

Differential efficacy of inhibition of mitochondrial and bacterial cytochrome bc_1 complexes by center N inhibitors antimycin, ilicicolin H and funiculosin

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Received 27 August 2007; received in revised form 16 October 2007; accepted 17 October 2007

Available online 1 November 2007

Abstract

We have compared the efficacy of inhibition of the cytochrome bc_1 complexes from yeast and bovine heart mitochondria and *Paracoccus denitrificans* by antimycin, ilicicolin H, and funiculosin, three inhibitors that act at the quinone reduction site at center N of the enzyme. Although the three inhibitors have some structural features in common, they differ significantly in their patterns of inhibition. Also, while the overall folding pattern of cytochrome b around center N is similar in the enzymes from the three species, amino acid sequence differences create sufficient structural differences so that there are striking differences in the inhibitors binding to the three enzymes. Antimycin is the most tightly bound of the three inhibitors, and binds stoichiometrically to the isolated enzymes from all three species under the cytochrome c reductase assay conditions. Illicicolin H also binds stoichiometrically to the yeast enzyme, but binds approximately 2 orders of magnitude less tightly to the bovine enzyme and is essentially non-inhibitory to the *Paracoccus* enzyme. Funiculosin on the other hand inhibits the yeast and bovine enzymes similarly, with $IC_{50} \sim 10$ nM, while the IC_{50} for the *Paracoccus* enzyme is more than 10-fold higher. Similar differences in inhibitor efficacy were noted in bc_1 complexes from yeast mutants with single amino acid substitutions at the center N site, although the binding affinity of quinone and quinol substrates were not perturbed to a degree that impaired catalytic function in the variant enzymes. These results reveal a high degree of specificity in the determinants of ligand-binding at center N, accompanied by sufficient structural plasticity for substrate binding as to not compromise center N function. The results also demonstrate that, in principle, it should be possible to design novel inhibitors targeted toward center N of the bc_1 complex with appropriate species selectivity to allow their use as drugs against pathogenic fungi and parasites.

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Keywords: bc_1 complex; Center N; Inhibitor; Yeast; Bovine; *Paracoccus denitrificans*; Antimycin; Illicicolin H; Funiculosin

1. Introduction

The cytochrome bc_1 complex is a homodimeric, multisubunit enzyme embedded in the inner mitochondrial membrane of oxygen utilizing eukaryotic cells and the plasma membrane of many gram negative bacteria [1]. The catalytic core of the enzyme is formed by the cytochrome b subunit, the Rieske iron–sulfur protein and the cytochrome c_1 subunit. In addition,

the mitochondrial enzyme contains 6–8 supernumerary subunits lacking any cofactors, while the bacterial enzyme contains none or one extra subunit [2]. The protonmotive Q cycle describes the mechanism by which the enzyme couples the transfer of two electrons from quinol to two cytochrome c to the translocation of four protons across the membrane [3,4]. The Q cycle involves two quinone binding sites in cytochrome b ; the center P (“ Q_P ”) site catalyzes quinol oxidation, with one electron transferred to the Rieske protein en route to cytochromes c_1 and c , while the second electron is transferred to the low potential b heme (b_L) and recycled inward through the membrane to the high potential heme (b_H) to the opposite site of the membrane, where it reduces quinone to form a tightly bound semiquinone at the center N (Q_N) site.

Abbreviations: b_L heme, low potential b heme; b_H heme, high potential b heme; DBH, decyl ubiquinol

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Several inhibitory compounds are known that target specifically the Q_P or Q_N site [5]. These have been of use in the structural and functional studies of the individual catalytic sites. In addition, the Q_P inhibitor atovaquone [6] is of pharmaceutical relevance for treatment of some parasitic and fungal infections. Only a few center N specific inhibitors are known to date. These include antimycin, which is a natural product of various species of *Streptomyces* [7], ilicicolin H, isolated from the imperfect fungus *Cylindrocladium ilicicola* [8,9], and funiculosin, produced by *Penicillium funiculosum* Thom [10,11]. Structurally the inhibitors are clearly different, but they also share some similarities [9]. Upon binding to the Q_N site, they all displace semiquinone [12]. While ilicicolin H and antimycin both possess a phenol ring, funiculosin and ilicicolin H share a pyridone ring system. Based on a similar optical effect of the latter two inhibitors on the cytochrome b_H spectrum, it was concluded that the ring system plays an important role in their binding [9]. On the other hand genetic studies in yeast indicate that the inhibitors have different modes of binding to the bc_1 complex [13].

In this study we have compared the inhibitory action of the Q_N inhibitors, antimycin, funiculosin and ilicicolin H on the cytochrome bc_1 complexes from three different species, *Saccharomyces cerevisiae*, *Bos taurus*, and *Paracoccus denitrificans*. We also examined the inhibitor sensitivity of yeast bc_1 complexes with center N mutations obtained from ilicicolin H resistant [14] and respiratory deficient revertants [15,16]. The results reveal striking differences in sensitivity of the enzymes from the different species, and comparing the cytochrome b sequences around the center N binding pocket allows some preliminary speculation regarding the structural basis of the differences in inhibitor efficacy between the species. The results also demonstrate the feasibility of designing new therapeutic

drugs, targeted to the bc_1 complex, with species selectivity based on structural differences in the center N of pathogens and host enzymes.

2. Materials and methods

2.1. Materials

Dodecyl maltoside was obtained from Anatrace. DEAE-Biogel and Tween-20 were obtained from Bio-Rad Laboratories. Antimycin, diisopropylfluorophosphate, horse heart cytochrome c , and decyl ubiquinone were purchased from Sigma Chemical Co. Dithionite was purchased from Fluka Biochemica. Decyl ubiquinol (DBH) was prepared as described before and quantified spectrophotometrically [17]. Funiculosin was a gift of Novartis, Basel, Switzerland. Ilcicolin H was obtained from the Merck sample repository. The Q_N inhibitors antimycin, ilicicolin and funiculosin were diluted in ethanol to a concentration of 50–200 μ M. The extinction coefficients used to calculate the concentration of the stock solutions were, for antimycin, $4.8 \text{ mM}^{-1} \text{ cm}^{-1}$ at 320 nm, for ilicicolin H, $5.3 \text{ mM}^{-1} \text{ cm}^{-1}$ at 349 nm, and for funiculosin $5.5 \text{ mM}^{-1} \text{ cm}^{-1}$ at 293 nm [5,9].

2.2. Purification of cytochrome bc_1 complexes

The wild-type yeast strain with the KM91 background and the cytochrome b mutants of that strain, M221Q, M221E and W30C were obtained from Dr. Anne-Marie Colson (Universite Catholique de Louvain-La-Neuve, Belgium) and Dr. Gael Brasseur (CNRS Marseille, France) [16,18]. The wild-type yeast strain with the W303 background and cytochrome b mutants of that strain, S20T, Q22E, Q22T and L198F, were described previously [14]. Bovine heart mitochondria were a gift from Dr. Chang-an Yu (Oklahoma State University).

Cytochrome bc_1 complexes from all of the yeast strains and bovine heart mitochondrial membranes were purified as previously described [19]. Purified cytochrome bc_1 complex from *P. denitrificans* was a gift from Dr. Bernd Ludwig (W. Goethe-Universität, Frankfurt am Main, Germany). The cytochrome c_1 concentration in the bc_1 complexes was determined from the difference spectrum of the ascorbate-reduced versus ferricyanide-oxidized enzymes, using an extinction coefficient of $17.5 \text{ mM}^{-1} \text{ cm}^{-1}$ at 554–539 nm. The cytochrome b concentration was determined from the difference spectrum

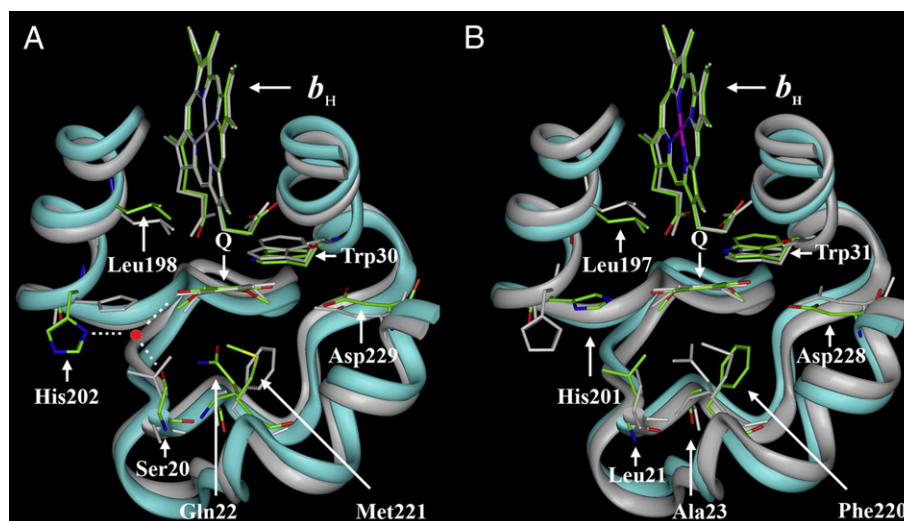


Fig. 1. Comparison of the yeast and bovine cytochrome b protein structures in the region of the proteins surrounding the Q_N site. Both panels show the b_H hemes, the ubiquinone ring and amino acid residues 16–38 and 194–230 of the yeast protein overlaid with residues 17–39 and 193–229 of the bovine protein. In panel A the carbon backbone of the yeast protein is colored cyan and the b_H heme ring, ubiquinone ring, and selected amino acids are labeled and colored. Oxygen atoms are red, nitrogen atoms are blue, carbon atoms are green and sulfur is yellow. The carbon backbone and other features of the bovine protein are grey. Panel A also depicts the water-mediated hydrogen bond between His-202, Ser-20, and the ubiquinone ring that is present in the yeast, but not in the bovine, structure. In panel B the carbon backbone of the bovine protein is colored cyan and the heme ring, ubiquinone ring, and selected amino acids are labeled and colored according to the same scheme as in panel A, while the yeast protein is depicted in grey. The figures were created from the coordinates of the yeast (1EZV, Ref. [22]) and bovine (1ppj, Ref. [24]) enzymes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

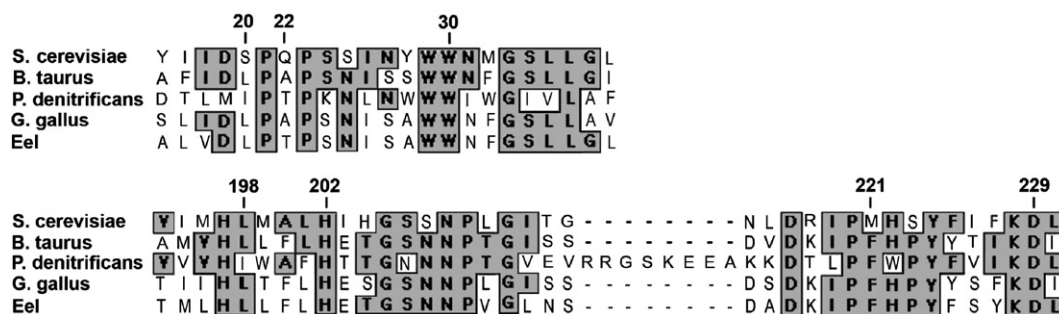


Fig. 2. Sequence alignment of the cytochrome *b* proteins from bovine, yeast, *P. denitrificans*, chicken and eel in the regions forming the Q_N site. The sequences from the latter two species were included since the enzymes from those species have been reported to be resistant to funiculosin, as discussed in the text. The alignment was constructed using ClustalW and yeast numbering of residues 16–38 and 194–230. In the bovine enzyme the Q_N site is formed by residues 17–39 and 193–229, and in the *P. denitrificans* enzyme by residues 31–53 and 208–252. The numbered arrows show the positions of the Q_N site yeast mutations that were analyzed, and the residues His-202 and Asp-229.

of dithionite-reduced versus ferricyanide-oxidized enzymes. The extinction coefficients used were $50 \text{ mM}^{-1} \text{ cm}^{-1}$ at 562–578 nm for the yeast and bovine enzymes [20,21], and $40 \text{ mM}^{-1} \text{ cm}^{-1}$ at 559–578 nm for the *P. denitrificans* bc_1 complex.

2.3. Ubiquinol-cytochrome *c* reductase assay and inhibition by the center *N* inhibitors antimycin, ilicicolin and funiculosin

Ubiquinol-cytochrome *c* reductase activities of the purified cytochrome bc_1 complexes from bovine heart, commercially available Red Star™ yeast, and *P. denitrificans* were measured at room temperature in an assay buffer containing 50 mM potassium phosphate, pH 7.0, 250 mM sucrose, 1 mM sodium azide, 0.2 mM EDTA, and 0.05% dodecyl maltoside. For the purified cytochrome bc_1 complexes from the other yeast strains, the assay buffer contained 50 mM potassium phosphate, pH 7.0, 1 mM sodium azide, 1 mM EDTA and 0.01% Tween-20. The dodecyl maltoside and Tween-20 were included in the assay buffers because they improved the stability of the diluted enzyme during the prolonged incubation required for equilibration with the inhibitors. The enzymes were diluted first to $\sim 1 \mu\text{M}$ in 400 μL of the same assay buffer and incubated on ice for 30 min. For measuring the activities, an aliquot of the enzyme was diluted from the $1 \mu\text{M}$ sample to a final concentration of 2.5 nM in an assay buffer containing 50 μM cytochrome *c* and 1 mM potassium cyanide and incubated for 1.5 min. The reaction was started by adding 50 μM DBH, and reduction of cytochrome *c* was monitored at 550–539 nm with the Aminco DW2a™ spectrophotometer in the dual wavelength mode. The extinction coefficient used to calculate the cytochrome *c* reduction was $21.5 \text{ mM}^{-1} \text{ cm}^{-1}$ at 550–539 nm. All measurements were done in duplicate.

For measuring the inhibitor titration curves, the activity was first measured without inhibitor and this was taken as 100% activity. The concentration of inhibitor in the enzyme solution was increased incrementally by adding aliquots from the inhibitor stock solutions to the $1 \mu\text{M}$ enzyme sample. After addition, the enzyme–inhibitor solution was gently mixed and incubated for 2 min on ice prior to measuring activity. The ethanol concentration in the solution did not exceed 5%.

3. Results

3.1. Comparison of the Q_N sites from yeast, bovine and *P. denitrificans* bc_1 complexes, and the location of yeast Q_N mutants

The Q_N site in the cytochrome bc_1 complex from yeast [22] and bovine [23,24] mitochondria is structurally highly conserved (Fig. 1). Comparing the sequences of residues¹ 16–38 and 195–230 that form the Q_N site shows that $\sim 50\%$ of the amino acids are identical. No structural information is available

for the complex from *P. denitrificans*, but the crystal structure of the complex from *Rhodobacter capsulatus* [25], which cytochrome *b* sequence is more than 80% conserved with that of *P. denitrificans*, also shows a conserved structure of the Q_N site. However the sequence identity of the bacterial enzyme with those of the yeast and bovine enzymes is slightly lower, ~ 35 and $\sim 45\%$, respectively. Interestingly, the sequence identity of the residues that are present within 5 Å of the ubiquinone (in the yeast structure) is no higher than the overall identity in the center *N* pocket. It is striking that, even with this degree of sequence variation, the distance and orientation of the ubiquinone ring relative to the b_H heme are essentially identical in the yeast and bovine enzymes (Fig. 1). Five of the most conserved residues near the ubiquinone are the axial histidine ligand to the heme iron, His 197, two residues implicated in quinone binding, His-202 and Asp-229, and Gly-33, Trp-30, and Lys-228 [26]. It is also striking that the conserved His-202 participates in a water-mediated hydrogen bond to the quinone ring in the yeast enzyme, but does not do so in the bovine enzyme where it is rotated closer to the quinone. We have studied here mutations of Trp-30, and four other residues, Ser-20, Gln-22, Leu-198, and Met-221 that are less conserved, but are within 5 Å of the ubiquinone ring.

Trp-30 forms a hydrogen bond with one of the propionate groups of the b_H heme (Fig. 1). The substitution of tryptophan by a cysteine in the W30C mutant has no natural equivalent. Leu-198 is conserved in the yeast and bovine enzymes (Figs. 1 and 2) and is located close to the b_H heme and the quinone. The analogous residue in *P. denitrificans* is an isoleucine. The substitution of leucine by phenylalanine in the L198F mutant is native in *Candida*, but other hydrophobic residues are also observed. Interestingly the L198F mutation was reported to confer resistance towards the three center *N* inhibitors, antimycin, funiculosin and ilicicolin H in the yeast enzyme [14]. It is not known whether the *Candida* bc_1 complex is intrinsically resistant to these center *N* inhibitors.

A hydrophobic or van der Waals interaction is observed between the residue at position 221 and the ubiquinone (Fig. 1). In the complex from bovine mitochondria, *P. denitrificans* and most other species the corresponding residue is a phenylalanine (Fig. 2) [26]. The occurrence of methionine in the yeast bc_1

¹ Yeast numbering of the cytochrome *b* amino acid sequence is used throughout, except when noted otherwise.

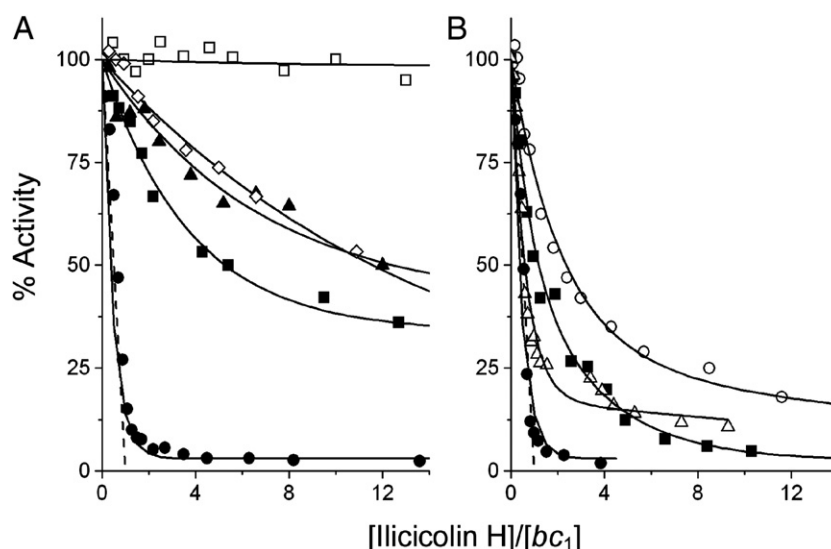


Fig. 5. Inhibition of ubiquinol-cytochrome *c* reductase activity of yeast cytochrome *b* mutants by ilicicolin H. The symbols used for the mutated enzymes in panels A and B are the same as in Fig. 4. The dashed lines show the theoretical titration curves for the stoichiometric binding of one inhibitor per bc_1 complex.

between a ubiquinone carbonyl group and the imidazole nitrogen [24] or indirectly through a water molecule [29]. The Leu-21 (bovine numbering) may also stabilize this histidine conformation. In the *P. denitrificans* enzyme the residues at positions 20 and 22 are isoleucine and threonine, respectively. The crystal structure of the *R. capsulatus* enzyme does not contain a ubiquinone bound at the Q_N site, but the histidine has the same configuration as in the bovine structure. Interestingly, substitutions of Ser-20 by a leucine, or Gln-22 by a threonine rendered the yeast strains resistant to ilicicolin H. Because the S20L mutant bc_1 complex was reported to be unstable [14], it has not been further characterized. However the S20T, Q22T and Q22E mutants have been investigated.

3.2. Inhibition of yeast, bovine and *P. denitrificans* bc_1 complexes by center *N* inhibitors

Fig. 3 compares the inhibition of cytochrome *c* reductase activities of yeast, bovine and *P. denitrificans* bc_1 complexes by antimycin, ilicicolin H and funiculosin. The titration curves in Fig. 3A show the inhibition of the three bc_1 complexes by antimycin. With one equivalent of antimycin more than 90% of activity is inhibited in the bovine and yeast enzyme, as shown before [9]. The *P. denitrificans* enzyme shows some residual activity at higher concentration of antimycin, but the inhibitor still effectively inactivates the enzyme. We previously reported that ilicicolin H is a potent inhibitor for yeast bc_1 complex, but not for the bovine enzyme [9]. Under the assay conditions used here, the residual catalytic activity is $\sim 10\%$ with one equivalent of ilicicolin H per bc_1 monomer. On the other hand, both the bovine and *P. denitrificans* enzymes are highly resistant towards this inhibitor (Fig. 3B), with IC_{50} values >100 nM for the bovine enzyme while the bacterial enzyme is barely inhibited even at the highest concentrations of inhibitor tested (data not shown). For the yeast enzyme, funiculosin is the least effective of the three inhibitors (Fig. 3C), and the titration curve for the bovine enzyme is essentially identical to that the yeast complex.

The *Paracoccus* enzyme is only weakly inhibited by this inhibitor.

3.3. Inhibition of yeast cytochrome *b* mutants

The results in Fig. 4 show that none of the mutations affects the inhibition of the isolated enzymes by antimycin; more than 90% of the catalytic activity is inhibited with a titer close to one inhibitor per bc_1 complex monomer. Thus the IC_{50} is still at least an order of magnitude less than 2.5×10^{-9} M. The slight hysteresis in the inhibitor titration of the bc_1 complex from wild-type yeast has been attributed by us to negative cooperativity between the two center *N* sites and inter-monomer electron transfer through the b_L hemes [30–32]. This same behavior is seen with some of the cytochrome *b* mutant enzymes (Fig. 4). The lack of hysteresis in some of the mutant enzymes suggests that the negative cooperativity might have been weakened due to some of the mutations.

Illicicolin H inhibits the cytochrome *c* reductase activity of the KM91 and W303 wild-type strains $\sim 90\%$ with a titer of one inhibitor per bc_1 monomer (Fig. 4), as is also observed with the bc_1 complex from commercial yeast (Fig. 3B). The S20T, Q22E, Q22T and L198F mutations in the yeast bc_1 complex that conferred resistance to ilicicolin H, indeed provide significant resistance towards this inhibitor (Fig. 5A). The Q22E mutant is completely resistant with up to 40 equivalents (100 nM) of inhibitor, whereas the other three mutations result in a gradual decrease in catalytic activity with increasing concentration of inhibitor. The M221E, M221Q and W30C mutants were not obtained as yeast strains with resistance to ilicicolin *in vivo*, but the purified enzymes from these mutants also do show resistance to inhibition by this inhibitor (Fig. 5B), although to a lesser extent than the other mutant enzymes.

With funiculosin the titration curves of the wild-type enzymes with the two genetic backgrounds appear to show some difference; the W303 strain is more resistant towards this

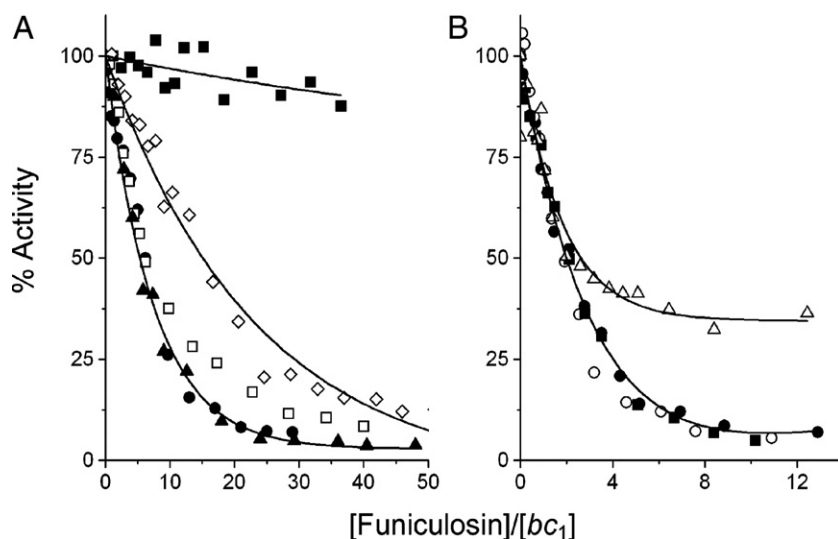


Fig. 6. Inhibition of ubiquinol-cytochrome *c* reductase activity of yeast cytochrome *b* mutants by funiculosin. The symbols used for the mutated enzymes in panels A and B are the same as in Fig. 4.

inhibitor than the KM91 strain, with half inhibition occurring at ~ 8 versus ~ 2 equivalents of inhibitor per *bc*₁ monomer, corresponding to IC₅₀ values of 20 and 5 nM respectively. However, with the exception of L198F and W30C, the mutations appear to have no significant effect on the sensitivity towards funiculosin. The *bc*₁ complex with the L198F cytochrome *b* mutation appears to be highly resistant, while the W30C mutation appears only to be more resistant at higher concentrations of funiculosin. Previous studies reported that both of these mutations confer resistance to this inhibitor [33,34].

4. Discussion

In the current study we have compared the efficacy of three center N inhibitors, antimycin, ilicicolin H and funiculosin towards the purified cytochrome *bc*₁ complexes from yeast, bovine heart, and *P. denitrificans*. We also tested the efficacy of the inhibitor against five variants of the yeast enzyme with mutations in cytochrome *b*. Comparison of the sequences and structures of the *bc*₁ complexes from the three species showed some common features for the binding of quinone and the activity of center N. On the other hand, there are a number of residues near the quinone that are not conserved. Previous structural studies in the bovine enzyme [29] and genetic studies in yeast [13] showed that the binding sites for different Q_N inhibitors do not entirely overlap. We have probed the degree of overlap more extensively with the center N mutations Ser-20, Gln-22, Trp-30, Leu-198 and Met-221 in the yeast cytochrome *b*. Analyzing these mutant enzymes allows some preliminary conclusions to be made regarding the differences in efficacy of the center N inhibitors against enzymes from the three different species.

Antimycin has been known to bind with high affinity to the bovine enzyme with an indirectly measured $K_d = 3.2 \times 10^{-11}$ M [35]. In our experiments (Fig. 3A) we did not detect any difference in the efficacy of inhibiting the catalytic activity of

the *bc*₁ complexes from the three species by this inhibitor. Structural studies of the chicken [36] and bovine [24,29] *bc*₁ complexes with antimycin bound show that the inhibitor is similarly docked very close to the *b*_H heme of cytochrome *b* in the two enzymes. Indeed, two of the most conserved residues (see Fig. 2 and Ref. [26]), Lys-228 and Asp-229, have been implicated in the strong binding of this inhibitor [29]. In the crystal structures of the chicken [36] and bovine [24,29] *bc*₁ complexes these two residues form hydrogen bonds with the inhibitor, directly or through structural waters. A hydrophobic interaction that is observed between ubiquinone and Met-221 (Phe in bovine and *P. denitrificans*, Figs. 1 and 2) may also contribute to the binding of this inhibitor, but this appears not to be essential (see below).

Likewise, none of the center N mutations in the yeast dramatically affects the sensitivity of the yeast enzyme for antimycin (Fig. 4). However, it should be noted that under the assay conditions employed in our experiments, an increase of as much as 50-fold from the $K_d = 3.2 \times 10^{-11}$ M estimated for the bovine enzyme would go undetected, since the concentration of enzyme is 2.5×10^{-9} M in the assay. This might explain why the L198F mutant, although resistant towards antimycin *in vivo* [14], was inhibited by a titer of ~ 1 antimycin per *bc*₁ monomer. The M221E and M221Q mutations also did not change the sensitivity to antimycin in our experiments, while a M221K mutation in the same parental strain blocked antimycin binding to the Q_N site [18].

We previously showed that the IC₅₀ value for inhibition of the cytochrome *c* reductase activity of the bovine *bc*₁ complex by ilicicolin H is ~ 100 -fold higher than for the yeast enzyme [9]. In the current experiments both the bovine and *P. denitrificans* enzymes appear to be highly resistant to this inhibitor (Fig. 3B). Thus the yeast enzyme may contain structural features important for the binding of this inhibitor that the other two are lacking, or the bovine and *P. denitrificans* enzymes may contain features that confer resistance to the binding of this inhibitor. Funiculosin

is the weakest of the three inhibitors with all three species enzymes. It differs from ilicicolin H in that only the *bc*₁ complex from *P. denitrificans* is less sensitive towards this inhibitor.

The L198F yeast cytochrome *b* mutation conferred cross-resistance towards all three inhibitors *in vivo* [14], but resistance was seen *in vitro* to only ilicicolin H and funiculosin, probably due to the high affinity of antimycin relative to enzyme concentration in the assay as discussed above. In particular, the effect of substitution of the leucine by a bulkier phenylalanine on the inhibition by funiculosin is dramatic. This suggests that this residue is an important binding determinant for this inhibitor. It is possible that an isoleucine at this position in the *P. denitrificans* enzyme may contribute to weakening of inhibition, but it is more likely that there are other determinants that are responsible for this difference. In this regard it is interesting to note that Degli Esposti and coworkers [26,37] proposed that valine at the position equivalent to Val-194 in the yeast cytochrome *b* is one of the residues responsible for the intrinsic resistance of fish, rabbit and horse cytochrome *bc*₁ complexes to funiculosin. However, the yeast and bovine enzymes, which have valine and alanine, respectively, at this position in their cytochrome *bs*, are equally sensitive to funiculosin (Figs. 2 and 3).

It should also be acknowledged that with all of the cytochrome *b* mutations the possibility remains that the effect of the mutational change is indirect, due to structural changes transmitted through the protein to a ligand-binding site some distance from the mutated residue. A precedent for such long-range effects might be found in mutational changes to Ala-126 in the yeast protein, which confer resistance to both center N and center P inhibitors (cf Ref. [37] and Table IV in Ref. [26]). The distinction between direct local steric effects and long-range indirect effects can only be addressed when crystal structures of the enzymes with ilicicolin and funiculosin bound become available.

The W30C mutation slightly lowers the sensitivity to inhibition of the yeast *bc*₁ complex by ilicicolin (Fig. 5), but has a more pronounced effect on funiculosin inhibition of the enzyme (Fig. 6). Trp-30 is itself not responsible for the difference in efficacy of funiculosin inhibition between the enzymes from *P. denitrificans* and the two other species, since it is conserved. However, the effects of the W30C mutation suggest that Trp-30 is part of a binding pocket for funiculosin, as was reported previously [34]. The W30C mutation was originally obtained as a second site mutation that corrects the respiratory deficient phenotype of the S206L mutant [16]. Two other second site mutations in the same revertant screen were obtained, N208Y and N208K, and the double mutations also confer resistance towards funiculosin [38]. The three residues (Trp-30, Ser-206 and Asn-208) are within 5 Å from each other. The temperature-sensitivity of the three revertant strains suggests a structural change has occurred that makes these *bc*₁ complexes more labile, and that may be near the funiculosin binding site and thus block the binding of funiculosin.

The Ser-20, Gln-22 and Met-221 residues are within 5 Å of each other, on the opposite site of the quinone ring with respect to Trp-30 and Leu-198 (Fig. 1). As was discussed above, Met-221 in the yeast enzyme and the phenylalanine residue at the

equivalent position in the bovine and *P. denitrificans* complexes (Figs. 1 and 2) stabilize the binding of the quinone at the Q_N site. The M221E and M221Q mutations render the yeast enzyme slightly resistant towards ilicicolin H (Fig. 5). One possible explanation is the importance of a hydrophobic interaction for the binding of ilicicolin H. However, the bovine and *P. denitrificans* enzymes are much less sensitive towards this inhibitor, even though a hydrophobic interaction may be present. The residues at positions 20 and 22 appear to be especially important for the binding of ilicicolin H, as discussed below. Thus, an alternative explanation for the results of the Met-221 variants is that these mutations have modified the conformation of Ser-20 and Gln-22 and thus weakened the binding of ilicicolin H.

Ser-20 and Gln-22 are two of the least conserved residues in the Q_N site of the three species. The results with the mutant yeast enzymes indicate that Ser-20 and Gln-22 play an important role in the different efficacies of ilicicolin H in inhibiting the *bc*₁ complexes from the three species (Fig. 5). The purified *bc*₁ complex from the S20T mutant and the enzyme in mitochondrial membranes from the S20L yeast strain [14] are both highly resistant towards ilicicolin H. Interestingly, leucine is the naturally occurring residue at the equivalent position in the bovine *bc*₁ complex, while another hydrophobic residue, isoleucine, is present in the enzyme from *P. denitrificans*. Both the Q22T and Q22E mutations also render the yeast enzyme less sensitive to ilicicolin H. Threonine is again a naturally occurring residue in one of the species, *P. denitrificans*.

A straightforward explanation of these comparisons is that Ser-20 and Gln-22 are critical for the binding of the ilicicolin H to the yeast *bc*₁ complex. In addition, the nature of the two residues at these positions appears to modulate the orientation of the imidazole ring of His-202. In the bovine and *R. capsulatus* (and possibly also *P. denitrificans*, see above) Q_N site the imidazole ring is rotated into the quinone binding site. This configuration may also weaken the binding of ilicicolin. Replacement of ilicicolin H by antimycin, followed by monitoring the spectral changes in the ferro-cytochrome *b*_H spectrum from the ilicicolin induced blue shift to the antimycin induced red shift, indeed is much faster in the mutant enzymes than in the wild-type enzymes (Ref. [9] and unpublished results).

We previously reported the effect of these yeast mutations on the quinol oxidation reaction at the Q_N site [28]. Although there were significant differences in the kinetics of quinol oxidation at center N, these differences did not compromise the catalytic function of the enzymes. With the exception of the W30C mutation, the impairment in center N kinetics in these mutants was attributed to weakened affinity for the quinol substrate at center N. The altered affinity for the substrate correlates well with the resistance of the mutants towards ilicicolin H. This suggests that ilicicolin H occupies a similar binding site as the ubiquinol substrate in the yeast *bc*₁ complex (Fig. 1) that involves the residues Ser-20, Gln-22, Leu-198 and Met-221. However ilicicolin is a much larger molecule than ubiquinol and structural perturbations by these mutations can have a more pronounced effect on the binding of the inhibitor. For example the L198F mutation has only a modest effect on the kinetics at center N, but increases the IC₅₀

for ilicicolin H 12-fold. On the other hand the binding site for funiculosin appears to be slightly different, where the important residues Leu-198 and Trp-30C are near the b_H heme. Ser-20, Gln-22 and Met-221 appear to play no direct role in the binding of this inhibitor, but are important for catalysis. The nature of the residues at positions 20 and 22 is an important determinant for binding of ilicicolin H and can account for the different efficacies of inhibition by this inhibitor in the three species.

In conclusion, it should also be noted that the species differences noted here might have important consequences for drug design. Atovaquone, a hydroxynaphthoquinone inhibitor of the cytochrome bc_1 complex, has been used therapeutically to treat *Plasmodium falciparum* malaria, *Pneumocystis carinii* pneumonia, and *Toxoplasma gondii* toxoplasmosis. This inhibitor kills these organisms by blocking ubiquinol oxidation at center P of the bc_1 complex, although its therapeutic effectiveness has been compromised by spontaneously arising mutations that confer resistance to the drug [39]. In principle, it should also be possible to design drugs targeted to the quinone reduction site at center N of the enzyme, provided that the inhibitory compounds display appropriate species selectivity toward the pathogen versus host enzymes. This would allow the advantages of a combination drug therapy directed toward a single essential enzyme target, thus minimizing the likelihood of side effects. The current results demonstrate that there are sufficient structural differences between fungal, bacterial, and mammalian center N sites to permit such drug design.

Acknowledgement

This research was supported by NIH Research Grant GM 20379.

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