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Purification and partial characterization of the xanthine-uric acid transporter (UapA) of *Aspergillus nidulans*

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ABSTRACT

UapA, the uric acid-xanthine permease from the filamentous ascomycete *Aspergillus nidulans*, is one of the most thoroughly characterized purine/H^+ transporters in eukaryotes. Detailed studies have addressed its regulation of expression, at both the transcriptional and post-translational levels, in response to physiological and developmental signals. An extensive kinetic profile towards a plethora of purines and mutational analyses have established models on how UapA recognizes the purine ring and revealed specific amino acid residues involved in proper folding, topogenesis, function and specificity. The present work describes for the first time the purification of the UapA transporter of *A. nidulans* through overexpression via the strong, ethanol-inducible, glucose-repressible, *alcA* promoter. Purification, almost to homogeneity, was achieved by Ni^{2+} affinity chromatography using a functional His-tagged UapA protein version. It is subsequently shown, by Circular Dichroism (CD) spectroscopy, that the purified protein is structured with a high α-helical content, as expected from the *in silico* predictions. The result of this work opens the way for further, analytical and biochemical studies on UapA at the protein level.

Introduction

Specific purine transporters are ubiquitously expressed in plants and animals allowing purine redistribution in specialized tissues which are unable to synthesize purines *de novo* [1–4]. In addition, most microorganisms, plants and animals are able to use external purines in their salvage pathways and/or as secondary nitrogen sources [5]. Purine transporters are responsible for the effective uptake and metabolism of purine analogs which are widely used as antimetabolites against a variety of different diseases and infections, ranging from anti-tumor and leukemia chemotherapy to anti-viral compound, antibiotics and drugs against parasitic diseases [3,6,7].

UapA is a high-affinity, high-capacity, xanthine-uric acid permease from the ascomycete *Aspergillus nidulans* and also the best studied purine transporter. The *uapA* gene has been cloned and its regulation of expression studied in great detail. UapA (MW 61080) is predicted to consist of 12 α-helical transmembrane segments (TMS),[1] separated by relatively short hydrophilic loops, and cytoplasmic N- and C-termini [8]. The protein is expressed through transcription, developmentally activated during conidiospore germination [9]. Physiological regulation of UapA during vegetative growth depends on the presence of purine and/or rich nitrogen sources in the growth media, and occurs through both transcriptional and post-translational mechanisms. In brief, the presence of purines induces the transcription of the *uapA* gene [10,11]. The presence of ammonium provokes the repression of *uapA* expression but also directly down-regulates UapA activity by endocytosis and sorting to the vacuole [12]. Finally, *uapA* is also specifically expressed in asexual and sexual compartments of *A. nidulans* [12]. Recently strong evidence was obtained that the unexpected presence of UapA on aerial structures of *A. nidulans* is associated with a cryptic role of purine transport and metabolism in fungal development (P. Borbolis, C. Gournas, D.G. Hatzinikolaou, A. Pantazopoulou and G. Diallinas, unpublished results).

UapA has also become a model paradigm of biochemical and genetic studies concerning structure–function relationships of the Nucleobase-Ascorbate Transporter (NAT) family. Kinetic studies of UapA-mediated transport, and also analogous studies with NAT proteins from other fungi [13,14] and from the Gram-negative bacterium *Escherichia coli* [14], have established molecular models describing how NAT members recognize the purine ring [8,13]. Genetic approaches have reinforced these models and revealed specific domains involved in purine selection, binding and transport [8,15–17]. Furthermore, similar genetic studies and the use of a

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[1] *Abbreviation used:* TMS, transmembrane segments; NAT, nucleobase-ascorbate transporter; GFP, green fluorescence protein; Cd, circular dichroism; ECL, enhanced chemiluminescence; PMSF, phenyl-methyl-sulfonyl fluoride; DDM, dodecyl-β-D-maltoside; MM, minimal medium; BSA, bovine serum albumin.

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Green Fluorescence Protein (GFP)-tagged version of UapA, have revealed determinants which are critical for proper permease folding and trafficking towards the plasma membrane [16,17].

The hydrophobic and polytopic nature of UapA, typical of transporters, has hindered its purification and analysis at the protein level. For most membrane proteins purification proves problematic due to low expression levels, extreme hydrophobicity and difficulties, such as cytotoxicity, associated with over-expression [18]. This is reflected in the fact that the number of membrane protein structures that have been solved so far, is very small compared to the thousands of solved structures of globular proteins. The attachment of hydrophilic tags and heterologous over-expression in bacteria or eukaryotic cells of the gene or cDNA encoding the desired membrane protein, offer alternatives to overcome these problems for some simpler membrane proteins, but the over-expression of complex membrane proteins still remains problematic [19,20]. Eukaryotic membrane proteins are not expressed in bacterial cells since bacteria do not usually target eukaryotic membrane proteins for insertion into the plasma membrane [21,22] and purification in heterologous systems might not reflect the physiological topology of the protein under study.

In this study we present an approach to purify, for the first time in literature, the UapA transporter through homologous overexpression in *A. nidulans*. To accomplish this we made use of the strong, ethanol-inducible, glucose-repressible, *alcA* promoter. The overexpressed protein has been purified to almost 100% homogeneity by affinity chromatography techniques. In addition, the purified protein was shown, by Circular Dichroism (CD), to conserve the expected α-helical structure. The result of this work opens the way for further studies on UapA at the protein level that might include the determination of its structure through X-ray crystallography.

Materials and methods

Chemicals

Restriction enzymes were from Takara. Enzyme sources were as cited in the text. Acrylamide and gel reagents were from Bio-Rad and Sigma, nitrocellulose membrane was from Whatman–Schleicher & Schuell, Anti-His HRP conjugates (Penta-His-HRP conjugate) and metal chelating resins (Ni-NTA Superflow) were from Qiagen, Supersignal West Pico Chemiluminescent substrate used for Enhanced Chemiluminescence (ECL) was from Pierce, Phenyl-methyl-sulfonyl fluoride (PMSF) and Protease inhibitor Cocktail for fungi and yeast were from Sigma, Dodecyl-β-D-maltoside (DDM) was from Applichem. All other chemicals were of the highest purity available.

Strains and media

The *A. nidulans* triple deleted strain Δ*ACZ* (*ΔuapA ΔuapC ΔazgA argB2 pabaA1*) carrying deletions of all three major purine transporters (*ΔuapA ΔuapC ΔazgA*) has been described before [12]. This strain also carries an arginine auxotrophic mutation (*argB2*) and a mutation leading to *p*-amino-benzoic acid requirement (*pabaA1*). Isogenic strains expressing UapA-His from its endogenous promoter or the *alcA* promoter, were constructed by transformation of strain Δ*ACZ* ([12] and herein, respectively, see below). The *E. coli* K-12 strain used in standard protocols was DH5α. Standard media for *A. nidulans* [23] and *E. coli* [24] were used. Supplements were added where necessary. Expression from the *alcA* promoter was induced by adding ethanol (50 mM) in *A. nidulans* Minimal Medium (MM) with 1 g/L fructose as sole carbon source and 5 mM urea as sole nitrogen source. Repression of the *alcA* promoter was achieved by replacing fructose with 10 g/L glucose.

Nucleic acid manipulations

Nucleic acid manipulations and labeling of DNA molecules used as *argB*- and *uapA*-specific probes in southern analysis were performed according to Sambrook *et al*., [24]. Total DNA was isolated from *A. nidulans* strains as described previously [10].

Plasmid constructions and strain selection

The *alc* expression construct (*alc*-UapA-His) was assembled as follows: A DNA fragment including the *uapA-His*-tag open reading frame and the 3′ untranslated region (*uapA-His-3′UTR*$_{uapA}$), was obtained from plasmid pAN510-His [12] by PCR using the NcoI-F and NcoI-R primers shown on Table I (Appendix A, on-line supplemental data). PCR was performed with Phusion DNA polymerase (FINNZYMES), using the following program: 30 s at 98 °C followed by 35 cycles consisting of 10 s at 98 °C, 30 s at 57.5 °C, 1 min at 72 °C and finally 10 min at 72 °C. The resulting PCR product was NcoI digested, gel extracted and ligated, in-frame, with the *alcA* promoter in a pGEM plasmid [pGEM-Alc; [25]]. The final plasmid, called pAN-AlcUapA-His, was introduced into the *A. nidulans* Δ*ACZ* strain by co-transformation with plasmid pAN-ArgB. Selection of stable transformants was based on complementation of the *argB2* mutation, as previously described [26]. Selected *A. nidulans* transformants that also incorporated intact *alcA-uapA-his* sequences were identified by PCR (data not shown), using appropriate sense and antisense primers His-F, His-R, UapA-7 and NcoI-R (Table I Appendix A, on-line supplemental data). The nature of pAN-AlcUapA-His integration events in selected transformants was analyzed by Southern analysis (data not shown). Four transformants, called T10, T22, T25 and T26, carrying, respectively, multiple, 4, 2 or single copies of *alcA-uapA-his* sequences were identified. These transformants were further tested for their ability to grow on various purine media as described in Results and discussion. Radiolabelled purine uptake measurements for the selected transformants were performed as described elsewhere [12,15].

Isolation of membrane protein

Total membrane protein was extracted from mycelia grown in liquid minimal media, harvested and ground in the presence of liquid nitrogen. The ground mycelial powder was suspended in cold extraction buffer [10 mM Tris, pH 7.5, 5 mM $MgCl_2$, 100 mM NaCl, 0.3 M sorbitol, 1 mM PMSF and a protease inhibitor cocktail (1:500)] and centrifuged for 10 min at 2000 rpm in a benchtop centrifuge to remove unbroken cells and cell walls. Membranes were pelleted from the supernatant by centrifugation at 18,000*g* for 1 h at 4 °C and either suspended in buffer A [10 mM HEPES, 250 mM sucrose, 1 mM PMSF, 1 mM DTT and protease inhibitor cocktail (1:500)] for SDS–PAGE and immunodetection or in buffer B [50 mM potassium phosphate buffer, pH 8, 150 mM NaCl, 1 mM PMSF, Protease inhibitor cocktail (1:500), 20% (v/v) glycerol and 12 mg/mL DDM] for solubilization and purification. Protein estimation was performed according to the method of Bradford [27] with Bovine Serum Albumin (BSA) as a standard.

Electrophoresis and immunoblot analysis

SDS–polyacrylamide gel electrophoresis was performed essentially as described by Laemmli [28]. Aliquots of the membrane protein were treated with two volumes of 1× sample loading buffer [64 mM Tris–HCl, pH 6.8, 3% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10% (w/v) glycerol, 0.002% (v/v) bromophenol blue] and incubated at 37 °C for 20 min prior to electrophoresis. Native-PAGE was performed using the same buffers omitting SDS and β-mercaptoethanol treatment. Protein bands were visualized

by Coomassie or silver staining. For immunoblotting, protein samples were fractionated on a denaturing SDS–PAGE gel and electrophoretically transferred onto a nitrocellulose membrane [29] in a Trans Blot cell filled with 20% (v/v) methanol, 192 mM glycine and 25 mM Tris. To block nonspecific binding sites, the blots were incubated with the specially formulated Blocking Reagent and Blocking Reagent Buffer, specific for anti-His HRP conjugate, for 1 h at room temperature. Immunodetection was performed with the anti-His HRP conjugate (Qiagen) in blocking solution for 1 h at room temperature (antibody dilution 1:2000). Blots were developed by the ECL method using the Supersignal West Pico Chemiluminescent substrate.

Solubilization and purification of UapA

Pelleted membranes were solubilized by resuspending in Nickel column buffer [50 mM sodium phosphate buffer, pH 8, 150 mM NaCl, 1 mM PMSF, protease inhibitor cocktail (1:500)] containing 1.2% w/v DDM. Solubilized proteins were separated from the insoluble ones by stirring the samples on ice for 30 min and then centrifuging at 4 °C for 20 min. To the soluble protein (supernatant) was then added 20% (v/v) glycerol (final concentration) and gently mixed. The detergent solubilized protein sample was either kept at −70 °C until required for use or loaded directly (1 ml ∼8 mg) onto a Ni^{2+}-loaded metal chelating resin [Ni-NTA Superflow (2.5 ml resin)] which had been pre-equilibrated for 1 h with several volumes of the wash buffer (50 mM sodium phosphate, pH 8, 300 mM NaCl, 20% (v/v) glycerol, 1% (w/v) DDM, 1 mM PMSF, 10 mM imidazole). The elution profile was evaluated by eluting the column with increasing concentrations of imidazole (10, 50, 100, 200, 250, 300, 400, and 500 mM) in wash buffer.

Desalting and sample concentration

In order to remove salts from the Ni-NTA column eluates, aliquots of the eluates (2.5 ml) were subjected to size exclusion chromatography on an Amersham Biosciences PD-10 gel filtration column (Sephadex G-25 resin). Each fraction was eluted from the column with 3.5 ml of distilled water and freeze-dried. Samples were next resuspended in buffer containing 10 mM HEPES, pH 7.5, 250 mM sucrose, 1 mM PMSF, 1 mM DTT and protease inhibitor cocktail (1:500) for electrophoretic analysis. For spectroscopic studies, purified and desalted UapA was concentrated by lyophilization and the lyophilized sample was resuspended in 50 mM sodium phosphate, pH 7.5, containing 20% glycerol and 0.1% DDM.

Circular dichroism

Circular dichroism analysis was performed on a Jasco J-815 CD Spectropolarimeter. Far-ultraviolet CD spectra of purified UapA (0.1 or 1 mg/mL) were recorded at 20 and 10 °C, in a quartz cell of 1-mm path length, at the wavelength range 250–180 nm, with a resolution of 0.2 nm. Values are given as ellipticity data, θ (mdeg).

Results and discussion

Overexpression of UapA from the alcA promoter

The *alcA* (alcohol dehydrogenase) promoter is a well-established tool for the over-expression of genes in *A. nidulans* [25] that is strongly induced by ethanol. In addition, it is also glucose-repressible, allowing the control of transcription of any gene fused to it. In this work, the *alcA* promoter was used to drive the over-expression of the UapA transporter.

The *uapA* gene, tagged at its C-tail with an epitope encoding 10-His residues, was placed under the control of the *alcA* promoter, in a plasmid called pAN-AlcUapA-His, as described in Materials and methods. This plasmid was introduced into the *A. nidulans* Δ*ACZ* triple knock-out strain, lacking all major purine transporters. Four transformants, called T10, T22, T25 and T26, carrying, respectively, multiple, 4, 2 or single copies of *alcA-uapA-his* sequences, as established by PCR and Southern analyses (data not shown), were selected for further analysis.

Fig. 1 shows the growth phenotypes of these transformants on purines, under physiological conditions that affect transcription from the *alcA* promoter. In particular, transformants were analyzed by tests, at 37 °C, under non-inducing (fructose), repressing (glucose) or inducing (fructose, ethanol) conditions for growth on uric acid or hypoxanthine, as sole nitrogen sources. Uric acid is a physiological substrate for UapA while hypoxanthine is not. The original recipient strain (Δ*ACZ*) cannot grow, neither on uric acid because it lacks UapA as well as the secondary uric acid-xanthine permease UapC [30], nor on hypoxanthine, as it lacks the major purine transporter AzgA [31]. As expected, all selected transformants were able to grow, similar to the wild-type strain, under ethanol-inducing conditions (fructose, ethanol), but could not grow on hypoxanthine. Growth on uric acid was abolished when glucose replaced fructose in the media (*alcA* repressing conditions). Finally, under conditions of glucose-repression and ethanol-induction, only T10, the multi-copy transformant could confer UapA-mediated growth on uric acid. This was expected, since it is known that the *alcA* promoter is not totally repressed by glucose, especially when ethanol is present in the medium. Evidently, in T10, the presence of multiple alcA-UapA-His copies enhanced the leakage of the alcA-driven expression of UapA.

No apparent growth defect was detected on media with nitrogen sources other than purines. Morphological growth defects could however, be detected under *alcA*-UapA induced conditions, at 25 °C. This could be rationalized considering the fact that at this temperature the *alcA* promoter is induced at even higher levels, compared to induction at 37 °C (unpublished observations). In that case, super-expression of UapA causes several defects in the

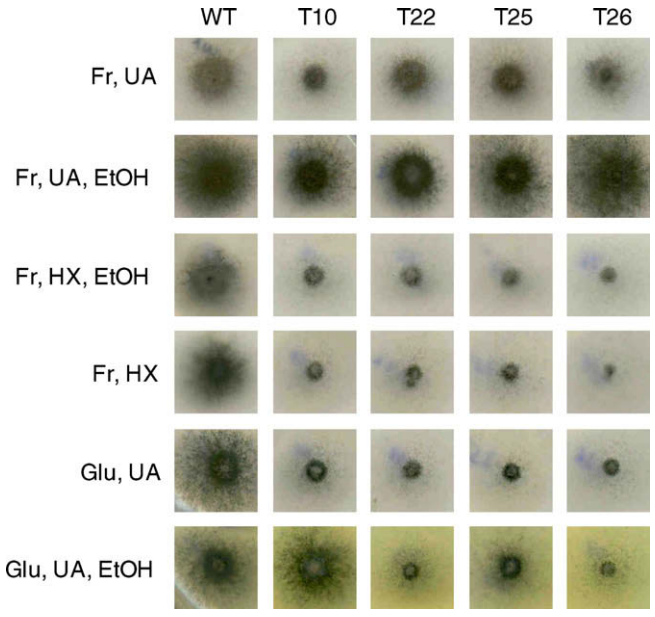

Fig. 1. Growth test (at 37 °C) of alc-UapA-His transformants (T10, T22, T25 and T26) on various solid minimal media. Transformants show normal growth on uric acid and fructose (*alcA* non-inducing) with growth greatly enhanced in the presence of ethanol (*alcA* inducing). No growth was observed on hypoxanthine or glucose (*alcA* repressing) medium. Fr, fructose (1 g/L); Glu, glucose (10 g/L); UA, uric acid (0.1 mg/mL); HX, hypoxanthine (0.1 mg/mL); EtOH, ethanol at 50 mM; WT, wild-type strain.

general mechanism of protein trafficking, as this was evidenced using a GFP-tagged version of *alcA*-UapA (unpublished observations).

Over-expression from the *alcA* promoter and addition of a His-tag at the UapA C-terminus did not affect the function and specificity of UapA. Transport kinetic assays using radiolabeled xanthine and competition experiments with other UapA substrates, have shown that the His-tagged version of UapA has physiological K_m values and a substrate profile which was practically identical to the wild-type protein. More specifically, the K_m (μM) values for xanthine were 8.0 ± 1.1 and 8.8 ± 1.7 for the wild-type and the His-tag strains, respectively, while the corresponding K_i (μM) values for uric acid were 12.0 ± 0.7 and 17.2 ± 1.9. The UapA transport capacity, reflected in apparent V_{max} values, is obviously dependent on the level of UapA protein present in the plasma membrane, which in turn depends on the physiological conditions used. Comparing V_{max} values for xanthine uptake in a strain expressing UapA from its native promoter under purine-inducing conditions, with those obtained with transformants T26 (single-copy) and T10 (multi-copies) under ethanol-inducing conditions, it was found that in the latter, expression capacities are increased approximately 4- and 10-fold, respectively. The fact that T10, despite having at least 10 copies of alC-UapA-His, has only 2.5-fold increased UapA-mediated transport compared to T26, is very probably due to saturation of transporter trafficking towards the membrane [12,17].

For further analysis and purification of UapA-His, we selected the multi-copy transformant T10, which was expected to produce the highest possible yield of the protein. Fig. 2 compares the efficiency of expression of the His-tagged version of UapA in T10 and an isogenic transformant expressing UapA-His under its natural promoter [12]. For both strains, optimal induction conditions were used (see Materials and methods). Crude membrane extracts, prepared from these strains as described in Materials and methods, were electrophoretically transferred to a nitrocellulose membrane and immunobloted with the anti-His HRP conjugate. UapA-specific bands were detected with apparent molecular mass of approx. 60 kDa, in good agreement with the predicted molecular mass of 61.1 kDa from the putative open reading frame. The results showed several-fold increase (>50-fold) in the UapA steady state level when expressed from the *alcA* promoter, as compared to the level obtained with the native promoter, even when the latter is induced with purines (Fig. 2). Thus, a significant overexpression of a functional UapA protein that should help with its purification has been achieved. It is worthy to note that maximum overexpression of *alc*-UapA-His occurred at 25 °C and led to growth defects (very slim and compact mycelia) which, however, are associated with general protein trafficking and are not important for the biochemical isolation of functional UapA.

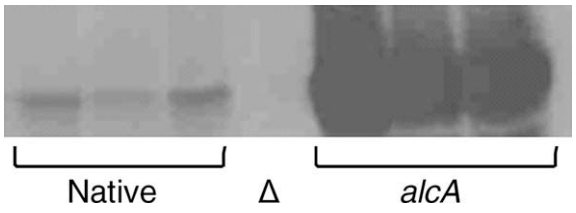

Fig. 2. Densitometric comparison of UapA expression from the native (UapA-His) and the strong alc (alc-UapA-His) promoters. An equal amount of total protein membrane extract (50 μg) isolated from the UapA-His and the pGEMalc-UapA-His strains (both grown under induced conditions) was electrophoresed, transferred to a nitrocellulose membrane and immunodetected using the anti-His HRP conjugate. Δ, represents the triple deleted strain.

Optimization of UapA solubilization

Prerequisite for purification is having the protein in a soluble form, to allow for proper interactions with the affinity ligand attached on the chromatographic resins. Hence, optimization of the detergent and the buffer composition is critical for obtaining maximum solubilization efficiency and stability of a protein in solution. To determine the optimal solubilization conditions for the UapA protein, total membrane proteins from the alc-UapA-His T10 strain were exposed to different concentrations of the detergent DDM, which has previously been used to solubilize a number of membrane proteins [32,33] and is very compatible with downstream studies. It should be noted that prior to the use of DDM, the membrane extract was exposed to a panel of different detergents (CHAPS, DDM, NP-40, Laurylsarcosyl, Tween, Triton X-100) at concentrations above their micellar concentrations (results not shown). Most of these, either precipitated the protein extract within a short period or enhanced the formation of aggregates on Western blot. Only CHAPS and DDM sustained the extract for an appreciable period of time and resulted in less aggregation as compared to the others. The DDM-solubilized samples revealed an optimal solubilization of the UapA protein on Western blots at a detergent concentration of 1% (w/v) DDM (Fig. 3).

UapA purification

The His epitope tag provides the dual possibility of detecting transporters *in vitro* and their purification through an immobilized metal affinity chromatographic column by making use of their coordinate binding to transition metals like Ni, Co, and Zn immobilized on a solid support. In the purification of UapA-His, the Ni-nitrilotriacetic acid resin [Ni-NTA Superflow, (Qiagen)] was used. DDM-solubilized (1% w/v) total protein extracts were prepared from the T10 strain as described and passed onto a previously equilibrated Ni^{2+}-loaded metal chelating resin (Ni-NTA Superflow). A serial elution of the column was performed using increasing concentrations of imidazole in the wash buffer in order to determine the imidazole concentration that elutes the His-tagged UapA protein from the column. Analysis of the eluted fractions on a Coomassie-stained SDS–PAGE gel after desalting and concentration by freeze–drying, revealed strong UapA specific bands in fractions that eluted at 200 and 250 mM imidazole concentrations with a

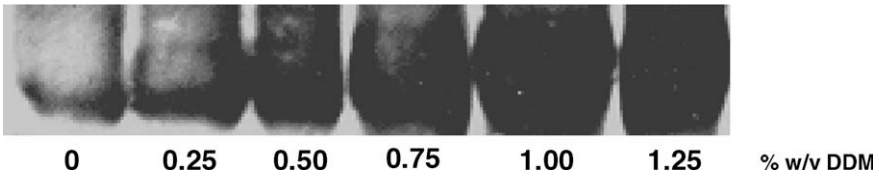

Fig. 3. Detergent solubilization of UapA: Total membrane extracts were resuspended in 1 vol. of extraction buffer containing various concentrations of DDM. Samples were incubated on ice for 30 min and centrifuged for 20 min at 4 °C at 12000*g*. The supernatant from each sample was collected and an equal fraction was electrophoresed on a 10% acrylamide gel, blotted to nitrocellulose membrane, and processed as an immunoblot with anti-His HRP antibody conjugate.

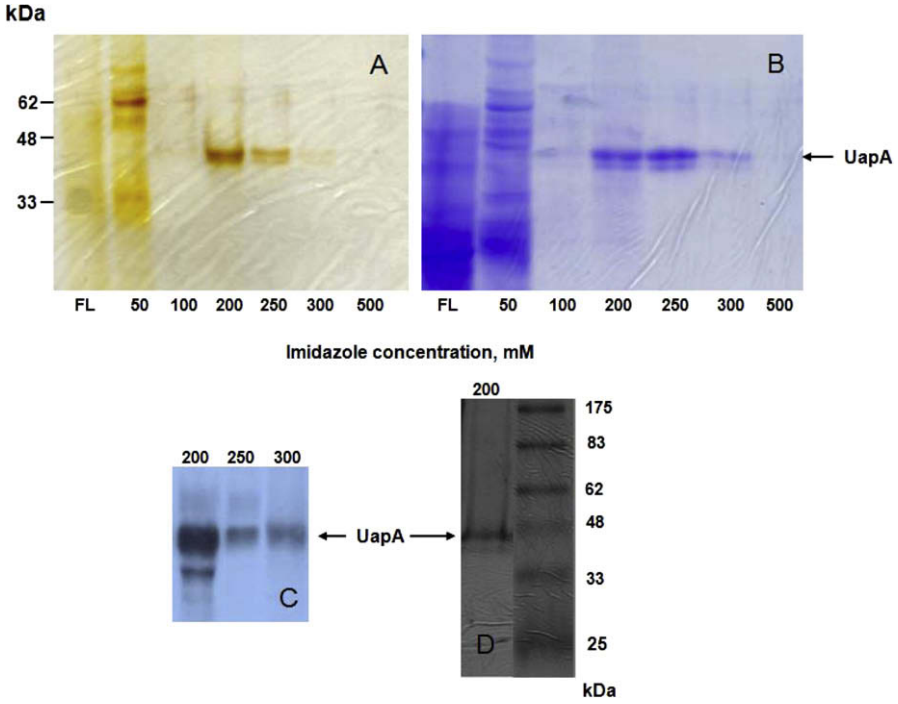

Fig. 4. Elution profile of UapA on Ni^{2+}-NTA column. The column was washed with column wash buffer and eluted with this buffer containing various concentrations of imidazole. Eluate samples were desalted on a PD-10 column (Amersham Biosciences) column and concentrated by lyophilization. Protein samples were resolved on 10% acrylamide gels that were either coomassie (A) or silver stained (B). Western blot analysis (C) for fractions at 200, 250 and 300 mM imidazole revealed strong UapA signals corresponding to the intensity of the silver staining. (D) shows the native-PAGE electrophoresis of the 200 mM imidazole fraction.

weak band eluting at 300 mM (Fig. 4A). These fractions were further analyzed on a silver stained gel to assess the degree of purity of the eluted proteins (Fig. 4B). From the densitometric scanning of these SDS–PAGE gels, UapA could be seen to elute at almost 100% homogeneity.

The fact that UapA elutes at 200–250 mM imidazole concentration in wash buffer indicates its very strong affinity with the Ni^{2+}-column, which could probably be due to the presence of the ten histidine residues at its C-terminus, as the binding strength of the Ni-resins increases with the number of histidine residues. Similar findings with 10-His-tagged membrane proteins have been reached for the bacterial transporters LacY [34] and YgfO [35]. The protein eluted as a doublet comprising a single sharp band at approximately 45 kDa co-purifying with an underlying band of relatively lesser intensity. Western blot analysis showed the two bands to be very reactive in immunoblots with the anti-His HRP conjugate giving the same pattern as in the SDS–PAGE gels (Fig. 4C). The fact that the purified UapA-His displays high electrophoretic mobility, i.e. it migrates at a position corresponding roughly to 15 kDa less than the predicted MW from its orf, is a common phenomenon among purified hydrophobic proteins [34,36,37]. Heterogeneity in size has been found to be common among hydrophobic transport proteins of yeast. Some have been attributed to their interaction with SDS, a case excluded here as native-PAGE electrophoresis under non-denaturing conditions of the purified UapA protein still gave a similar pattern with the two bands (Fig. 4D). This shows that the two forms of UapA appearing in both denaturing and non-denaturing conditions might correspond to two alternative chemical forms of the transporter.

Out of approximately 47 mg of membrane protein that were pelleted from each batch of 2.5 g of dry mycelium, 0.6 mg of purified UapA was obtained, representing 1.3% of the total pelleted membrane protein. It is unlikely, that this yield (which is based on the pelleted membrane protein) could be further optimized since the optimum solubilization conditions have been determined and applied, while the purification procedure practically involved only one chromatographic step at conditions pre-imposed from the use of the histidine epitopes. The use of ultracentrifugation at high g values would obviously increase the overall yield (based on the mycelia biomass) since it would result in close to quantitative membrane protein pelleting but the much larger amount of precipitated membrane proteins may had resulted in a more laborious purification scheme. In conclusion, the high expression levels obtained through the use of the *alcA* promoter in combination with the 10-His epitope, enabled us to develop, for the first time in literature, a rapid and reproducible purification procedure for this

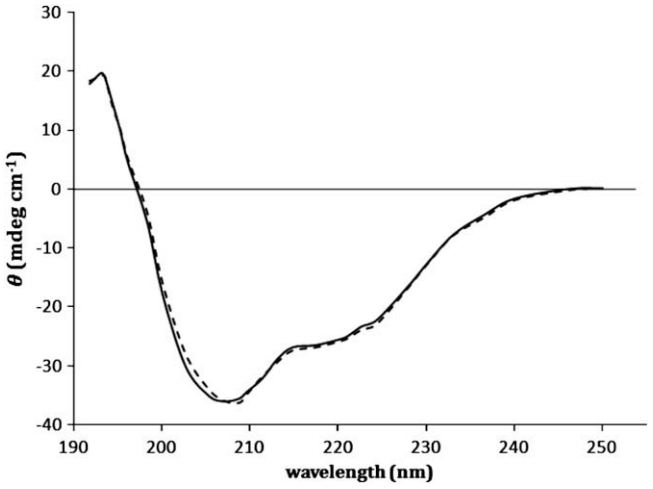

Fig. 5. Far-ultraviolet CD spectra of purified UapA in a buffer of 50 mM NaP_i, pH 7.5, 20% glycerol, 0.1% DDM, recorded at 20 °C (continuous line) and 10 °C (broken line). Background values obtained with buffer alone are negligible in the wavelength range shown and were not subtracted.

highly hydrophobic protein that yields sufficient UapA amounts even without quantitative membrane protein pelleting.

Circular dichroism (CD) of purified UapA

At either 20 or 10 °C, purified UapA yields a CD spectrum (Fig. 5) characteristic of a protein which assumes a predominantly α-helical structure [38]. Mean residue ellipticity values and, therefore, the α-helical content, could not be determined with accuracy, due to the problems associated with the inaccuracy of standard colorimetric protein assays and high protein hydrophobicity [39]. In any event, however, the observed α-helical signature confirms *in silico* algorithm predictions for the secondary structure of UapA (of which roughly half of the 574 residues are predicted to fall within one of 12 transmembrane α-helical segments) and is a common feature among secondary active transporters, as shown experimentally in several cases by X-ray crystallography [40–42], CD spectra [33,43] and specific site-directed techniques [44,45].

Conclusions

UapA was overexpressed from its native cellular environment and purified to near-homogeneity. Based on circular dichroism, the purified UapA sample displays a predominantly α-helical structure, as expected from the *in silico* prediction algorithms. The findings set the stage for a more analytical structural study of this eukaryotic prototype of purine-transporting NATs which is of importance for the elucidation of purine-transporter interactions at high resolution.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pep.2008.08.012.

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