High affinity binding of albicidin phytotoxins by the AlbA protein from *Klebsiella oxytoca*

Lianhui Zhang, Jinling Xu and Robert G. Birch

Author for correspondence: Robert G. Birch. Tel: +61 7 3365 3347. Fax: +61 7 3365 1699.
e-mail: r.birch@botany.uq.edu.au

Department of Botany,
The University of
Queensland, Brisbane QLD
4072, Australia

Albicidins are a family of phytotoxins and antibiotics which play an important role in the pathogenesis of sugarcane leaf scald disease. The *albA* gene from *Klebsiella oxytoca* encodes a protein which inactivates albicidin by heat-reversible binding. Albicidin ligand binding to a recombinant AlbA protein, purified by means of a glutathione *S*-transferase gene fusion system, is an almost instant and saturable reaction. Kinetic and stoichiometric analysis of the binding reaction indicated the presence of a single high affinity binding site with a dissociation constant of 6.4×10^{-8} M. The AlbA–albicidin complex is stable from 4 to 40 °C, from pH 5 to 9 and in high salt solutions. Treatment with protein denaturants released all bound albicidin. These properties indicate that AlbA may be a useful affinity matrix for selective purification of albicidin antibiotics. AlbA does not bind to *p*-nitrophenyl butyrate or α -naphthyl butyrate, the substrates of the albicidin detoxification enzyme AlbD from *Pantoea dispersa*. The potential exists to pyramid genes for different mechanisms in transgenic plants to protect plastid DNA replication from inhibition by albicidins.

Keywords: albicidin inactivation, albicidin binding protein, phytotoxin and disease resistance, affinity matrix, binding kinetics

INTRODUCTION

Albicidins, a family of phytotoxins and antibiotics produced by the phytopathogen *Xanthomonas albilineans*, appear to play an important role in systemic invasion and symptom development in sugarcane leaf scald disease (Birch & Patil, 1987a; Zhang & Birch, 1997). Albicidins are bactericidal at nanomolar concentrations against a range of Gram-positive and Gram-negative bacteria (Birch & Patil, 1985b). Inhibition of prokaryote DNA replication is the primary mechanism of action (Birch & Patil, 1985a, 1987b). The major antibacterial component (albicidin) has been partially characterized as a compound with three to four aromatic rings and a molecular mass of 842 Da (Birch & Patil, 1985b).

The albicidin resistance gene, *albA*, cloned from *Klebsiella oxytoca*, encodes a 25.8 kDa basic protein (isoelectric point 10.92) of 206 aa, which inactivates albicidin by binding (Walker *et al.*, 1988). A second basic albicidin-binding protein (isoelectric point 10.86) is encoded by the *albB* gene cloned from *Alcaligenes*

denitrificans. There is no significant homology between the two proteins except at the N terminus (56% identity and 100% similarity over 16 aa) which could be the functional albicidin-binding domain (Basnayake & Birch, 1995). Binding of albicidin to both proteins is reversible by heat denaturation, but the albicidin binding mechanism is unknown. Here we report the purification of a recombinant AlbA protein, kinetic analysis and the stability of albicidin binding. The results indicate potential applications of AlbA in engineering disease resistance and in the purification of albicidins.

METHODS

Bacteria and antibiotics. Bacteria, growth conditions, albicidin purification and quantification were as described previously (Zhang & Birch, 1997). The single peak of albicidin after HPLC was used in kinetics and stoichiometry studies. For other experiments, the mixture of albicidins obtained after HW-40(S) chromatography was used.

Construction of a glutathione *S*-transferase (GST)–AlbA fusion protein expression plasmid and purification of AlbA. The *albA* coding region was amplified using PCR. The forward primer was 5' CTC GGA TCC ATG AAA ATG TAC GAT CGC TG 3' and reverse primer was 5' ATC GAG CTC

Abbreviation: GST, glutathione *S*-transferase

TGA GCT TCT ACC CGG ACC 3'. The primers have a *Bam*HI site and a *Sac*I site at their 5' ends, respectively. Plasmid clone pJM1076 (Walker *et al.*, 1988) was used as template. The amplified PCR product of 706 bp was digested by *Sac*I, blunt-ended by Klenow fragment treatment, then digested with *Bam*HI before cloning into the *Bam*HI/*Sma*I-linearized GST fusion vector pGEX-2T (Smith & Johnson, 1988). The resultant construct, pGSTK54H, contains the chimaeric *albA* gene fused in-frame to the GST gene under the control of the IPTG-inducible *tac* promoter.

Escherichia coli DH5 α (pGSTK54H) was cultured and induced by IPTG. The expression of fusion protein was detected by monitoring GST activity and AlbA was purified according to the manufacturer's instructions (Pharmacia) with some modifications described in Results. Briefly, the bacterial culture was pelleted by centrifugation; cell extracts were prepared by ultrasonication and applied to a glutathione Sepharose 4B affinity column. The GST–AlbA fusion protein was bound to the affinity column matrix and AlbA was separated from GST by digestion with the protease thrombin for 16 h at room temperature. Following digestion, the eluate containing pure AlbA was collected and analysed by SDS-PAGE. Purified AlbA, dissolved in PBS containing 20% (v/v) glycerol, lost 20% of its albicidin binding activity after 1 week at -20°C , so it was freeze-dried and kept at -20°C . Protein concentrations were determined by the dye-binding method (Bradford, 1976) using bovine serum albumin for calibration.

The glutathione Sepharose 4B affinity column was regenerated by washing thrice with 2.5 bed vols of alternating high pH (0.1 M Tris/HCl + 0.5 M NaCl, pH 8.3) and low pH (0.1 M sodium acetate + 0.5 M NaCl, pH 5.3) buffers, followed by re-equilibration with 10 bed vols of PBS. We found under these conditions the column can be regenerated and reused at least seven times without noticeable loss of binding capacity.

Binding assay. For kinetic studies, AlbA was dissolved in TMM buffer (10 mM Tris/HCl, pH 7.0, 10 mM MgCl₂, 2 mM 2-mercaptoethanol) and albicidin was dissolved in TMM buffer containing 5% (v/v) methanol. Incubation mixtures of 100 μl , containing 2 μg AlbA (or 4 μg GST–AlbA fusion protein) and 1.25–100 ng albicidin were incubated at 25°C for 10 min before a quantitative assay of free albicidin. Bound albicidin was calculated by subtracting the amount of free albicidin from the amount of albicidin added.

The effects of pH, temperature and other chemicals on the binding activity of AlbA were determined in incubation mixtures containing 30 ng albicidin. The effect of pH was tested in buffers ranging from pH 2.2 to 8.0 (prepared with different proportions of 0.2 M sodium phosphate and 0.1 M citric acid) and pH 9.0 (0.2 M Tris/HCl). Chemicals prepared in TMM buffer or other buffers, as indicated, were pre-incubated with AlbA for 10 min before adding albicidin. To test stability of the AlbA–albicidin complex, the pH of the mixture was adjusted using either 0.2 M sodium phosphate/0.1 M citric acid or 0.2 M Tris/HCl buffer and incubation was continued at 25°C for 30 min before bioassay. The same conditions were used to test the effects of different chemicals on the stability of the AlbA–albicidin complex. To determine temperature stability, the mixture was incubated at different temperatures for 30 min before assay.

Gel filtration experiments. To study binding speed, 5 μg AlbA was mixed for 20 s with 30, 80 or 120 ng albicidin in a final volume of 50 μl , and the mixture was added immediately to a Bio-Spin 30 chromatography column (Bio-Rad) and centrifuged at 1100 *g* for 2 min at room temperature. The eluted solution (approximately 50 μl) was mixed with 450 μl 1%

(w/v) SDS in water to denature AlbA. The solution was centrifuged at 12000 *g* for 5 min and the amount of albicidin in the solution was determined. The protein content was determined by the dye-binding method before dilution with 1% SDS.

This gel filtration technique was also used to test whether AlbA could bind esterase substrates *p*-nitrophenyl butyrate and α -naphthyl butyrate. Mixtures containing 500 μM esterase substrate and 5, 30 or 75 μg AlbA were incubated at room temperature for 10 min before adding to Bio-Spin 30 columns for centrifugation. The eluted solution was added to 400 μl 1% SDS in water and incubated for 5 min to denature the protein. Incubation was continued for 5 min following the addition of 10 μl 2 M NaOH to hydrolyse esters. Hydrolysed *p*-nitrophenol was measured at 410 nm, and α -naphthol was determined by the diazonium salt method (Bardi *et al.*, 1993).

RESULTS

Optimization of expression of AlbA in *E. coli*

Upon addition of IPTG to the culture medium, strain DH5 α (pGSTAlbA) expressed a fusion protein with a predicted molecular mass of 52 kDa, from which the recombinant AlbA protein with four extra amino acids at the N terminus was released by incubation with thrombin (Fig. 1). GST activity is tolerant to C-terminal fusion and was monitored to optimize conditions for production of the GST–AlbA fusion protein. In cultures grown at 37°C , GST activity peaked 4–5 h after IPTG induction (Fig. 2a). It has been reported that heat-shock protein and proteases induced at 37°C cause degradation of proteins with abnormal conformations in *E.*

Fig. 1. (a) Map of the GST–AlbA fusion protein expression plasmid pGSTAlbA. (b) DNA and protein sequences in the fusion region of GST and AlbA. The start codon (ATG) in the native *albA* gene is underlined and the arrow indicates the cleavage site of the site-specific protease thrombin.

Fig. 2. (a) Production and stability of the GST-AlbA fusion protein, indicated by GST activity in crude cell extracts from IPTG-induced cultures grown at 30 °C (■) or 37 °C (●). (b) Concentration dependence of albicidin binding by AlbA. (c) Scatchard plot of the binding reaction between albicidin and AlbA. Purified AlbA (2 μg) was incubated with various amounts of albicidin. (d–g) Effect of pH (d), temperature (e), methanol (f) and SDS (g) on albicidin binding by AlbA.

coli (Sherman & Goldberg, 1992). At 30 °C GST activity was maximal 12 h after adding IPTG and was stable until at least 16 h. The yield of fusion protein at 30 °C was about 15% higher than that at 37 °C (Fig. 2a). The yield of recombinant AlbA protein was about 50 mg from an overnight 5 l bacterial culture.

AlbA purified using the routine GST affinity procedure was contaminated with about 10% of other proteins ranging from 20 to 80 kDa in size, as judged by band intensities on SDS-PAGE gels stained with Coomassie brilliant blue R-250. These contaminants were eliminated by centrifuging the sonified cell extracts twice at

12000 *g* for 20 min at 4 °C, and by washing the loaded glutathione Sepharose 4B column with 20 bed vols of 1×PBS before adding thrombin solution (Fig. 3). The purity of AlbA was estimated to be more than 99.5% because Coomassie brilliant blue R-250 staining can reveal bands containing as little as 0.1 μg protein.

Stoichiometry, kinetics and specificity of binding

The binding of albicidin to AlbA is a saturable process (Fig. 2b). Analysis of the binding data by the Scatchard plot method (Scatchard, 1949) indicated a single high affinity binding site per AlbA molecule with a dis-

Fig. 3. SDS-PAGE analysis of GST-AlbA fusion protein expression and purity of one-step glutathione Sepharose 4B affinity column purified proteins. Lanes: 1, protein molecular mass standards; 2, crude cell extract of *E. coli* DH5 α (pGSTAlbA) without IPTG induction; 3, crude cell extract of *E. coli* DH5 α (pGSTAlbA) with IPTG induction; 4, GST-AlbA fusion protein released from affinity column by glutathione; 5, purified AlbA released from affinity column by thrombin; 6, purified GST protein. The protein samples were run in a stacking (4.5 %)/resolving (12 %) SDS-PAGE gel.

sociation constant (K_d) of 6.4×10^{-8} M. At saturation, the maximum capacity of AlbA was $27.8 \text{ ng albicidin } (\mu\text{g AlbA})^{-1}$ (Fig. 2c). Based on a molecular mass of 842 Da for albicidin, it can be estimated that 0.85 mol albicidin was bound per mol AlbA. The purified GST-AlbA fusion protein showed similar strong affinity to albicidin (K_d 7.7×10^{-8} M).

Albicidin added to AlbA solution and immediately centrifuged through a size-exclusion spin column was quantitatively recovered as AlbA-albicidin complex, indicating that the binding reaction was probably completed within 30 s at room temperature. Under the same conditions, there was no detectable binding of the esterase substrates *p*-nitrophenyl butyrate or α -naphthyl butyrate to AlbA.

Effect of pH and temperature on binding and stability of the AlbA-albicidin complex

The maximal binding capacity of AlbA was maintained from pH 6 to 9, with a sharp decrease from pH 5 to 4 and a second plateau to pH 2 (Fig. 2d). The AlbA-albicidin complex was stable at temperatures from 4 to 40 °C, but all bound albicidin was released within 10 min incubation at temperatures above 65 °C (Fig. 2e). Very similar results were obtained when the pH and temperature treatments were applied during or after binding.

Effect of chemical reagents on stability of the AlbA-albicidin complex

Sodium phosphate buffer did not interfere with albicidin binding by AlbA, in contrast to AlbB (Basnayake & Birch, 1995). The AlbA-albicidin complex was stable in solutions of Tris/HCl, NaCl and potassium phosphate

Table 1. Effect of different solutions and chemicals on the stability of the AlbA-albicidin binding complex

Treatment	Free albicidin (%) [*]
Water	0
Sodium acetate (0.1 M, pH 5.2)	0
Sodium acetate (0.3 M, pH 5.2)	20
Tris/HCl (0.5 M, pH 7.4)	0
Tris/HCl (0.5 M, pH 8.3)	0
NaCl (2.5 M)	0
Sodium phosphate (0.45 M, pH 7.5)	0
Methanol (80 %)	100
SDS (1 %)	100
Urea (5.5 M)	0
Guanidinium thiocyanate (2 M)	99

^{*}Albicidin released within 30 min at 25 °C after treatment of the AlbA-albicidin complex with the indicated solution.

up to at least 0.45 M (Table 1). Some albicidin was released by 0.3 M, but not by 0.1 M sodium acetate (pH 5.2). Methanol (70 %), guanidinium thiocyanate (2 M) or SDS (0.2 %) released almost all albicidins, but urea, another protein denaturant, had no effect at concentrations up to 5.5 M. More detailed study showed substantial release of albicidin at concentrations as low as 30 % methanol and 0.01 % SDS (Figs 2f and g).

DISCUSSION

Albicidin binds rapidly (<30 s) to a single high affinity binding site (K_d 6.4×10^{-8} M) on AlbA, the albicidin binding protein from *K. oxytoca*. pH dependency indicates that binding could be due in part to electrostatic interactions. The sharp increase in albicidin binding from pH 4 to 5 is in the pK_a range of a histidine side chain. Histidine residues are involved in substrate binding to the dihydrofolate reductase from *Lactobacillus casei* (Matthews *et al.*, 1979) and in isocitrate lyase from *Phycomyces blakesleeianus* (Rua *et al.*, 1995). There is no histidine residue in the conserved N terminus of AlbA and AlbB binding proteins (Basnayake & Birch, 1995). There are six histidine residues in AlbA (Walker *et al.*, 1988). Among them H-66, H-124 and H-140 are completely buried in α -helix with very poor solvent accessibility as predicted by the PHDacc program (Rost & Sander, 1994). The H-64, H-77 and H-182 residues have a moderate level of solvent accessibility and therefore are more likely to be involved in albicidin binding, especially H-182 which is surrounded by a stretch of 14 hydrophobic amino acid residues located in a loop region. Albicidin shows little water solubility but can be easily dissolved in some organic solvents (Birch & Patil, 1985a), so a hydrophobic region in AlbA may contribute to albicidin binding.

Albicidin enters *E. coli* via the Tsx nucleoside uptake pore (Birch *et al.*, 1990), but nucleosides and nucleotides

did not delay inactivation of albicidin by competition for the binding site in AlbA (Walker *et al.*, 1988). The albicidin detoxification enzyme, AlbD, from *Pantoea dispersa* has structural features similar to known esterases and shows strong esterase activity toward substrates such as *p*-nitrophenol butyrate and α -naphthyl butyrate (Zhang & Birch, 1997), but AlbA does not bind to these esterase substrates. Thus Tsx, AlbA and AlbD appear to recognize different structural features of albicidin.

The high affinity, speed, stability and specificity of albicidin binding by AlbA support the potential of *albA* as a novel phytotoxin and disease resistance gene. It has been estimated that the albicidin concentration in tissues surrounding invaded xylem could be up to 500 ng g⁻¹ (Birch & Patil, 1987b), requiring at least 15 μ g AlbA g⁻¹ in transgenic plant tissues for total inactivation of albicidins. This is within the range of minor cellular proteins and novel proteins expressed in transgenic plants, and is not expected to impose a serious metabolic load. The stability of the AlbA–albicidin complex in plant cells is unknown, but in bacterial cells there does not appear to be any turnover resulting in restoration of antibiotic activity. An efficient genetic transformation system exists for sugarcane (Bower *et al.*, 1996). The characterization of AlbA and AlbD proteins, with different binding specificities and inactivation mechanisms against albicidin, indicates the possibility to pyramid these genes to confer increased albicidin phytotoxin and leaf scald disease resistance in transgenic sugarcane plants.

Rapid, high affinity, specific binding of albicidins, which is reversible by 70% methanol or 0.2% SDS, also indicates potential for use of AlbA in affinity chromatography to purify albicidin antibiotics. The optimized procedures for fusion protein expression and purification of AlbA described here allow large-scale production of highly pure AlbA, which may in turn simplify the purification of albicidins.

ACKNOWLEDGEMENTS

This project is partially supported by ARC grants, and fellowships from The University of Queensland and ARC to L.Z.

REFERENCES

- Bardi, L., Dell'Oro, V. & Delfini, C. (1993). A rapid spectrophotometric method to determine esterase activity of yeast cells in an aqueous medium. *J Inst Brew* **99**, 385–388.
- Basnayake, W. V. S. & Birch, R. G. (1995). A gene from *Alcaligenes denitrificans* that confers albicidin resistance by reversible antibiotic binding. *Microbiology* **141**, 551–560.
- Birch, R. G. & Patil, S. S. (1985a). Preliminary characterisation of an antibiotic produced by *Xanthomonas albilineans* which inhibits DNA synthesis in *Escherichia coli*. *J Gen Microbiol* **131**, 1069–1075.
- Birch, R. G. & Patil, S. S. (1985b). Antibiotic and process for the production thereof. USA Patent 4525354, 25 June 1985.
- Birch, R. G. & Patil, S. S. (1987a). Correlation between albicidin production and chlorosis induction by *Xanthomonas albilineans*, the sugar cane leaf scald pathogen. *Physiol Mol Plant Pathol* **30**, 199–206.
- Birch, R. G. & Patil, S. S. (1987b). Evidence that an albicidin-like phytotoxin induces chlorosis in sugar cane leaf scald disease by blocking plastid DNA replication. *Physiol Mol Plant Pathol* **30**, 207–214.
- Birch, R. G., Pemberton, J. M. & Basnayake, W. V. S. (1990). Stable albicidin resistance in *Escherichia coli* involves an altered outer membrane nucleoside uptake system. *J Gen Microbiol* **136**, 51–58.
- Bower, R., Elliott, A. R., Potier, B. A. M. & Birch, R. G. (1996). High-efficiency, microprojectile-mediated co-transformation of sugarcane, using visible or selectable markers. *Mol Breed* **2**, 239–249.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein using the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
- Matthews, D. A., Alden, R. A., Freer, S. T., Xuong, N.-H. & Kraut, J. (1979). Dihydrofolate reductase from *Lactobacillus casei*, stereochemistry of NADPH binding. *J Biol Chem* **254**, 4144–4151.
- Rost, B. & Sander, C. (1994). Conservation and prediction of solvent accessibility in protein families. *Proteins* **20**, 216–226.
- Rua, J., Soler, J., Busto, F. & De Arriaga, D. (1995). The pH dependence and modification by diethyl pyrocarbonate of isocitrate lyase from *Phycomyces blakesleeanus*. *Eur J Biochem* **232**, 381–390.
- Scatchard, G. (1949). The attractions of proteins for small molecules and ions. *Ann N Y Acad Sci* **51**, 660–672.
- Sherman, M. Y. & Goldberg, A. L. (1992). Involvement of the chaperonin DnaK in the rapid degradation of a mutant protein in *Escherichia coli*. *EMBO J* **11**, 71–77.
- Smith, D. B. & Johnson, K. S. (1988). Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* **67**, 31–40.
- Walker, M. J., Birch, R. G. & Pemberton, J. M. (1988). Cloning and characterization of an albicidin resistance gene from *Klebsiella oxytoca*. *Mol Microbiol* **2**, 443–454.
- Zhang, L. & Birch, R. G. (1997). The gene for albicidin detoxification from *Pantoea dispersa* encodes an esterase which attenuates pathogenicity of *Xanthomonas albilineans* to sugarcane. *Proc Natl Acad Sci USA* **94**, 9984–9989.

Received 19 June 1997; accepted 16 September 1997.