might prove to be a valuable tool to assay expression, purification, and/or address structure–function relationships of other, low-abundance membrane transport proteins in the system of *A. nidulans*.

This is the first report on the construction of a functional tagged Cys-less transporter, expressed from its endogenous locus, and the application of an NEM-labelling assay in the *A. nidulans* system. Further than the recent

study on a Cys-less *A. nidulans* NrtA nitrate transporter (Unkles et al., 2005), our work introduces both an avidin-affinity purification silent tag and a sulfhydryl-specific alkylation assay for the Cys residues of the *A. nidulans* PrnB proline transporter. This development reinforces research of PrnB and related proteins by allowing employment of Cys-scanning mutagenesis in conjunction with site-directed sulfhydryl labelling to study structure–function relationships in a more systematic manner. As a first application, we have used the background of the Cys-less AAS PrnB-tag to study amino acid residues K245, F248 and Q219 that were suggested previously (Tavoularis et al., 2003) to be important for PrnB-mediated proline transport. More specifically, replacements K245L and F248L were shown to decrease significantly the affinity of PrnB for proline (4- and 8-fold higher K_m values, respectively). Substitutions of Q219 to R or H increased the transport capacity of PrnB transporter (6- to 7-fold increased V_{max}) without affecting significantly K_m values. Results presented here, show that replacement of residues K245 and F248 with Cys in a Cys-less background leads to a total loss-of-function PrnB transporter, further strengthening the hypothesis that these residues, which map on the same side of putative α-helix TMS6, are significant for high affinity PrnB-mediated proline uptake. In contrast, replacement of Q219 with Cys in the Cys-less background and NEM treatment showed no sensitivity to inactivation. Q219 maps in the extracellular loop between TMS6 and TMS7 and it was previously shown that substitutions of Q219 with R or H had a significant (6-fold) up-effect on the capacity of PrnB to transport proline (Tavoularis et al., 2003). However, results presented here strongly suggest that residue Q219 is not critical for PrnB-mediated proline uptake. Taken together, the data highlight TMS6 of PrnB and its flanking sequences as first priority target for systematic Cys-scanning analysis using the genetic tools established herein.

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