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Rapid report

Genetic evidence for the existence of two quinone related inhibitor binding sites in NADH-CoQ reductase

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Abstract

Using the NADH-CoQ reductase of *Rhodobacter capsulatus* as a model for the mitochondrial Complex I, we have for the first time isolated bacterial mutants resistant to piericidin-A, a classical inhibitor of the mitochondrial enzyme. Their sensitivity to other inhibitors directed towards the quinone binding domain of complex I gives direct genetic evidence for the existence of two inhibitor binding sites.

Keywords: Complex I; NADH-CoQ reductase; Piericidin; Quinone binding site; *Rhodobacter capsulatus*

The mitochondrial NADH-CoQ reductase or Complex I catalyses the first step of mitochondrial respiration. Bovine Complex I is composed of 43 subunits divided into two parts: an hydrophilic domain bearing the catalytic NADH binding site and a hydrophobic one where proton pumping must take place [1]. Based on the observation of bound semiquinones associated with Complex I it has been proposed that quinone binding site(s) might take part in an elaborate proton pumping mechanism reminiscent to the Q-cycle [2]. Alternatively, according to the 'dual gated model', two quinones bound to two different quinone sites are gating proton transfer through a redox switch from the quinone to semiquinone state [3]. Despite the fact that the existence of several quinone binding sites is strongly suggested by characterization of Complex I inhibitors [3–8], the relationship between quinone and inhibitors binding sites still remains unclear. The membranous domain of Complex I which must house the quinone binding site(s) is essentially composed of the seven mitochondrially encoded ND subunits. Genetic approaches are therefore difficult even in lower eucaryotes. Moreover, it is rather difficult to study these highly hydrophobic proteins by classical biochemical approaches. For these reasons we developed a bacterial model of Complex I in *Rhodobacter capsulatus*. Indeed, the bacterial type-I NADH-CoQ reductases (NDHs) are multimeric enzymes which present seven subunits equivalent to the mitochondrial NDs [9–12]. The genes for these bacterial NDs are clustered in the bacterial *nuo* operon. Unlike the type-I NDH of *Escherichia coli* [5], the enzyme of *R. capsulatus* is highly sensitive to the potent inhibitor of mitochondrial complex I, piericidin-A. We therefore used this model to isolate bacterial piericidin resistant mutants.

Piericidin-A was isolated as described by Tamura

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[13] and was purified by HPLC on an Hypersil column. In preliminary experiments, we observed that growth of W1 (a home-made kanamycin resistant derivative of *R. capsulatus* B10 strain) on RcV malate [14], in the dark, at 30°C, was severely repressed by the presence of 5 μM piericidin-A but was never completely prevented. We noted the presence of some colonies growing far more efficiently than the others. Unfortunately, characterization of these strains proved disappointing as they were not piericidin resistant at the complex I level. Other independant observations made in the laboratory: complex I deficient mutants are able to grow aerobically on RcV malate, in the dark, using the end of the respiratory chain to energize theirs membranes [15]. However, at the difference with wild type, these mutants are unable to grow in the presence of myxothiazol. As piericidin inhibition should reproduce a situation similar to complex I deficiency, we infered from our experiments with complex I deficient mutants that addition of myxothiazol together with piericidin should strengthen the piericidin resistance screening. In practice, we spread about $4 \cdot 10^9$ bacteria per RcV malate plate on four plates containing 2.5 μM piericidin-A and 10 μM myxothiazol. This screening allowed the isolation of about 20 colonies per plate. As myxothiazol totally inhibits photosynthetic growth of *R. capsulatus* by blockade of the cyclic photosynthesis [16], it was easy to eliminate mutants resistant to this inhibitor in a second screening: 40 colonies isolated on the piericidin-myxothiazol medium were grown in parallel in photosynthetic conditions plus myxothiazol or in dark-aerobic conditions. This second screening showed that 36 of the isolated colonies were actually myxothiazol resistant. However, the four colonies unable to grow in 'myxothiazol-photosynthesis' conditions appeared to be resistant to piericidin-A and sensitive to myxothiazol. These mutants were called PiA, PiB, PiC and PiD, respectively. The following study deals with the characterization of mutants PiC and PiD.

High concentrations of both piericidin and myxothiazol are reported to cross-inhibit Complex III and Complex I of mitochondria [17]. Furthermore, the piericidin-A concentration required for complete inhibition of *R. capsulatus* growth is about 10^4 times the apparent K_i of the type-I NDH measured on isolated membranes (see below). To confirm that the piericidin-A resistance seen on plates was truly associated with Complex I, the effect of piericidin on the NADH oxidase activity was titrated on isolated membranes of Pi mutants and of wild type strain. As shown in Fig. 1 the NADH oxidase activity associated with Pi mutant membranes is definitely resistant to piericidin-A. This piericidin resistant oxidase activity could have resulted in the mutants, from the expres-

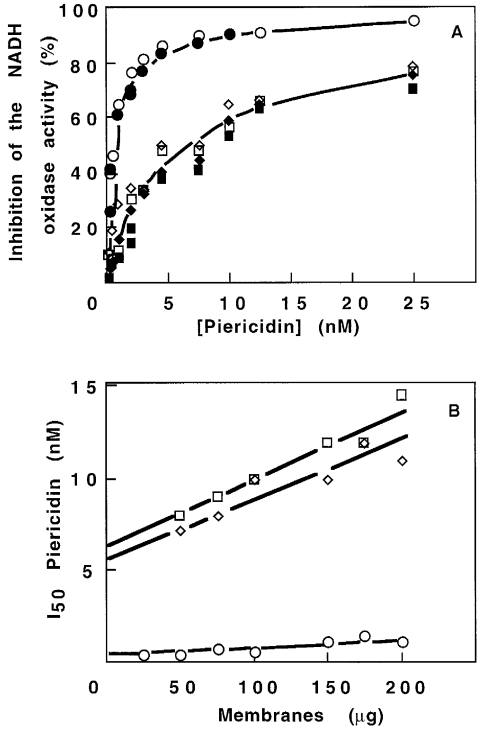

Fig. 1. Sensitivity of the NADH oxidase activity of wild-type and P_i mutant membranes to piericidin A. Panel A: titration of the effect of piericidin on the NDH of P_i mutants. 0.1 mg membranes aliquots of wild-type control (circles) or mutants PiC (squares) and PiD (diamonds) were incubated for 5 min at 30°C, in the presence of variable amounts of piericidin. The NADH oxidase activity was then recorded spectrophotometrically at 30°C ($\epsilon_{340} = 6200\ M^{-1} \cdot cm^{-1}$). To check for the possible contribution of type-II NDH in the observed resistance the experiment was repeated with either NADH (open symbols) or deaminoNADH (closed symbols) as a substrate. Panel B: effect of the protein concentration on the apparent I_{50} for piericidin of PiC and PiD mutants. This panel presents a synthesis of a series of experiments conducted mainly as in panel A but with variable amounts of added membranes. The circles represent I_{50} values estimated on wild-type membranes, the squares are associated with PiC and the diamonds with PiD. Extrapolation to zero protein gives apparent K_i values of 0.2 nM, 5.5 nM and 6.5 nM for the NDHs of wild-type control, PiC and PiD, respectively.

sion of a type-II NDH which is a small homodimeric enzyme encountered in some bacterial species and unrelated to the mitochondrial complex I [9]. However, all the present observations were unmodified when NADH was replaced by deamino-NADH which is specifically oxidized by the type-I NDH (Fig. 1A). The maximal inhibition of NADH oxidase activity associated with mutant and wild type membranes reached 95%. Thus for both mutants, the resistance must result simply from a decrease of the affinity of type-I NDH for piericidin-A. From titrations of the I_{50} of piericidin as a function of membrane protein concentration [4], we calculated an apparent K_i of 0.2 nM, 5.5 nM and 6.5 nM for the NDHs of W1, PiC and PiD, respectively (Fig. 1B). The mutants K_i are thus about 30 times the apparent K_i displayed by the wild type NDH. These apparent K_i values were obtain for an incubation time with inhibitors deliberately limited to 5 min as the NADH oxidase activity strongly decreases when membranes are kept diluted at 30°C. Similar measurements made after 1 h revealed that the activity was reduced by more than a half with a concomitant decrease of inhibitors sensitivity by a factor 1.5–2. Nevertheless, the ratio of apparent affinity between wild type and resistant mutants remained unchanged. We observed that the piericidin resistance could be transferred by homologous recombination between the wild type genome and a suicide plasmid bearing all the 3′ part of the *nuo* operon of mutants PiC and PiD. Different kinetic

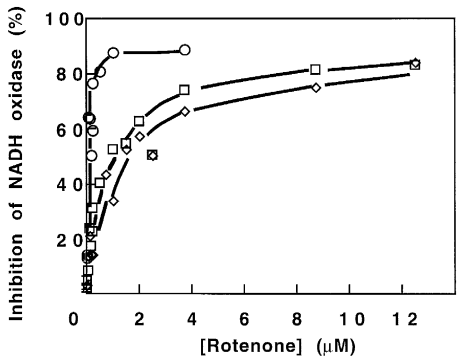

Fig. 2. Effect of rotenone on the NADH oxidase activity of mutant membranes. This figure illustrates the sensitivity of our mutants to rotenone. NADH-oxidase activity was recorded on 0.1 mg of membranes of wild-type control (circles), PiC mutant (squares) or PiD mutant (diamonds) after preincubation for 5 min at 30°C with various concentrations of inhibitor.

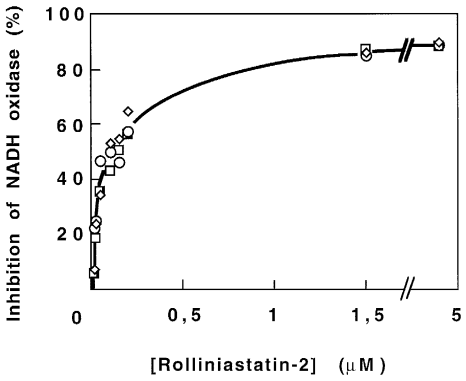

Fig. 3. Effect of rolliniastatin-2 on the NADH oxidase activity of mutant membranes. This figure illustrates the sensitivity of our mutants to rolliniastatin-2. NADH-oxidase activity was recorded on 0.1 mg of membranes of wild-type control (circles), PiC mutant (squares) or PiD mutant (diamonds) after preincubation for 5 min at 30°C with various concentrations of inhibitor.

data have suggested the presence of two classes of inhibitors for mitochondrial Complex I, which can be exemplified by rolliniastatin (site A) and rotenone (site B) [3–8]. Although classically described as site A inhibitor for the mitochondrial complex I, piericidin is considered to bind also the rotenone binding site [3,4]. We thus further studied the effect of the two 'site specific' inhibitors: rotenone and rolliniastatin-2, in order to confirm (or unvalidate) the existence of these two classes of inhibitors binding sites and, to determine which of these sites was affected in our resistant mutants. Titrations of the inhibition of NADH oxidase activity on the membranes of PiC and PiD mutants clearly demonstrated that the piericidin resistant mutants are also resistant to rotenone (Fig. 2) but display an unmodified sensitivity to rolliniastatin-2 (Fig. 3). Thus, according to the present knowledge on Complex I inhibitor binding sites (see above), our mutants are clearly altered in the B site shared by rotenone and piericidin-A but not altered in the A site inhibited by rolliniastatin-2 [3,4,18]. These observations constitute the first genetic evidence to validate the existence of the A and B sites predicted by enzymologic studies. In theory, minor genetic alteration can lead to an extensive reorganization of the overall structure of a protein. However, the fact that our mutants keep a high enzymatic activity (about 65% and 80% of wild type activity for PiC and PiD, respectively) and display a very selective change in

their sensitivity to Complex I inhibitors indicates that the structural alterations must be limited.

To try and get a better picture of the inhibitor binding sites, our promising genetic approach is developed in three directions: first we are physically mapping the genetic alterations associated with the resistance observed in the isolated Pi mutants. This mapping is an original and elegant way to allow the physical characterization of the inhibitors binding sites. Secondly, in collaboration with other groups, we are pursuing the kinetic characterization of the sensitivity of our mutants to other known inhibitors of Complex I to help clarify the classification of the inhibitors binding sites. Thirdly, other inhibitor resistant mutants have to be isolated to get a full panorama of the inhibitor binding sites.

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