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The cytochrome bc_1 complex inhibitor Ametoctradin has an unusual binding mode

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ABSTRACT

Ametoctradin is an agricultural fungicide that selectively inhibits the cytochrome bc_1 complex of oomycetes. Previous spectrophotometric studies using the purified cytochrome bc_1 complex from *Pythium* sp. showed that Ametoctradin binds to the Q_o-site of the enzyme. However, as modeling studies suggested a binding mode like that of the substrate ubiquinol, the possibility for a dual Q_o- and Qi-site binding mode was left open.

In this work, binding studies and enzyme assays with mitochondrial membrane preparations from *Pythium* sp. and an *S. cerevisiae* strain with a modified Q_i-site were used to investigate further the binding mode of Ametoctradin. The results obtained argue that the compound could bind to both the Q_o- and Qi-sites of the cytochrome bc_1 complex and that its position or binding pose in the Q_i-site differs from that of Cyazofamid and Amisulbrom, the two Q_i-site-targeting, anti-oomycetes compounds. Furthermore, the data support the argument that Ametoctradin prefers binding to the reduced cytochrome bc_1 complex. Thus, Ametoctradin has an unusual binding mode and further studies with this compound may offer the opportunity to better understand the catalytic cycle of the cytochrome bc_1 complex.

1. Introduction

Ametoctradin is an agricultural antifungal compound by BASF Crop Protection. It is commercially available under its brand name Initium®. Ametoctradin is highly effective against late blight and downy mildews in a wide range of specialty crops [1]. On the molecular level, Ametoctradin is an inhibitor of the respiratory chain bc_1 complex of oomycetes. Treatment of oomycete pathogens with Ametoctradin causes a rapid decrease of oxygen consumption and intracellular ATP levels. In consequence, it inhibits highly energy demanding processes like zoospore formation and release, zoosporangia release, germination and motility [1].

The bc_1 complex is a multimeric enzyme, with three subunits forming the catalytic core that contain the redox-active groups (hemes and [2Fe-2S]), i.e. cytochrome c_1, the iron-sulfur protein and the mitochondrially-encoded cytochrome *b*. It catalyzes the transfer of electrons from quinol to cytochrome *c* and the vectoral translocation of protons across the inner mitochondrial membrane. The complex has two distinct quinone-binding sites (Q_o, quinol oxidation site, and Q_i, quinone reduction site) that are formed by cytochrome *b*.

bc_1 complex inhibitors have been developed as treatment against most major crop pathogens. Unfortunately, soon after the introduction of this important class of agricultural fungicides, resistant isolates of several crop pathogens were reported [2]. Since then, resistant isolates of more than thirty different crop pathogens have emerged carrying various target-site mutations in their cytochrome *b* genes [3–6]. However, developments of resistant isolates and target site mutations as well as cross resistance depend on the binding site and the binding mode of the individual inhibitors. An in-depth understanding of a compound's binding to the bc_1 complex would help develop better strategies to prevent the evolution of resistance.

To elucidate the binding mode of inhibitors, crystallographic studies are the gold standard. However, this approach is unfeasible for oomycete-specific inhibitors such as Ametoctradin, because of the technical difficulties in isolating suitable bc_1 complex from oomycete species. Other approaches can be used, such as spectrophotometric binding studies that make use of the change in cytochrome spectra upon reduction and/or inhibitor binding.

A prior spectrophotometric study using purified bc_1 complex from *Pythium* sp. suggested that Ametoctradin binds to the Q_o-site in a manner that may be like the inhibitor Stigmatellin [7,8], and may resemble that of Ascochlorin and nonyl-4-hydroxyquinoline-N-oxide

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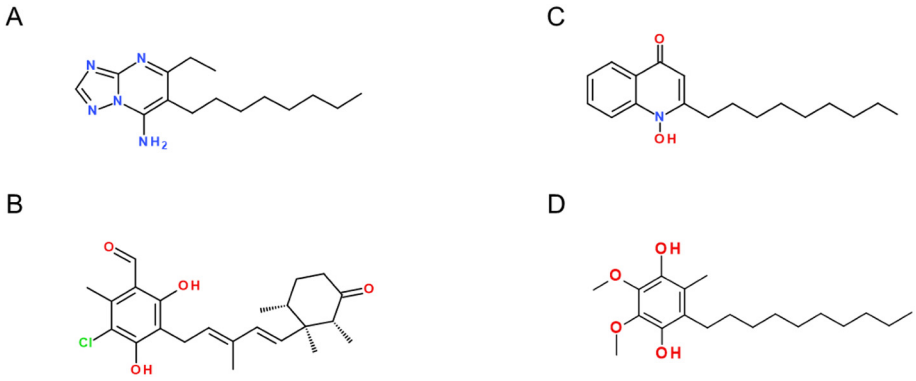

Fig. 1. Chemical structures of quinol analogs. A) Ametoctradin, B) Ascochlorin, C) Nonyl-4-hydroxyquinoline-*N*-oxide (NQNO) and D) Decylubiquinol.

(NQNO), two quinol analogs that can bind to both Q_o- and Q_i-sites [9,10]. Since the chemical structure of Ametoctradin (Fig. 1) and the modeling studies suggested a binding mode like ubiquinol, the possibility for a dual site binding mode of Ametoctradin was left open [7].

In this work, to further investigate the binding mode of Ametoctradin, spectrophotometric binding studies with the *Pythium* bc_1 complex in mitochondrial membrane preparations were carried out including classic double kill studies, in which combinations of inhibitors are used to analyze the binding site of novel inhibitors [9,11,12]. Mitochondrial membrane preparations confer an advantage over the purified complex for this approach. In studies with isolated bc_1 complex, re-oxidation of reduced cytochrome *b* by reversal of the bifurcated reaction is often observed [9,12], which may add complexity if time resolved analysis at early time points is unavailable [7]. By contrast, this re-oxidation of the bc_1 complex was not observed when mitochondrial membrane preparations were used [11] because cytochrome *c* oxidase keeps the high-potential chain oxidized and prevents the non-enzymatic reduction of cytochrome c_1, which is required for the reversal of the bifurcated reaction in the Q_o-site. In parallel, yeast (*S. cerevisiae*) mutants with a modified Q_i-site rendered sensitive to Ametoctradin and mutants with point mutation in the Q_o-site were analyzed. The data suggest that Ametoctradin has an unusual binding mode that is clearly different from those of the other oomycete-specific inhibitors Cyazofamid and Amisulbrom as well as from classic inhibitors such as Strobilurins (i.e. Azoxystrobin) and Antimycin A.

2. Experimental procedures

2.1. Decylubiquinol-cytochrome c oxidoreductase assay

Mitochondrial membranes were prepared as described below. Concentration of bc_1 complex in the mitochondrial samples was determined from dithionite-reduced minus oxidized (ferricyanide) optical spectra, using an extinction coefficient of 25.6 $mM^{-1} \times cm^{-1}$ for the average absorbance of the b_L and b_H hemes at 562 nm − 575 nm [13,14]. Mitochondrial membrane preparations were added to obtain a final concentration of ~6 nM bc_1 complex. Decylubiquinol-cytochrome *c* oxidoreductase activities were determined at room temperature by measuring the reduction of cytochrome *c* (final concentration of 50 μM) at 550 nm versus 540 nm over 4 minute time-course in 10 mM potassium phosphate pH 7, 0.01% (w/v) lauryl-maltoside and 1 mM KCN using an extinction coefficient of 21.1 $mM^{-1} \times cm^{-1}$ [15,16]. Measurements were initiated by the addition of decylubiquinol, a synthetic analog of ubiquinol (final concentration of 50 μM). Rates were determined from the linear phase of the reaction. The half-maximal inhibition concentrations (IC50) were determined by dose response measurements. Each experiment was repeated three times (with nine replicates for each measurement) and the values obtained were averaged and standard errors were calculated. An inhibition smaller 50% at 10 μM inhibitor concentration (highest concentration in the assay) was rated as no inhibition.

2.2. Yeast mutants

The cytochrome *b* mutants were generated by side-directed mutagenesis and mitochondrial transformation [17,18]. In all experiments, control and mutants have identical nuclear and mitochondrial genomes with the exception of the mutations introduced in the cytochrome *b* gene. Growth experiments were performed using strains derived from AD1-9. AD1-9, kindly provided by M. Ghislain, UCL, Belgium, lacks several membrane transporters, which renders the cells more sensitive to inhibitors [18]. Mitochondrial membrane preparations were prepared from diploid strains. The mutant PFQi3 combines nine amino-acid substitutions at the Q_i-site, namely I190G, A191L, A192C, I195F, L198F, S207T, M221F, F225L and I226L. The Q_o-site mutants harbor a single amino acid substitution, I147V, T148I, L150F, S152A or L275F [18,19].

2.3. Isolation of mitochondrial membrane preparations from Pythium

A 500 ml pre-culture of *Pythium sp.* (10 g/l malt extract, 4 g/l yeast extract and 4 g/l glucose) was inoculated from a culture dish and grown over night at 24 °C and shaking at 140 rpm. The next morning, 9 l of culture (9 × 1 l in 2 l flasks) were started from the pre-culture. *Pythium* was grown for 3 days at 24 °C and 140 rpm. The mycelium was harvested by filtering and was subsequently immersed in 500 ml buffer (650 mM mannitol, 5 mM EDTA, 2 g/l bovine serum albumin, 50 mM potassium phosphate, pH 7.4). The total volume was ~1 l. After addition of 1 mM PMSF and 1 volume of glass beads (0.5 mm), the mycelium was lysed in a glass mill for 12 min at 3500 rpm. The glass beads were removed by filtering and cell debris was removed by centrifugation for 20 min at 10000 × *g*. The membrane fraction together with the mitochondrial membranes was pelleted by centrifugation for 2 h at 50000 × *g*. The pellets were stored at −80°.

2.4. Isolation of mitochondrial membrane preparations from S. cerevisiae

10 ml pre-cultures of the *S. cerevisiae* strains (10 g/l yeast extract, 20 g/l bacto tryptone and 10 g/l glucose) were inoculated from a culture dish and grown over night at 28 °C and shaking at 160 rpm. The next morning, 5 l of culture (5 × 1 l in 2 l flasks) were started for each yeast from the pre-cultures using ethanol as the carbon source (10 g/l yeast extract, 20 g/l bacto tryptone and 16 g/l ethanol). The yeasts were grown for 44 h at 28 °C and 160 rpm. The cells were harvested by centrifugation and subsequently immersed in 250 ml buffer (650 mM mannitol, 5 mM EDTA, 2 g/l bovine serum albumin, 50 mM potassium phosphate, pH 7.4). After addition of 1 mM PMSF, the cells were lysed using a high-pressure homogenizer (Avestin). Cell debris was removed