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Specificities of the two center N inhibitors of mitochondial bc_1 complex, antimycin and funiculosin: strong involvement of cytochrome *b*–asparagine-208 in funiculosin binding

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Abstract Funiculosin, a center N inhibitor of the bc_1 complex, induces a blue-shift in the cytochrome *b* spectrum. A thermosensitive revertant [Coppee, J.Y. et al., J. Biol. Chem. 269 (1994) 4221–4226] isolated from a cytochrome *b* respiratory-deficient mutant, exhibits a red-shift instead of the blue-shift retained in the original mutant and shows resistance to this inhibitor. Replacing cytochrome *b*-Asparagine-208 by Lysine in this revertant, keeping the original mutation S206L, leads, when mitochondria are incubated at non-permissive temperature, to complete loss of bc_1 complex activity and funiculosin-binding, while the antimycin-binding is conserved. These data suggest some inhibitor site specificity and close proximity between the funiculosin-binding site and the catalytic center N domain (Q_N).

Key words: Funiculosin resistance; Cytochrome bc_1 complex; Red-shift; Cytochrome *b* mutant; Catalytic center N; *Saccharomyces cerevisiae*

1. Introduction

The role of the bc_1 complexes in the electron transfer coupled to proton translocation across the membrane has been best described to date by the widely accepted Q-cycle scheme introduced by Mitchell [1] and reviewed by Trumpower [2]. This mechanism requires two distinct quinone reaction sites, the hydroquinone oxidation (Q_P) and the quinone reduction (Q_N) centers, which are located on opposite sides of the membrane. Two classes of inhibitors are linked to each of these centers: one set of compounds, exemplified by myxothiazol and reviewed by Link et al. [3], blocks the electron transfer through the Q_P side; another set of inhibitors, exemplified by antimycin blocks the Q_N center.

Cytochrome *b* is the only subunit of the bc_1 complex which is coded by mitochondrial DNA. The first folding model for cytochrome *b* was proposed by Widger et al. [4] and Saraste [5]. Knowledge about drug-resistance mutations (reviewed in [6]) has suggested, on the basis of other calculations, a modified version of this model with eight instead of nine hydrophobic helices spanning the inner mitochondrial membrane [7–9]. This revised model fits all the available data on mutants, in particular the assignment of the axial ligands to cytochrome b_{562} (b_H) in the vicinity of the Q_N center, and to cytochrome b_{565} (b_L) in the vicinity of the Q_P center [10,11].

The bc_1 complex inhibitors have strong effects on the spectra of the heme close to the respective binding sites and minor effects on the distant heme [12]. For instance, the center N inhibitors antimycin and funiculosin have been found spectroscopically and potentiometrically to affect mainly heme b_H [12], while myxothiazol and stigmatellin affect heme b_L [13,14]. These inhibitors induce a spectral shift of the cytochrome *b* hemes, generally a red-shift (bathochromic effect), which, according to Link et al. [3] is proportional to the amount of bound inhibitor. These effects on the optical spectra have previously been observed on the three absorption bands (α, β, γ) of both oxidized and reduced cytochrome *b* in submitochondrial particles and bc_1 complex of beef-heart by Kamenskiĭ et al. [15]. These authors described a red-shift with antimycin and a blue-shift with funiculosin.

We report here the spectral shifts caused by these two inhibitors in reduced cytochrome *b* of the wild-type strain and in a cytochrome *b* respiratory-deficient mutant, and the striking modifications observed with a thermosensitive revertant modified in the vicinity of heme b_{562}. These results yield some new insights on the Q_N center.

2. Materials and methods

2.1. Strains

The wild-type strains and the original mutants were from the Gif collection.

Parental strain (wild-type box$^+$): the diploid KM91 was obtained by crossing 777–3A α *ade1 op1 rho*$^+$ *mit*$^+$ with KL14–4A α *his1 trp1 OP1 rho*°. The original *rho*$^+$ *mit*$^-$ respiratory-deficient mutant 777–3A/M4721 was isolated from the haploid strain 777–3A α *ade1 op1 rho*$^+$ *mit*$^+$ [16] and the mutation was mapped within the cytochrome *b* gene [11]. The haploid strain 777–3A/M4721 was crossed with KL14–4A α *his1 trp1 OP1 rho*° to form the diploid KM 208. Its mt DNA sequence has been determined and a single missence mutation S206L has been identified [11]. The respiratory competent revertant S206L–N208K has been selected from the respiratory-deficient S206L (KM208) [17]. The experiments were carried out with the diploid strains, which are all isogenic except for the cytochrome *b* mutations.

Similarly, the original *rho*$^+$ *mit*$^-$ respiratory deficient mutant 777–3A/M4410 was isolated from the haploid strain 777–3A [16] and the mutation mapped within the cytochrome *b* gene [11]. This strain was crossed with IL126–1C/52 *a ura1 OP1 rho*° to form the diploid PS493 and a single mutation M221K has been identified [11]. The parental strain (box$^+$) obtained using the same protocol was named PS409.

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Abbreviations: Q_N center, ubiquinone reduction center of the mitochondrial ubiquinol cytochrome *c* oxidoreductase (cytochrome bc_1 complex or complex III) on the negative side of the inner mitochondrial membrane; Q_P center, ubiquinol oxidation center of ubiquinol cytochrome c oxidoreductase on the positive side of the inner mitochondrial membrane; DBH_2, decylubiquinol (2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinol); Me_2SO, dimethyl sulfoxide. *Enzyme:* Ubiquinol cytochrome *c* oxidoreductase: EC 1.10.2.2. (bc_1 complex).

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2.2. Media, growth conditions, preparation of mitochondria and cytochrome content determination

They were as described in ref. [18]. Mitochondria were used without adding any detergent so as to preserve the membrane integrity.

MR3 buffer: 0.65 M Sorbitol, 10 mM KH_2PO_4, 2 mM EDTA, 0.1 mM $MgCl_2$, 0.3% BSA, pH 6.5.

2.3. Shift measurements

Red- or blue-shift spectra were recorded in an Amino-DW2A spectrophotometer as the spectral difference between the dithionite reduced enzyme with and without a saturating amount of inhibitor. The wavelengths were calibrated with a Holmium filter. Mitochondria were first reduced by adding a few grains of sodium dithionite and the base line was recorded. Inhibitor was then added to the sample cuvette and the same volume of solvent to the reference cuvette. The spectra were recorded at 25°C in the MR3 buffer.

2.4. bc_1 complex activity and inhibitor titration

DBH_2-cytochrome *c* reductase activity was determined spectrophotometrically [19] in isolated mitochondria by measuring the reduction of cytochrome *c* (40 μM) with the dual wavelength-stirred reaction cuvette procedure at 550 nm using 540 nm as a reference. The reaction was observed at 25°C in a phosphate buffer (50 mM phosphate-K, 50 μM EDTA, pH 7.4), in the presence of 2 mM KCN with a synthetic analog of ubiquinol (100 μM), decylubiquinol (2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinol) as electron donor. The slight chemical reduction of cytochrome c which occurred with decylubiquinol in the absence of mitochondria was substracted from the enzymatic assay with mitochondria.

The I_{50} values for inhibitors, defined as the concentration required to reduce the decylubiquinol cytochrome *c* reductase activity by 50%, were deduced from the inhibitor titration curves obtained at increasing concentrations of each inhibitor. With each aliquot of mitochondria, the inhibitor was incubated for 3 min before the measurement was performed, as that it could penetrate into the membrane. I_{50} was calculated in moles of inhibitor per mole of cytochrome *b* by measuring the spectral cytochrome *b* content in each strain (not shown; as in ref [11]). In each strain the relative inhibitor titer $(I_{50})_r$ has been taken to be the ratio between I_{50} with the mutated strain and I_{50} with the wild type strain. This parameter indicates the resistance of the strain to the inhibitor, in comparison with the wild type strain.

2.5. Chemicals

Inhibitors were used in the form of stock ethanolic or Me_2SO solutions. Antimycin and decylubiquinone were from Sigma, myxothiazol from Boehringer and funiculosin was a generous gift from Sandoz Laboratory.

3. Results

3.1. General characteristics of the strains

The cytochrome *b* mutant S206L did not grow on non-fermentable substrates (glycerol, ethanol, ...) but continued to show a low bc_1 complex activity, while the antimycin binding site was not modified [11]. This mutation in position 206 was localized at the edge of the transmembrane α-helix IV, at the beginning of the extramembranous loop between α-helices IV and V (matrix side). A thermosensitive revertant S206L–N208K has been selected from this mutant, which recovered respiratory growth and shows efficient respiratory activities [17].

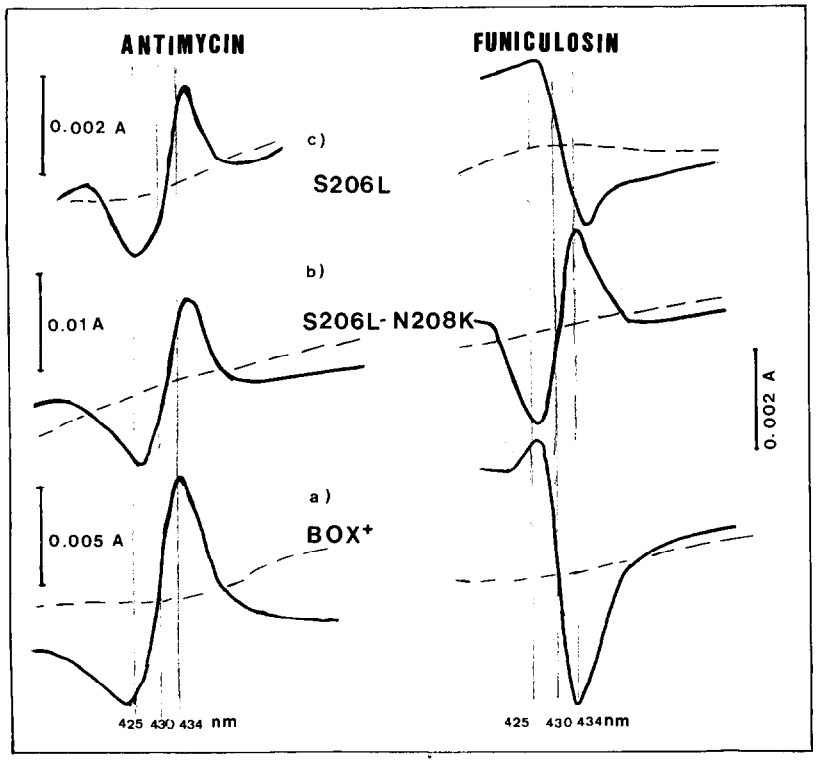

Fig. 1. Spectral shift of reduced cytochrome *b* induced by the binding of two center N inhibitors in the wild type strain, a cytochrome *b* respiratory-deficient mutant and a revertant. The sample and reference cuvettes were reduced with dithionite. The broken line indicates the difference spectra without any added inhibitor; the inhibitor was added to the sample cuvette in saturating amounts in relation to the cytochrome *b* content (10 μM with antimycin, 20 μM with funiculosin) and the same volume of solvant was placed in the reference cuvette. In each strain, the intensity of the shift is dependent on the cytochrome *b* content. The strains were: (a) wild type BOX^+, (b) revertant S206L–N208K and (c) original cytochrome *b* mutant S206L. Cytochrome *b* content – in antimycin assays: BOX^+, 0.95 nmol/ml; S206L–N208K, 1.13 nmole/ml; S206L, 0.89 nmol/ml; in funiculosin assays: BOX^+, 0.84 nmol/ml; S206L–N208K, 0.9 nmol/ml; S206L, 0.89 nmol/ml.

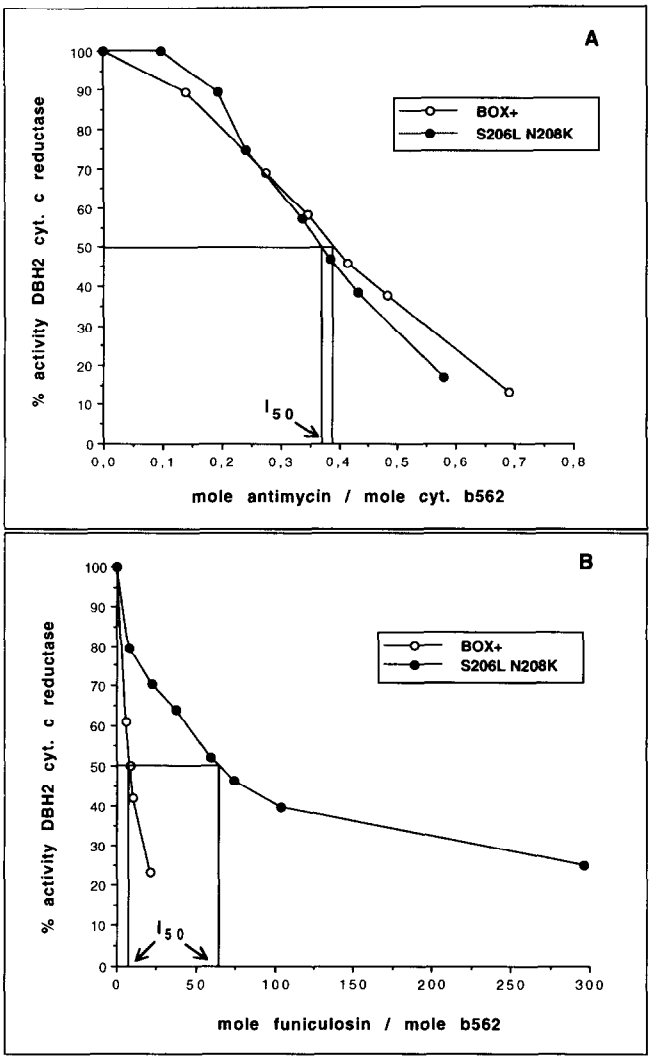

Fig. 2. Inhibition of the DBH_2 cytochrome *c* reductase activity by funiculosin and antimycin. The activity was measured in the dual wavelength mode at 550–540 nm at 25°C in a stirred cuvette; 100% activity was 77 s^{-1} and 53 s^{-1} with the wild type and revertant, respectively. (A) The inhibitor titer I_{50} with antimycin was 0.39 mol antimycin/mol cytochrome b_{562} in the case of the wild type strain. The relative inhibitor titer $(I_{50})_r$ with the revertant (I_{50} revertant/I_{50} wild type) was 0.95 in the case of antimycin. (B) The inhibitor titer I_{50} with funiculosin was 8.4 mol of funiculosin per mole of cytochrome b_{562} in the case of the wild type strain. The relative inhibitor titer with the revertant was 7.5 in the case of funiculosin.

Like mutant S206L, the cytochrome *b* mutant M221K did not grow on respiratory substrates. This strain synthesized a cytochrome *b* content which was about 70–80% that of its parental strain (box^+), but did not retain bc_1 complex activity [20]. However, no subunit of its bc_1 complex was found to be missing, as shown by the electrophoretic profile of the isolated complex [19].

3.2. Red- or blue-shift spectra

With the parental strain KM91, the difference spectra observed after adding saturating amounts of antimycin or funiculosin to the reduced mitochondria were similar in shape to those previously observed in the α and γ absorption bands of bovine heart cytochrome *b* [15,21]. These S-shaped curves crossed the baseline at 562 nm in the case of the α-band and 430 nm in the case of the γ-band. The spectrum recorded after adding antimycin had a minimum at 425 (γ-band) and 559 nm (α-band) and a maximum at 434 nm and 565 nm: this long wave maximum was interpreted as reflecting a shift of cytochrome b_{562} toward longer wavelengths (bathochromic effect). As the Soret bands were by far the most intense and as they were reproducible, they were used in this study in order to compare the action of the inhibitors with the various strains. After adding funiculosin, the spectrum was a mirror-image of that observed in the presence of antimycin, with a short wavelength shift of the b_{562} spectrum (hypsochromic effect) (Fig. 1a). Similar results were obtained with the cytochrome *b* respiratory-deficient mutant S206L, except that the maximum and the middle point were displaced towards the red by about 1.5 nm, which is in agreement with the fact that heme b_{562} in this mutant is selectively shifted in the same way by 1.5 nm [11] (Fig. 1c). Antimycin had the same effect on the revertant S206L–N208K, as on the wild type and mutant, namely a red-shift of cytochrome *b*, while a different situation occurred with funiculosin: instead of the usual blue-shift generated by this inhibitor, a red-shift was observed in this double mutated strain (Fig. 1b). As the mutation S206L alone in the original mutant did not modify the type of action of funiculosin on the cytochrome *b* spectra, this spectral change from an hypsochromic to a bathochromic effect is attributed to the replacement of asparagine 208 by lysine in addition to the original mutation.

3.3. Inhibitor titrations of wild-type and revertant

The inhibitory action of antimycin and funiculosin at the bc_1 complex level was illustrated by Fig. 2. From these curves, the I_{50} values were determined as described in section 2.4. The relative inhibitor titer with the revertant S206L–N208K was

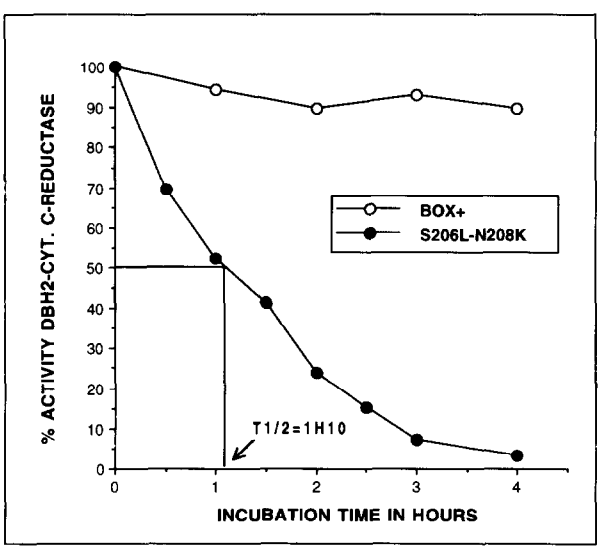

Fig. 3. Effect of incubation at a non permissive temperature (37°C) on the complex III activity of mitochondria isolated from wild type and thermosensitive revertant cells grown at a permissive temperature (28°C). The DBH2 cytochrome *c* reductase activity was measured at 37°C; 100% activity was 128 s^{-1} with the wild type BOX^+ and 70 s^{-1} with the revertant. The $t_{\frac{1}{2}}$ (50% activity decrease) was 1 h 10 min in the case of the thermosensitive strain S206L–N208K.

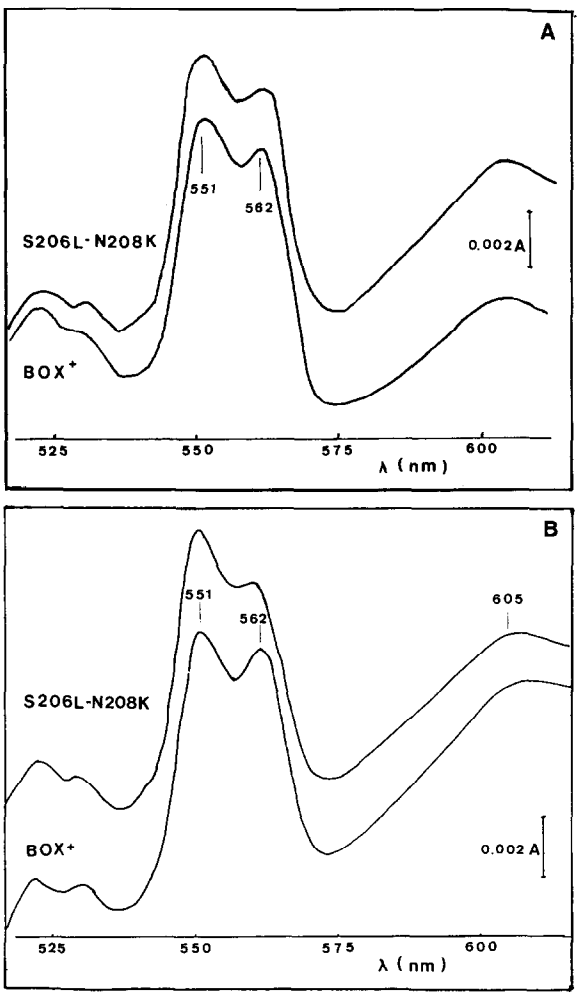

Fig. 4. Effect of incubation at a non-permissive temperature (37°C) on the cytochrome spectra. Difference spectra of mitochondria were recorded in the split mode between 500 and 650 nm before (A) and after (B) incubation for 6 h at 37°C. The sample cuvette was reduced with dithionite and the reference cuvette was oxidized with ferricyanide. Mitochondria of the wild type strain BOX$^+$ and of the revertant S206L–N208K were suspended at 1 mg/ml in the MR3 buffer. Spectral cytochrome *b* content: (A) before incubation: 0.38 nmol/ml with BOX$^+$; 0.35 nmol/ml with the revertant S206L–N208K; (B) after incubation: 0.34 nmol/ml with BOX$^+$; 0.31 nmol/ml with the revertant S206L–N208K.

found to be equal to 1 with antimycin and 7.5 with funiculosin. These values reflect the resistance of this revertant towards the inhibitors, directly evaluated at the bc_1 complex level: no resistance was observed with antimycin and about 7-fold more resistance than with the wild-type was shown with funiculosin.

3.4. *Thermosensitivity of cytochrome bc_1 complex activity in mitochondria of the revertant*

The growth of the thermosensitive revertant S206L–N208K on the respiratory substrates (ethanol or glycerol) is abolished at 37°C. Mitochondria were isolated from the wild type yeast (box$^+$) and the revertant cells grown at permissive temperature (28°C) on galactose medium. Under these conditions, the revertant synthesized a cytochrome *b* content which was quasi-identical to that of the wild-type, (about 90%) and exhibited the same growth yield [17].

To determine whether the activity of the bc_1 complex is affected by the temperature when the cells are grown at their permissive temperature (28°C), (i.e. under the conditions where the complex is functional), the DBH_2-cytochrome *c* reductase activity was measured as a function of the incubation time at a non-permissive temperature (37°C). As shown in Fig. 3, the in vitro bc_1 complex activity of the wild-type was quite stable, while the mitochondria of the revertant are susceptible to be inactivated at the high temperature: all the activity was abolished after exposure of the mitochondria from this strain at 37°C for 4 h (Fig. 3).

In order to check whether the cytochrome *b* heme is still present in that strain when the activity has been abolished, the difference spectrum of mitochondria of the revertant and that of the wild-type strain were recorded (dithionite reduced versus oxidized samples), before and after exposure for 6 h at 37°C (Fig. 4). No or slight decrease (about 10% as in Fig.4) in the spectral cytochrome *b* was observed with both strains, and the ratio of cytochrome *b* content with the revertant to that with the parental strain remained to be about 90% after like before the incubation: this means that incubation at the non-permissive temperature does not affect the attachment of cytochrome *b* heme. In the same way, the various subunits of the bc_1 complex were still present (G. Brasseur, P. Brivet-Chevillotte article in preparation).

3.5. *Thermosensitivity of the two center N inhibitor binding to the bc_1 complex*

Blue- or red-shifts induced by the inhibitors reflect their binding to cytochrome *b*. They were always determined in this study at saturating concentration of inhibitor (while a titration curve would require lower amounts of inhibitor [22]). As it was confirmed that cytochrome *b* heme is still present after abolition of the bc_1 complex activity in the revertant, it was possible to qualitatively test the binding of the inhibitors depending on whether or not the shift occurred, after incubation at the non-permissive temperature and by making comparisons with the wild type strain. After exposure for 5 h at 37°C, the red-shift resulting from antimycin binding was observed in the revertant as well as in the wild-type mitochondria (Fig. 5). On the other hand, the (red) shift observed with funiculosin completely disappeared in the revertant, while the blue-shift persisted in the wild-type mitochondria (Fig. 5). These data therefore suggest that antimycin binding is not strongly modified in the revertant, while funiculosin binding seems to be abolished, as is the

Table 1
Summary of the bc_1 complex activity and inhibitor binding observed after incubating mitochondria isolated from the wild type strain and the thermosensitive revertant for 5 h at 37°C

Strains	DBH2-Cyt-*c* reductase activity	Funiculosin binding (shift)	Antimycin binding (shift)	Myxothiazol binding (shift)
Wild type	+	+	+	+
S206L–N208K	–	–	+	+

The DBH_2 cytochrome *c* reductase activity was measured as in Fig. 2. As the intensity of the spectral shift is generally proportional to the bound inhibitor [3], the inhibitor binding was estimated depending on whether or not the shift was induced in cytochrome *b* after incubation at non-permissive temperature, as in Fig. 5.

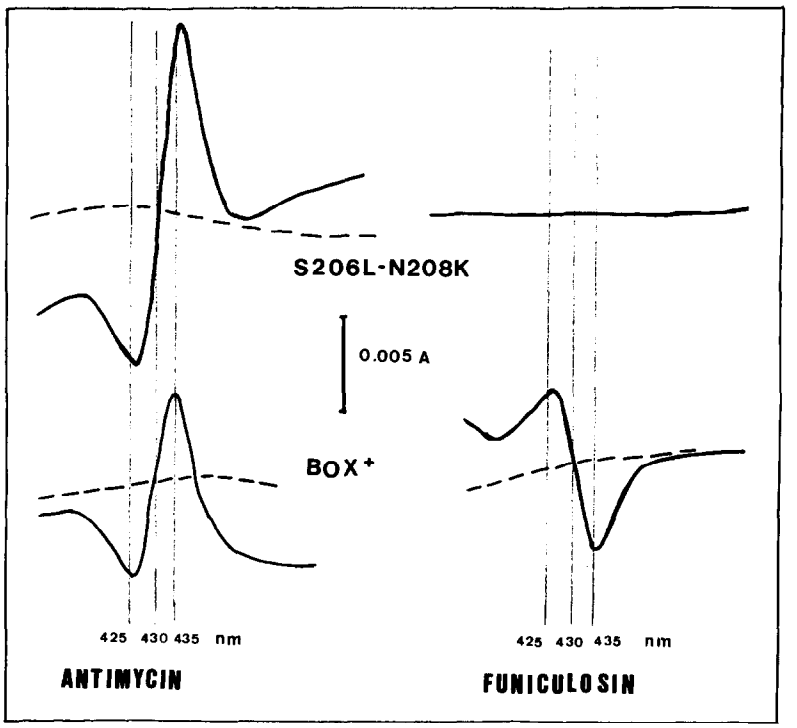

Fig. 5. Spectral shift of reduced cytochrome *b* induced by the binding of antimycin or funiculosin in the wild type and thermosensitive revertant mitochondria, incubated for 5 h at 37°C. Conditions were as in Fig. 1, except that the mitochondria were incubated for 5 h in the MR3 buffer at 37°C. Wild type strain = BOX^+; cytochrome *b* revertant = S206L–N208K. Cytochrome *b* content – in antimycin assays: BOX^+, 1.16 nmol/ml; S206L–N208K, 1.53 nmol/ml; in funiculosin assays – BOX^+, 1.37 nmol/ml; S206L–N208K, 0.94 nmol/ml.

bc_1 complex activity, after incubation at non-permissive temperature. These results are summarized in Table 1.

3.6. *Another case: mutant M221K*

In this strain, the replacement of methionine by lysine which did not pertub a correct assembly of the bc_1 complex, induced the loss of electron transfer through this complex. Unlike what occured with the revertant S206L–N208K, the antimycin binding was strongly affected [20], since the dissociation constant (as measured by fluorescence) increased from about 10^{-12} M with the wild type, to about 10^{-3} M with this mutant. With a large excess of inhibitor a (diminished) red-shift was still observed however with antimycin, while the spectral blue-shift induced by funiculosin in the wild type strain completely disappeared in that mutant (Fig. 6). So in this center N mutant, like in the thermosensitive inactivated center N revertant S206L–N208K, a concomitant loss of activity and of the shift due to the funiculosin binding was observed. On the other hand, the center P inhibitor myxothiazol induced an identical red-shift in both strains (not shown).

4. Discussion

With the revertant S206L–N208K, a red-shift of cytochrome b_{562} heme was induced by saturating amounts of funiculosin instead of the blue-shift usually observed with the wild type species: this switch from a hypsochromic to a bathochromic effect reflects a considerable change in the electronic surroundings of heme b_{562}, and in the binding niche of funiculosin. As the original mutant S206L like the wild type strain, exhibits a blue-shift, this reversal of the shift direction must have been due to the addition of a second mutation whereby asparagine 208 was replaced by a charged lysine. A similar change from a blue to a red-shift was also observed (unpublished results) with another proximal revertant isolated from mutant S206L [17], namely S206L–N208Y and with a distal one S206L–W30C; but the strain constructed with the single mutation W30C [17] exhibits a blue-shift and has wild type characteristics (data not shown). These data suggest that the nature of the effect induced by funiculosin (hypso or bathochromic) is depending on the interraction of a second mutation with the original S206L mutation. One common feature between these three reversions is that they have either a hydrophylic amino-acid in replacement of a hydrophobic one in position 30 (W30C) or a bulkier one in position 208 (N208K or N208Y): this second mutated amino acid might compensate the loss of the well-conserved hydrophylic amino acid serine in the original mutant S206L and allows restoration of bc_1 complex activity [17]; it also confers thermosensitivity to the revertant and the observed red-shift with funiculosin. With antimycin on the contrary, no change is observed in the (red) shift induced by this inhibitor with the revertant S206L–N208K (or with any of the other strains tested).

From the titration curves of the two inhibitors at the complex III level, it was deduced that the revertant S206L–N208K behaves like the wild type strain in response to antimycin ($(I_{50})_r = 1$) but is resistant to funiculosin ($(I_{50})_r = 7$). No resistance to either antimycin [11] or funiculosin has been found to occur with the original mutant S206L, while a resistance to funiculosin was observed with another revertant of this mutant,

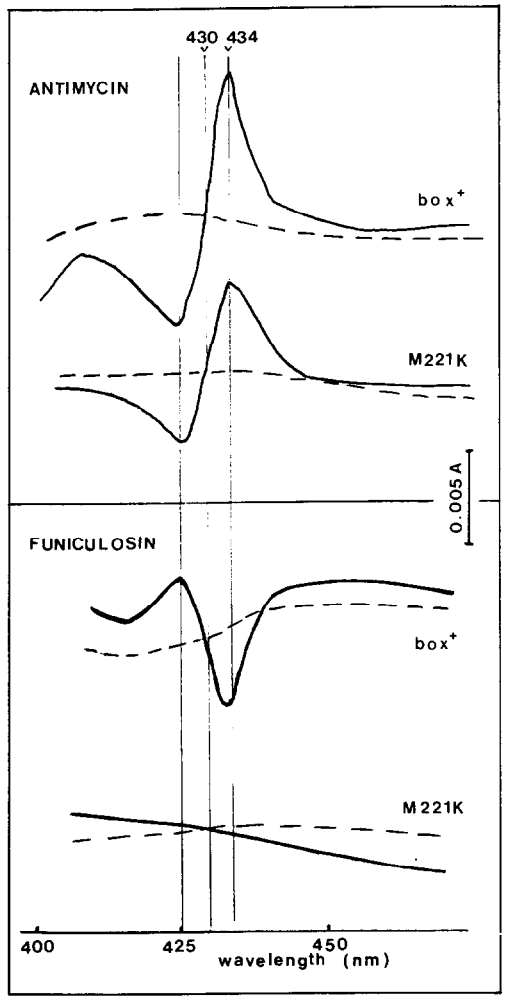

Fig. 6. Spectral shifts of dithionite reduced cytochrome *b* induced by antimycin and funiculosin binding in the wild type BOX^+ and the cytochrome *b* respiratory-deficient mutant M221K. The antibiotics were added at saturating level: 5 μM antimycin and 20 μM funiculosin. Cytochrome b_{562} content of each strain: 1.2 nmol/ml.

namely S206L–N208Y [23]. The replacement of asparagine 208 by either lysine or tyrosine specifically alters the funiculosin binding. These data confirm the involvement of asparagine 208 in funiculosin binding site.

A long incubation of the thermosensitive revertant at non-permissive temperature leads to the inactivation of the bc_1 complex activity (Fig. 3), and this inactivation shows up particularly clearly in the reduction kinetics of cytochrome *b* by the center N pathway (Brasseur G. and Brivet-Chevillotte P., article in preparation). Under these conditions, the red-shift induced by saturating amounts of either antimycin (Fig. 5) or the center P inhibitor myxothiazol (data not shown) was not strongly affected, so that it can be assumed, that these inhibitors still bind to cytochrome *b*. The shift induced by funiculosin completely disappeared on the contrary in the revertant S206L–N208K, so that it might be concluded that the structural perturbation of the tertiary structure induced by incubation at non-permissive temperature involved not only the loss of bc_1 complex activity but also seems to abolish the funiculosin binding. An almost similar situation with the concomitant loss of activity (due to center N modification) and likely funiculosin binding has been observed with the cytochrome *b* respiratory-deficient mutant M221K (Fig. 6).

In addition to showing that asparagine 208 and methionine 221 are involved in the funiculosin binding site, these data taken as a whole suggest that:

(i) *At least part of the funiculosin binding site is very close to the b_{562} heme.* This is in agreement with the first location of a funiculosin resistance mutation in yeast, which was found to be L198F [24]. Moreover, Degli Esposti et al. [25] hypothesized that position 194 might be involved in funiculosin binding because in the horse, which is naturally resistant to funiculosin, alanine194 is replaced by valine. These two positions (194, 198) are close to histidine197 linking the b_{562} heme to the transmembrane helix IV. Besides, funiculosin is the only bc_1 inhibitor which has been found to induce a large shift on the midpoint potential of heme 562 (by about 100 mV [12]); this might be explained by the close proximity of its binding site with this heme.

(ii) *The antimycin and funiculosin binding sites partly overlap but are not identical.* Indeed, co-resistance to these two inhibitors has been identified so far only in position G37 with *M. musculus* [26], *Paramecium* [27], *S. cerevisiae* [28] and *S. pombe* (A37) [29], in position M221 in yeast (mutant M221K described above) and in position G232 in the mouse [30]. The amino-acid in the latter position is not conserved and threonine is present instead of glycine in yeast. These two areas (37 and 221–232) are neighbouring ones within the 3D structure of the protein [17]. No antimycin resistance were observed in the 197–208 region, where funiculosin resistant mutants have been identified [23,24].

(iii) *The funiculosin binding site is in the close vicinity of the catalytic center N site.* Center N inhibitors block cytochrome b_{562} heme oxydation by quinone species in the steady-state electron transfer. With the thermosensitive revertant incubated at non-permissive temperature, mutations in positions 206 and 208 located at the edge or even inside transmembranous helix IV might initially favour a local distorsion of the tertiary structure of cytochrome *b* and probably increase the distance inside and/or between the helices, so that the electron transfer is blocked and/or the quinone binding site affected. Due to its location (at 6–10Å from the N-side protein [31]) and to its sensitivity to antimycin, the EPR-detectable semiquinone $Q^{\bullet}i^-$ (or $Q^{\bullet}n^-$) binding site might be close to the antimycin binding site which was estimated to be 17 Å from cytochrome b_{562} heme [32]; this heme b_{562} was established to be located approximately in the middle of the membrane (estimated to be 40–50 Å in width) [33]. Rich et al. [12] hypothesized that this EPR-detectable $Q^{\bullet}i^-$ might be linked to the reduced form of cytochrome b_{562}, while other redox form of EPR-silent ubiquinone species might be present at the Q_N site and interract with heme b_{562}, as previously suggested by Salerno et al. [34]. The funiculosin binding site spread over from the close vicinity of heme b_{562} to the antimycin binding site (near positions 221–232). The concomitant loss of the bc_1 complex activity (due to perturbation in the center N side either near position 208 or 221) and of the shift induced by the binding of funiculosin (suggesting loss or drastic perturbation of this binding) strongly suggest the close

proximity between the funiculosin binding site and the catalytic center N domain.

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