RESEARCH ARTICLE

Detection of a homotetrameric structure and protein–protein interactions of *Paracoccidioides brasiliensis* formamidase lead to new functional insights

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Abstract

Paracoccidioides brasiliensis causes paracoccidioidomycosis, a systemic mycosis in Latin America. Formamidases hydrolyze formamide, putatively plays a role in fungal nitrogen metabolism. An abundant 45-kDa protein was identified as the *P. brasiliensis* formamidase. In this study, recombinant formamidase was overexpressed in bacteria and a polyclonal antibody to this protein was produced. We identified a 180-kDa protein species reactive to the antibody produced in mice against the *P. brasiliensis* recombinant purified formamidase of 45 kDa. The 180-kDa purified protein yielded a heat-denatured species of 45 kDa. Both protein species of 180 and 45 kDa were identified as formamidase by peptide mass fingerprinting using MS. The identical mass spectra generated by the 180 and the 45-kDa protein species indicated that the fungal formamidase is most likely homotetrameric in its native conformation. Furthermore, the purified formamidase migrated as a protein of 191 kDa in native polyacrylamide gel electrophoresis, thus revealing that the enzyme forms a homotetrameric structure in its native state. This enzyme is present in the fungus cytoplasm and the cell wall. Use of a yeast two-hybrid system revealed cell wall membrane proteins, in addition to cytosolic proteins interacting with formamidase. These data provide new insights into formamidase structure as well as potential roles for formamidase and its interaction partners in nitrogen metabolism.

Introduction

Paracoccidioides brasiliensis, a dimorphic fungus of the phylum *Ascomycota*, is a major human pathogen with a broad distribution in Latin America (Restrepo, 1985). *Paracoccidioides brasiliensis* grows as a saprophytic mold in the environment, but undergoes phase transition to a yeast form at mammalian physiological temperatures. The fungus, which is the etiologic agent of paracoccidioidomycosis, is primarily a respiratory pathogen, infecting the host through the inhalation of airborne propagules from the mycelia phase. In the pulmonary alveolar epithelium, through differentiation, the mycelia switch to the parasitic yeast form that can spread to multiple organs and tissues (Franco, 1987).

Paracoccidioides brasiliensis nitrogen metabolism is poorly understood. Formamide aminohydrolases (FMD, EC 3.5.1.49) catalyze the highly specific hydrolysis of formamide to produce ammonia and formate (Skouloubris *et al.*, 1997). Some microorganisms are able to use formamide as a nitrogen source. For example, *Aspergillus nidulans* possess a formamidase gene (*fmd*S), which allows the fungus to utilize formamide as the sole nitrogen source (Fraser *et al.*, 2001). *Paracoccidioides brasiliensis* overexpresses the gene encoding formamidase, with higher amounts of transcripts in the saprobe mycelia phase (Felipe *et al.*, 2005). A 45-kDa protein species fractionated by liquid isoelectric focusing and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was identified by amino acid sequencing to be

P. brasiliensis formamidase. We also demonstrated that cellular extracts from yeast, mycelium and of the recombinant formamidase displayed the ability of converting formamide into ammonia and formic acid. In addition, formamidase of *P. brasiliensis* reacts with immune sera from patients with paracoccidioidomycosis, providing an association of the protein with fungal pathogenesis (Borges *et al.*, 2005).

In this study, we observed a 180-kDa protein present in fungal cells that was reactive with a polyclonal antibody produced against recombinant formamidase. The enzyme was purified in two chromatographic steps, and the protein was identified by MS as formamidase. Using an immunocytochemical assay, the fungal formamidase was localized to the *P. brasiliensis* cytoplasm and cell wall. The biological role of formamidase in *P. brasiliensis* is unknown. Our previous data indicated that the *P. brasiliensis* formamidase presents high and specific affinity for formamide, suggesting that the protein plays a role in *P. brasiliensis* nitrogen metabolism. In order to better understand the biological role of formamidase, we searched for potential macromolecules that may interact with formamidase using the yeast two-hybrid system and coimmunoprecipitation assays. We identified proteins interacting with formamidase localized in the cytosol and the cell wall/membrane. These data provide a new insight into the structure and function of the *P. brasiliensis* formamidase as well as identify protein-binding partners that have not been known before to interact with formamidase.

Materials and methods

Maintenance of *P. brasiliensis*

Paracoccidioides brasiliensis isolate 01 (ATCC MYA-826) was grown as described previously (Barbosa *et al.*, 2006). The yeast phase and mycelium were grown at 36 and 22 °C, respectively, in Fava–Netto's medium (1% w/v peptone; 0.5% w/v yeast extract; 0.3% w/v proteose peptone; 0.5% w/v beef extract; 0.5% w/v NaCl; 4% w/v glucose; 1% w/v agar, pH 7.2).

Preparation of mycelia and yeast cell protein extracts

Yeast and mycelium protein crude extracts were prepared as described previously (Borges *et al.*, 2005). For the preparation of total cell homogenate, mycelium and yeast cells were frozen and ground with a mortar and pestle in the presence of protease inhibitors: 50 μg mL^{-1} *N*-α-ρ-tosyl-L-lysine chloromethylketone; 1 mM 4-chloromercuribenzoic acid; 20 mM leupeptin; 20 mM phenylmethylsulfonyl fluoride; and 5 mM iodoacetamide in a homogenization buffer (20 mM Tris-HCl, pH 8.8; 2 mM $CaCl_2$). The mixture was centrifuged at 12 000 ***g*** at 4 °C for 10 min, and the supernatant was collected. The protein content of samples was determined according to Bradford (1976).

Heterologous protein expression and generation of polyclonal antibody

The production and purification of the recombinant formamidase was performed as described (Borges *et al.*, 2005). cDNA encoding formamidase (GenBank accession number AY63575) was cloned into the SalI/NotI restriction sites of pGEX-4T-3 (GE Healthcare®, Chalfont St Giles, UK). The recombinant protein was expressed in the soluble form by the *Escherichia coli* XL 1 Blue MRF' and purified by affinity chromatography under nondenaturing conditions. The soluble fraction was applied to a Gluthatione Sepharose™ 4B resin column (GE Healthcare®). The resin was washed three times in 1 × phosphate-buffered saline (PBS) (0.14 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) and the recombinant protein was cleaved by addition of thrombin protease (50 U mL^{-1}). The purity and size of the recombinant protein were evaluated by running the molecule on 12% SDS-PAGE, followed by Coomassie blue staining.

The recombinant formamidase was used to generate a specific mice polyclonal antibody. The purified protein (300 μg) was injected into mice with Freud's adjuvant, three times at 2-week intervals. The serum, containing a specific anti formamidase polyclonal antibody, was collected and stored at –20 °C. The mice were also bled before immunization to obtain preimmune serum.

Protein fractionation by electrophoresis and Western blot analysis

The total protein extract of *P. brasiliensis* yeast cells was separated by isoelectric focusing, as described (O'Farrell, 1975). Gels were loaded with the proteins in sample buffer containing 9.5 M urea, 1.6% v/v ampholines 5.0–8.0, 0.4% v/v ampholines 3.5–10.0, 2% v/v nonionic detergent Nonidet P-40 and 5.0% v/v β-mercaptoethanol. The strips were treated for 30 min with equilibration buffer (0.08 M Tris-HCl pH 6.0, 5% v/v β-mercaptoethanol, 2.3% w/v SDS, 1% v/v glycerol and 0.01% w/v bromophenol blue). The second dimension was performed on a 5–15% gradient polyacrylamide gel, as described by Laemmli (1970). Immunoblot reactions with sera from immunized or control mice were carried out for 2 h at room temperature with gentle shaking. The membranes were washed three times with 0.1% v/v Tween 20 in PBS and subsequently incubated for 1 h at room temperature with goat anti-mouse IgG coupled to alkaline phosphatase (Sigma Aldrich Co., St. Louis, MO). The blots were developed with 5-bromo-4-chloro-3-indolylphosphate/nitrobluetetrazolium.

Native PAGE was carried out using the GE Healthcare® mini gel equipment, basically according to the previously described methods (Laemmli, 1970). Coomassie brilliant blue was used to stain protein bands. Determination of native molecular mass was performed by native PAGE with varying cross-linking degrees of polyacrylamide, according to Ferguson's method, using 6%, 7% and 8% native gels (Ferguson, 1964).

Enzymatic assay for formamidase

The formamidase activity was measured by monitoring the production of ammonia, as described (Skouloubris *et al.*, 1997). Protein samples (500 ng) were added to 200 μL of formamide substrate solution at a final concentration of 100 mM in 100 mM phosphate buffer, pH 7.4, and 10 mM EDTA. The reaction mixture was incubated at 37 °C for 30 min. Subsequently, 400 μL of phenol-nitroprusside and 400 μL of alkaline hypochlorite solution (Sigma Aldrich Co.) were added. The samples were incubated at 50 °C for 6 min and the $A_{625\,nm}$ was read. The amount of ammonia released was determined by comparing with a standard curve. One unit (U) of formamidase was defined as the amount of enzyme required to hydrolyze 1 μmol formamide $\cdot min^{-1}\,mg^{-1}$ total protein.

Purification of the *P. brasiliensis* formamidase

The total protein extract of *P. brasiliensis* yeast cells was obtained as described above. The extract was resuspended in a buffer containing 25 mM Tris-HCl, pH 7.5, and applied onto a 10-mL DEAE Sepharose column (GE Healthcare®). The column was equilibrated with the same extract resuspension buffer. Proteins were eluted using a linear gradient: 0.1–1 M NaCl for 30 min, followed by 1.0 M NaCl for 10 min, at a flow rate of 0.5 mL min^{-1}. Fractions (2 mL) were collected and aliquots were tested for formamidase activity, as described above. The enzymatically active fractions were applied onto a 10-mL phenyl sepharose column (GE Healthcare®), previously equilibrated with a buffer containing 50 mM Na_2HPO_4 and 0.5 M $(NH_4)_2SO_4$, pH 7.0. Protein flowing through the column was collected and assayed for formamidase activity, as described above. Fractions containing activity were pooled and stored for further analysis.

Proteolysis and peptide mass fingerprinting

Two protein species of 180 and 45 kDa that had been separated by SDS-PAGE were excised from the gel and soaked in 50 μL acetonitrile. The solvent was removed under vacuum and incubated in 100 mM NH_4HCO_3 buffer containing 10 mM 1,4-dithiothreitol for 1 h at 56 °C under gentle agitation. The above buffer was removed and replaced by 55 mM iodoacetamide in 100 mM NH_4HCO_3 for 45 min at room temperature in the dark. The gel pieces were then subjected to alternate 5-min washing cycles with NH_4HCO_3 and acetonitrile, dried down, swollen in 50 μL of 50 mM NH_4CO_3 containing 12.5 ng mL^{-1} sequencing-grade modified porcine trypsin (Promega, Madison, WI) and incubated at 37 °C overnight. The resulting tryptic peptides were extracted by adding 20 μL of 5% v/v acetic acid and removing the solution. This was repeated once. The extracts were pooled, dried under vacuum and then solubilized in 0.1% v/v trifluoroacetic acid for MS analysis. The 180-kDa protein tryptic fragments were analyzed using a MALDI-TOF mass spectrometer (Reflex IV, Bruker Daltonics, Karlsruhe, Germany). The 45-kDa protein tryptic digest sample was analyzed using a MALDI-Synapt MS™ mass spectrometer (Waters-Micromass, Manchester, UK). The peptide mass list obtained for each spectrum was searched using MASCOT (http://www.matrixscience.com) against the SwissProt database (http://expasy.org/sprot).

Immunocytochemistry of formamidase

Yeast cells were fixed in a mixture containing 4% w/v paraformaldehyde, 0.5% v/v glutaraldehyde and 0.2% w/v picric acid in 0.1 M sodium cacodylate buffer at pH 7.2 for 24 h at 4 °C. The cells were rinsed several times using the same buffer, and free aldehyde groups were quenched with 50 mM ammonium chloride for 1 h, followed by staining in a solution containing 2% w/v uranyl acetate in 15% v/v acetone for 2 h at 4 °C (Berryman & Rodewald, 1990). Dehydration was performed in a series of ascending concentrations of acetone (30–100% v/v) and the material was embedded in LR Gold resin (Electron Microscopy Sciences, Washington, PA). The ultrathin sections were collected on nickel grids, preincubated in 10 mM PBS containing 1.5% w/v bovine serum albumin (BSA) and 0.05% v/v Tween 20 (PBS-BSA-T) and incubated for 1 h with the polyclonal antibody to the recombinant formamidase (diluted 1 : 100). Cells were washed with PBS-BSA-T, and incubated for 1 h at room temperature with the labeled secondary antibody mouse IgG, Au conjugated (10 nm average particle size; 1 : 20 dilution; Electron Microscopy Sciences). The grids were washed with PBS-BSA-T and distilled water, stained with 3% w/v uranyl acetate and lead citrate. Cells were imaged using a Jeol 1011 transmission electron microscope (Jeol, Tokyo, Japan). Controls were incubated with mouse preimmune serum at 1 : 100, followed by incubation with the labeled secondary antibody. The gold particles were quantified in three independent preparations of yeast cells. The particles were counted for the total cell distribution, as well as in the cytoplasm and the cell wall, as described previously (Barbosa *et al.*, 2006).

cDNA library construction and two-hybrid assays

A cDNA library was obtained using RNA extracted from *P. brasiliensis* yeast cells. The cDNAs were synthesized using the SMART PCR cDNA synthesis kit (Clontech Laboratories, Palo Alto, CA) and cloned into the prey vector pGADT7 in order to perform yeast two-hybrid screens using the Matchmaker Two-Hybrid System 3 (Clontech). To identify potential protein–protein interactions with formamidase, the cDNA encoding formamidase (Borges *et al.*, 2005) was subcloned into the bait vector pGBKT7. The generation of transformants was obtained by introducing the bait vector into *Saccharomyces cerevisiae* yeast strain Y187 (*MAT*α, *trp1-901*) and the prey vector into the *S. cerevisiae* strain AH109 (*MAT*a, *leu2-3*). The experimental protocol was performed according to the Matchmaker GAL4 Two-Hybrid System 3 manual and the Yeast Protocol Handbook (Clontech). Following cell mating, the *S. cerevisiae* diploids containing the two vectors were selected from plates containing SD/–Leu/–Trp minimal media. To exclude false-positive clones, the colonies were replicated using high-stringency plates containing SD–Ade/–His/–Leu/–Trp minimal media. The screening of positive clones was accomplished by detecting the blue/white color of the substrate 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside. Adenine and histidine were the reporter genes expressed together with *lacZ* (α-galactosidase reporter gene). PCR colony assay was performed on the clones using AD-LD 5′ and AD-LD 3′ supplied oligonucleotides for the pGADT7-Rec bait plasmid. The PCR products of the identified transformants were subjected to DNA sequencing using a MegaBACE 1000 sequencer (GE Healthcare®) for automated sequence analysis. Sequence homologies to the genes of interest were performed by searching the GenBank database using the BLAST algorithm (http://www.ncbi.nlm.nih.gov).

In vitro translation and coimmunoprecipitation assays

The cDNA encoding formamidase and the identified cDNAs that potentially interact with formamidase were synthesized using the TNT® Coupled Reticulocyte Lysate System (Promega). The PCR products of selected colonies were used as a template for the *in vitro* transcription/translation. The proteins were *in vitro* synthesized and labeled with ^{35}S-methionine (Perkin-Elmer, Wellesley, MA) using rabbit reticulocyte lysate. The reaction was incubated at 30 °C for 2 h. Next, 2.5 μL of the translated samples were loaded onto an SDS gel for the analysis of the translated products.

The translated formamidase was fused to a c-myc epitope (c-myc-FMD) and the translated proteins were fused to a hemagglutinin epitope (HA-Prey) and were mixed at 25 °C for 1 h. The mixture was incubated with protein A agarose beads, and with monoclonal c-myc antibody in 1 × PBS at 25 °C for 1 h. After washing, beads containing proteins were resuspended in SDS-loading buffer containing 50 mM Tris-HCl, pH 6.8, 100 mM 1,4-dithiothreitol, 2% w/v SDS, 0.1% w/v bromophenol blue and 10% v/v glycerol, followed by boiling at 80 °C for 5 min. The proteins were separated on a (4–12%) SDS-PAGE linear gradient. The gel was fixed with 20% v/v ethanol and 10% v/v acetic acid for 30 min, and incubated in 20 mL of fluorographic reagent NAMP 100 (Amplify Fluorographic Reagent – GE Healthcare®). The gels were dried at 80 °C for 90 min under vacuum, followed by autoradiography. Controls were performed for each assay preparation. Each assay was repeated three times using a different batch of *in vitro* translated products for validation.

Results

Polyclonal antibody recognition of *P. brasiliensis* formamidase

Antibodies were generated against purified recombinant formamidase by mouse immunization. Total protein extracts of *P. brasiliensis* yeast cells and mycelium, and the purified recombinant formamidase were obtained. The proteins were separated by gel electrophoresis, blotted onto a membrane and treated with the polyclonal antibody (Fig. 1a, lanes 1–4). A single protein species was detected in the total protein extract from yeast cells (Fig. 1a, lane 1) and mycelium (Fig. 1a, lane 2). In addition, the recombinant purified formamidase was recognized as a single protein species by the polyclonal antibody (Fig. 1a, lane 4). The antibody did not react with the total protein extracts from *E. coli* (Fig. 1a, lane 3). No cross-reactivity was detected when the same samples were incubated with the mouse preimmune serum (Fig. 1a, lanes 5–8). A single protein species was detected in a two-dimensional Western blot assay of yeast cells, with a molecular mass of 45 kDa and a p*I* of 6.3 (Fig. 1b, panel 1). Again, no cross-reactivity was detected when incubated with the preimmune serum (Fig. 1b, panel 2).

Identification of a *P. brasiliensis* formamidase tetramer

Protein fractionation by gel electrophoresis was performed on the yeast cell protein extracts without heating the samples. This resulted in the detection of numerous small-molecular-weight components in addition to a 180-kDa protein species, as shown in Fig. 1c. The protein of 180 kDa was also detected by a Western blot using the polyclonal antiformamidase antibody (Fig. 1d, lane1). A protein species of 45 kDa was detected in the same protein extract when subjected to a high heat (95 °C) treatment (Fig. 1d, lane 2), suggesting that the 180-kDa protein could represent a tetramer of units of the 45-kDa formamidase.

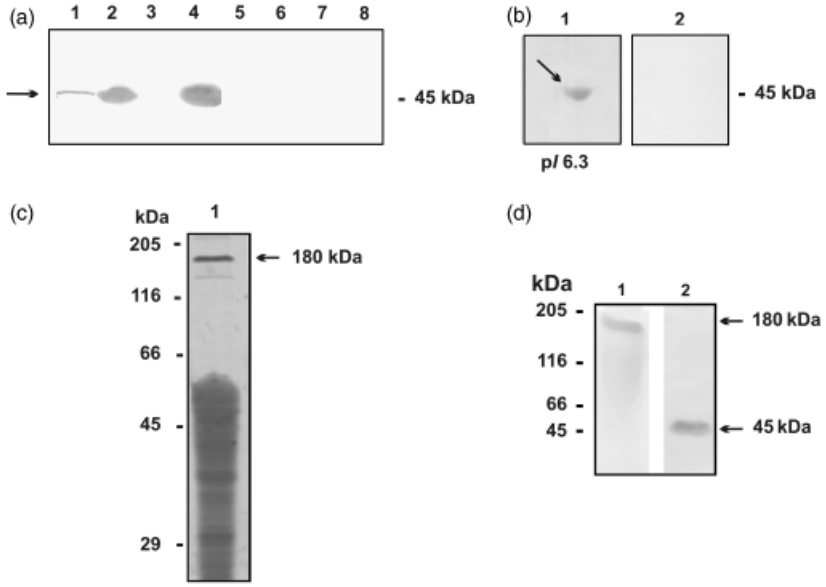

Fig. 1. Production and characterization of the polyclonal antibody anti-*Paracoccidioides brasiliensis* formamidase and fractionation of *P. brasiliensis* protein extracts. (a) Protein fractionation by one-dimensional gel electrophoresis and Western blot analysis. Protein extracts from yeast cells (20 μg, lanes 1 and 5), from mycelium (20 μg, lanes 2 and 6), total extracts of *Escherichia coli* XL1-blue cells (lanes 3 and 7) and the purified recombinant formamidase (5 μg, lanes 4 and 8) were fractionated and transferred to a membrane. Western blot analysis was performed with the antiformamidase antibody, 1 : 1000 diluted (lanes 1–4) or mouse preimmune serum, 1 : 1000 diluted (lanes 5–8). After reaction with the anti-mouse IgG alkaline phosphatase-coupled antibody (diluted 1 : 2000), the reaction was developed with 5-bromo-4-chloro-3-indolylphosphate/nitrobluetetrazolium. The molecular size is indicated. (b) Fractionation of *P. brasiliensis* protein extracts by two-dimensional gel electrophoresis and Western blot analysis. 1, Protein extracts from yeast cells (50 μg) after reaction with the polyclonal antiformamidase antibody. 2, The same extract as in 1 reacted with the preimmune serum. (c) *Paracoccidioides brasiliensis* total yeast cell protein extract nondenatured by heat. The proteins were stained by Coomassie blue R-250. (d) *Paracoccidioides brasiliensis* total yeast cell protein extracts, nondenatured (lane 1) and denatured by heat (lane 2), were fractionated and transferred to a membrane. Western blot analysis was performed as described above. Molecular sizes are indicated.

The purification of the 180-kDa protein reactive to the antiformamidase polyclonal antibody was performed using a combination of ion exchange and hydrophobic interaction chromatography (Supporting Information, Table S1). Using formamide as a substrate, the formamidase activity was monitored. The activity eluted as a single peak in two chromatographic steps. Formamidase was purified 24-fold, with a 41% yield (Table S1). The homogenous preparation is shown as a single protein species on SDS-PAGE (Fig. 2a and b). A phenyl sepharose-separated fraction was loaded onto an SDS-PAGE gel (Fig. 2a). A protein species of 180 kDa was observed in the nondenatured sample (Fig. 2a, lane 1), whereas a protein of 45 kDa was obtained in the same sample subjected to heat (Fig. 2a, lane 2). Both protein species reacted with the antibody antiformamidase (Fig. 2b). Taken together, the results support the hypothesis that the purified 180-kDa protein is a tetramer of four identical subunits of 45 kDa as the temperature disrupts subunit organization. The purified formamidase migrated as a protein of 191 kDa in native PAGE (Fig. 2c, lane 1), thus revealing that the enzyme forms a homotetrameric structure in its native condition. On the other hand, the heat-denatured sample migrated as a protein with 43.3 kDa, corresponding to the monomer of formamidase (Fig. 2c, lane 2).

To validate that the 180- and 45-kDa protein species were the same species (Fig. 2a, lanes 1 and 2, respectively), both bands were digested in-gel with trypsin. The resulting peptides were analyzed by MS. The mass spectra for the 180 and 45 kDa are shown in Fig. 2d and e, respectively. Experimental masses were searched against the theoretical digest of the predicted formamidase of *P. brasiliensis*. Ten peptides were matched to the 180 kDa (Table 1; Fig. 2d), while the same 10 peptides were also matched for the 45-kDa species (Fig. 2e, Table 1). The mass spectrum for the 45-kDa species matched with all the MS peaks detected for the 180-kDa species. These data provide additional support that the 45- and 180-kDa species represent, respectively, monomers and tetramers of the formamidase of *P. brasiliensis*.

Cellular localization of the *P. brasiliensis* formamidase

We explored the cellular localization of the formamidase using the polyclonal antibody antiformamidase in combination with

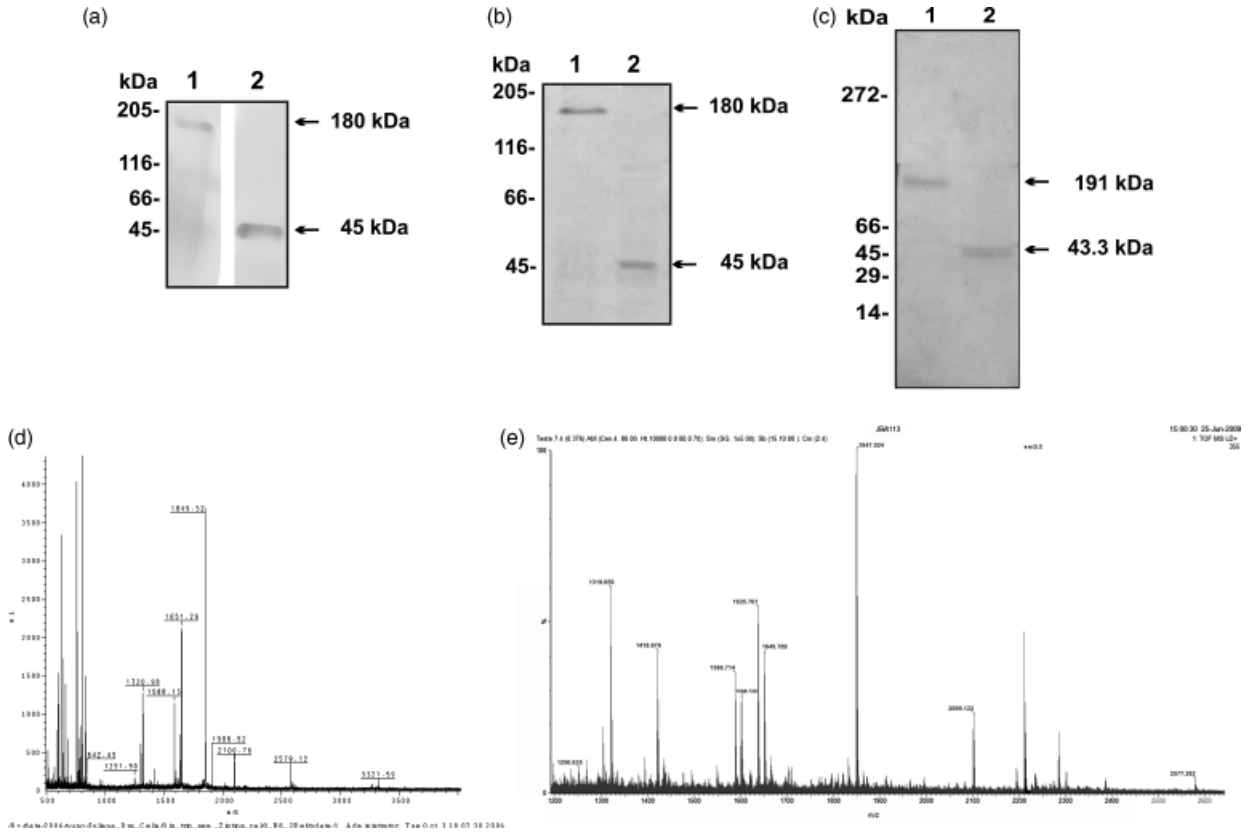

Fig. 2. Purification and characterization of the *Paracoccidioides brasiliensis* formamidase. (a) SDS-PAGE (12%) of phenyl sepharose fraction nondenatured (lane 1) or denatured by heat (lane 2). The proteins on the gel were stained by Coomassie blue R-250. (b) Reactivity of the protein fraction from phenyl sepharose to the polyclonal antibody, as determined by Western blot analysis. The phenyl sepharose eluted fraction nondenatured (lane 1) or denatured by heat (lane 2) was fractionated, transferred to a membrane and reacted to the antiformamidase antibody. (c) Native PAGE (6%) of phenyl sepharose fraction nondenatured (lane 1) or denatured by heat (lane 2). The proteins on the gel were stained by Coomassie blue R-250. (d, e) Peptide mass fingerprinting of trypsin digested 180- and 45-kDa protein species, respectively.

immuno-electron microscopy. Yeast cells were processed by postembedding with gold particles. Formamidase was detected in the cytoplasm and the cell wall (Fig. 3a and b). The control sample exposed to the preimmune serum was free of the label (Fig. 3c). The quantification of gold particles was performed in three independent preparations of yeast cells. Gold particles were counted for the total cell distribution, as well as for the cytoplasm and the cell wall separately. A similar number of particles were detected in the cytoplasm and the cell wall (data not shown).

Detection of proteins interacting with *P. brasiliensis* formamidase

To identify proteins interacting with the *P. brasiliensis* formamidase, the yeast two-hybrid assay was used to screen for protein interactions in a *P. brasiliensis* cDNA library. The cDNA library was constructed from RNAs obtained from *P. brasiliensis* yeast cells. The positive mating clones were subjected to PCR, and the products were subjected to DNA sequencing. Eight positive cDNAs were obtained. The positive cDNAs encoded proteins that were putative *P. brasiliensis* formamidase-interacting molecules, as summarized in the Table 2. Some cDNAs were redundant such as those encoding for homologues of polyubiquitin (Ubq10), FKBP-type peptidyl–prolyl isomerase (Fkbp) and protein kinase C (PkC). In addition, cDNAs encoding homologues to ribosomal protein Rps4, 2-oxoglutarate dehydrogenase E1subunit, calnexin (Cne1), cysteine protease (Atg4) and cell wall protein glycosyl hydrolase (Dfg5 like) were detected. Figure 4 shows the products of *dfg5*, *ubq10* and *fkbp*, confirmed by the coimmunoprecipitation assays. The formamidase is depicted as a 45-kDa protein species; the protein species observed to interact with formamidase were as follows: Dfg5-like, 16 kDa (Fig. 4, lane 1); Ubq10, 18 kDa (Fig. 4, lane 2); and Fkbp, 20 kDa (Fig. 4, lane 3). Negative controls were performed using the c-myc antibody with *in vitro* synthesized proteins Dfg5-like, Ubq10 and Fkbp. This confirmed the specific binding of c-myc antibody with formamidase (Fig. 4, lanes 4–6).

Table 1. Identification of *Paracoccidioides brasiliensis* formamidase by peptide mass fingerprinting

Tryptic peptide mass (Da)			
Experimental data (in-gel digestion)*	Experimental data (in-gel digestion)†	Expected data (*in silico* digestion)	Identified amino acid sequence
1251.204	1250.635	1250.297	^{12}VDLHKPASEQK^{22}
1420.000	1418.676	1418.993	^{46}IECLDWTGGQIK^{57}
1600.123	1598.728	1599.116	^{116}NGGGFLDEFYPNAAK^{130}
1588.132	1586.714	1587.125	^{131}AIWDFEGIFCSSR^{143}
2579.118	2577.282	2578.110	^{150}FAGLIHPGILGCAPSAEVLAEWNR^{173}
2100.763	2099.122	2099.755	^{188}VVAKPPEPINVHAGSASDAIKA^{208}
1651.256	1649.789	1650.248	^{218}TIPGRPEHGGNCDIK^{232}
1849.518	1847.924	1848.511	^{295}SPIFHPGPVEPQFSPGR^{311}
1637.213	1635.761	1636.206	^{312}YLTFEGFSVDHHGK^{325}
1320.977	1319.659	1319.969	^{326}QHFLDATVAYR^{336}

*The 180-kDa and †the 45-kDa protein species were digested with trypsin, and masses of resulting peptides were determined by MS and compared with the theoretical ones produced by *in silico* digestion of proteins found in the SwissProt databases (http://expasy.org/sprot/). Experimental mass was obtained with an accuracy of 0.1– 0.2 Da.

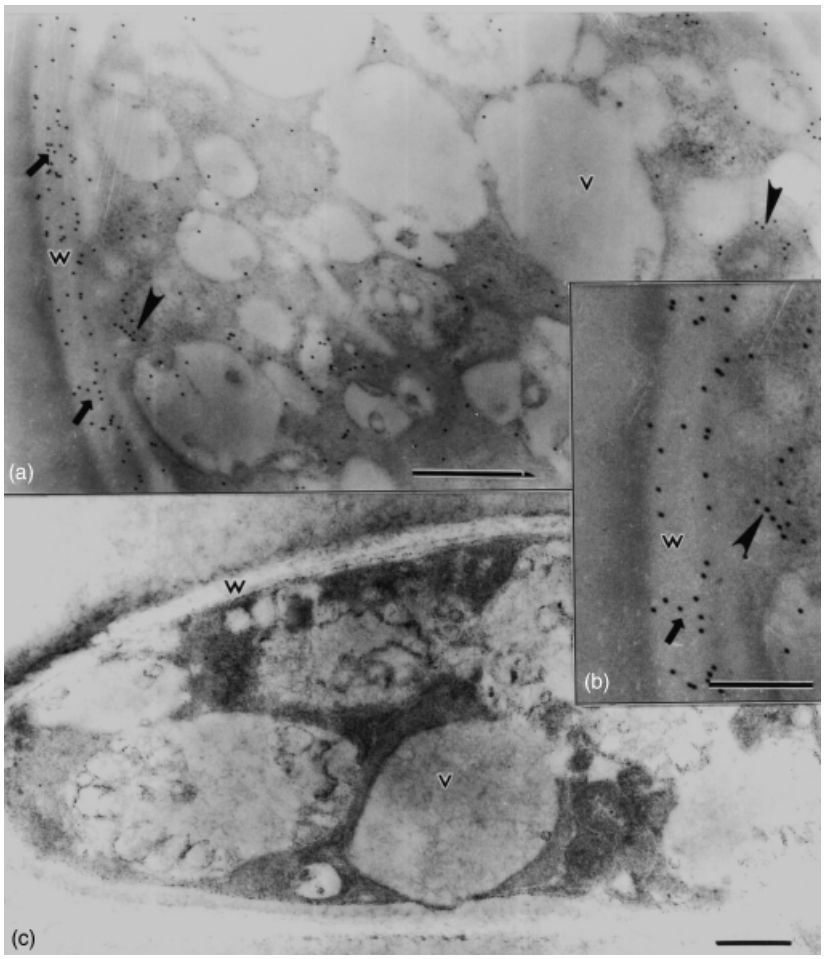

Fig. 3. Immunoelectron microscopy detection of the formamidase in *Paracoccidioides brasiliensis* yeast cells using the postembedding method. (a) Transmission electron microscopy of yeast cells. The arrowheads indicate gold particles. (b) Magnification of the cell wall/membrane. (c) Negative control is exposed to the preimmune serum and is free of label. Scale bars = 0.5 μm (a and b) and 0.2 μm (c). w, cell wall; v, cytoplasmic vacuole.

Discussion

Nitrogen metabolism is required for microorganisms to survive. Virtually nothing is known regarding *P. brasiliensis* formamidase, which belongs to the amidase/nitrilase family. Formamidase is suspected to play a role in nitrogen metabolism in other organisms. The homodimeric structure was first reported by Wyborn *et al.* (1994), it is now generally

Table 2. cDNAs identified in yeast two-hybrid assays for which the cognate proteins putatively interact with the *Paracoccidioides brasiliensis* formamidase

Gene product	Best BLAST hit/accession number*	Redundancy
Polyubiquitin (Ubq10)	*Neurospora crassa*/XP_958803.1	3
FKBP-type peptidyl–prolyl isomerase (Fkbp)	*Aspergillus clavatus*/XP_001274819	5
Protein kinase C (PkC)	*Aspergillus fumigatus*/XP_753454.1	5
40S ribosomal protein S4 (Rps4)	*Ajellomyces capsulatus*/XP_001537815	2
2-Oxoglutarate dehydrogenase E1 component, mitochondrial precursor	*Ajellomyces capsulatus*/XP_001544488	2
Calnexin (Cne1)	*Paracoccidioides brasiliensis*/ABB80132.1	1
Cysteine protease (atg4)	*Coccidioides immitis*/XP_001248363	1
Cell wall glycosyl hydrolase Dfg5 (Dfg5)	*Paracoccidioides brasiliensis*/DQ534495	1

*GenBank accession numbers (http://www.ncbi.nlm.nih.gov).

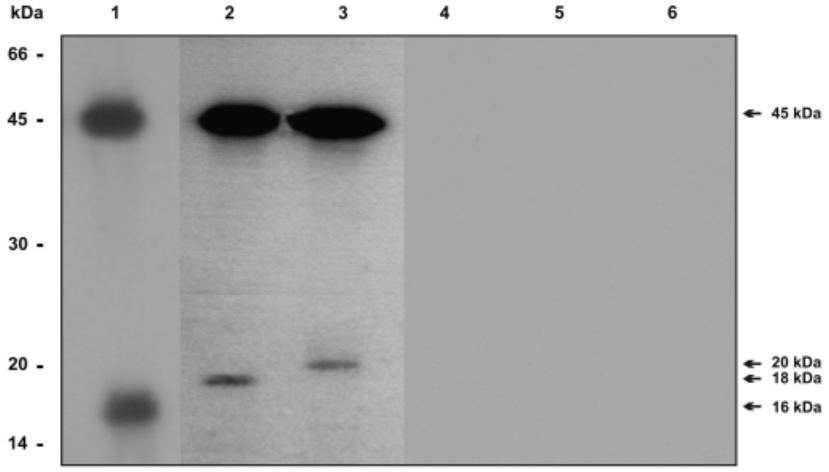

Fig. 4. Coimmunoprecipitation of *Paracoccidioides brasiliensis* proteins putatively interacting with *P. brasiliensis* formamidase. The proteins were *in vitro* synthesized and labeled with ^{35}S-methionine. The translated formamidase fused to the c-myc epitope (c-myc-FMD) and the translated proteins fused to the hemagglutinin epitope (HA-Prey) were mixed and the mixture was incubated with protein A-agarose beads and the monoclonal c-myc. The proteins were separated by SDS-PAGE. The gel was fixed, dried under vacuum and autoradiography was obtained. Dfg5-like protein (lane 1), Ubq10 (lane 2) and Fkbp protein (lane 3). Negative controls were performed; lanes 4–6, the same proteins as in 1–3.

accepted that the active *Methylophilus methylotrophus* formamidase is a homotrimer in solution (Wyborn *et al.*, 1996). A homohexameric structure was reported for the *Helicobacter pylori* crystallized formamidase (Hung *et al.*, 2007). The data presented here suggest that *P. brasiliensis* formamidase is arranged in a tetrameric structure. Western blot analysis under reducing conditions revealed a 180-kDa protein species in the *P. brasiliensis* yeast cells. Furthermore, heat-denatured protein samples revealed a protein species with 45 kDa, as demonstrated previously (Borges *et al.*, 2005). In order to better characterize both protein species reactive with the polyclonal antibody antiformamidase, we performed a combination of ion exchange and hydrophobic interaction chromatography. After two steps of purification, an almost homogenous enzyme preparation was observed with a 24-fold increase in specific activity. The data suggest that the formamidase could represent an important proportion of the total protein in *P. brasiliensis* cells. Accordingly, transcriptional analysis evidenced that the expressed sequence tags encoding formamidase were highly abundant in fungal cells (Felipe *et al.*, 2005). This purification procedure yielded a homogeneous preparation of the formamidase enzyme with molecular masses of 45 and 180 kDa, as determined in SDS-PAGE. Tryptic digestion of both protein species provided identical MS spectra, strongly supporting the suggestion that the 180-kDa formamidase is a tetramer comprised of four identical monomers. The purified formamidase migrated as a protein of 191 kDa in native PAGE, thus also confirming the enzyme tetrameric structure in its native state.

Immunocytochemical analysis showed that the subcellular localization of the *P. brasiliensis* formamidase is the cytoplasm and the cell wall. The localization of some classic cytoplasmatic molecules lacking an N-terminal signal peptide in other cellular compartments is not uncommon, as described by our laboratory for *P. brasiliensis* glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (Barbosa *et al.*, 2006) and triosephosphate isomerase (TPI) (Pereira *et al.*, 2007). Molecules that lack an N-terminal signal peptide sequence have been described in the cell wall of *S. cerevisiae*, in addition to their usual cytoplasmatic localization (Nombela *et al.*, 2006). Cytoplasmic proteins GAPDH, TPI and formamidase have recently been detected in extracellular vesicles secreted by *Histoplasma capsulatum* (Albuquerque *et al.*, 2008) and *Cryptococcus neoformans* (Rodrigues *et al.*, 2008). The data presented here for other fungal species

support our experimental results of the detection of an enzyme located in the cytoplasm and in the cell wall.

In addition to discovering that formamidase was localized to the cell wall, it was observed that formamidase interacted with cell wall proteins such as Dfg5 in addition to several proteins involved in folding and processing such as Cne1, Fkbp and Ubq10. We hypothesize that the cell wall protein Dfg5 (Castro *et al.*, 2008; present work) may interact with formamidase at the fungal surface, where both are located. Furthermore, Fkbp, Rps4 and 2-oxoglutarate dehydrogenase, which we observed to interact with *P. brasiliensis* formamidase, were also detected in the extracellular vesicles secreted by *H. capsulatum* (Albuquerque *et al.*, 2008). The formamidase-interacting protein Cne1 was also found in the extracellular vesicles secreted by *C. neoformans* (Rodrigues *et al.*, 2008). In summary, these interactions are most likely linked to the localization of *P. brasiliensis* formamidase in the cell wall of the fungus.

The localization of *P. brasiliensis* formamidase in the cell wall could contribute to *P. brasiliensis* antigenic properties. Cell wall localization of formamidase could also play a putative role of in nitrogen metabolism as this enzyme produces ammonia, as described for *H. pylori* (van Vliet *et al.*, 2003). *Helicobacter pylori* has evolved strategies to respond to acidic growth conditions, including the overexpression of the gene encoding formamidase (Bury-Moné *et al.*, 2004). We could speculate that ammonia may be involved in tissue damage and in acid resistance to host tissues, as well as being a source for nitrogen assimilation in *P. brasiliensis*. The interaction of formamidase with a subunit of 2-oxoglutarate dehydrogenase that we detected in this study could reinforce its role in the fungal metabolism of nitrogen.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Purification of the formamidase of *Paracoccidioides brasiliensis*.

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