Energy-linked spectral shift of ferrocytochrome *b* in beef heart submitochondrial particles

Wolfram S. Kunz and Alexander Konstantinov*

A.N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow 117234, USSR

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Cytochrome b *Energy-linked spectral shift* *Q-cycle* *2-*n*-nonyl-4-hydroxyquinoline* N*-oxide*

1. INTRODUCTION

A respiratory chain inhibitor antimycin has long been known to bring about a shift of the ferrous cytochrome *b*-562 α-absorption band by about 1–2 nm to the red [1–3]. This red shift was sometimes considered to be a specific effect of antimycin [4,5] since it had not been observed in animal mitochondria with an antimycin-type inhibitor HOQNO [3]. At the same time, rather similar perturbations of the *b* cytochrome α-band by antimycin, HOQNO and mucidin have been described in yeast mitochondria [6,7].

Here, we report on the spectral changes of *b* cytochromes brought about by HOQNO and mucidin in beef heart SMP. A spectral response very similar to that induced by HOQNO is also observed upon addition of ATP to dithionite-reduced SMP. The effects of the ligands and of the energization are tentatively ascribed to redistribution of ferrocytochrome *b*-566/*b*-562 between 2 conformational states.

2. METHODS

Phosphorylating Mg, Mn, succinate and ATP-submitochondrial particles (SMP) were prepared from beef heart mitochondria essentially as in [8]. Spectral measurements were made in an Aminco-DW2^{2TM} instrument in standard rectangular 1-cm quartz cells at 30°C. Antimycin and 2-*n*-nonyl-4-hydroxyquinoline *N*-oxide (HOQNO) were from Serva. Dithionite (Lab. grade) was purchased from Merck. Mucidin was a generous gift from Dr V. Musilek (Institute of Microbiology, Acad. Sci. ČSSR, Prague).

3. RESULTS

Fig.1 compares the effects of antimycin, HOQNO and mucidin on the absorption spectrum of the dithionite-reduced SMP. In agreement with the earlier reports [1–3] antimycin induces a derivative-shaped spectral response centered at ~562–563 nm (spectrum a) which is known to originate from the red shift of the ferrous cytochrome *b*-562 α-absorption band.

It can be seen that two other ligands of site 2, HOQNO and mucidin, also give rise to spectral changes in the vicinity of *b* cytochrome α-band. Spectral shifts of ferrocytochrome *b* brought about by mucidin and HOQNO were observed previously in yeast SMP [6,7]. However, the effects of these two antibiotics were reported to be of about the same size as the antimycin-induced response, which is clearly not the case with the beef heart preparation. The small magnitude of the HOQNO-induced spectral changes may explain why the effect of this inhibitor had not been noticed in [3].

* To whom correspondence should be addressed

Abbreviations: HOQNO, 2-*n*-alkyl-4-hydroxyquinoline *N*-oxide; SMP, submitochondrial particles

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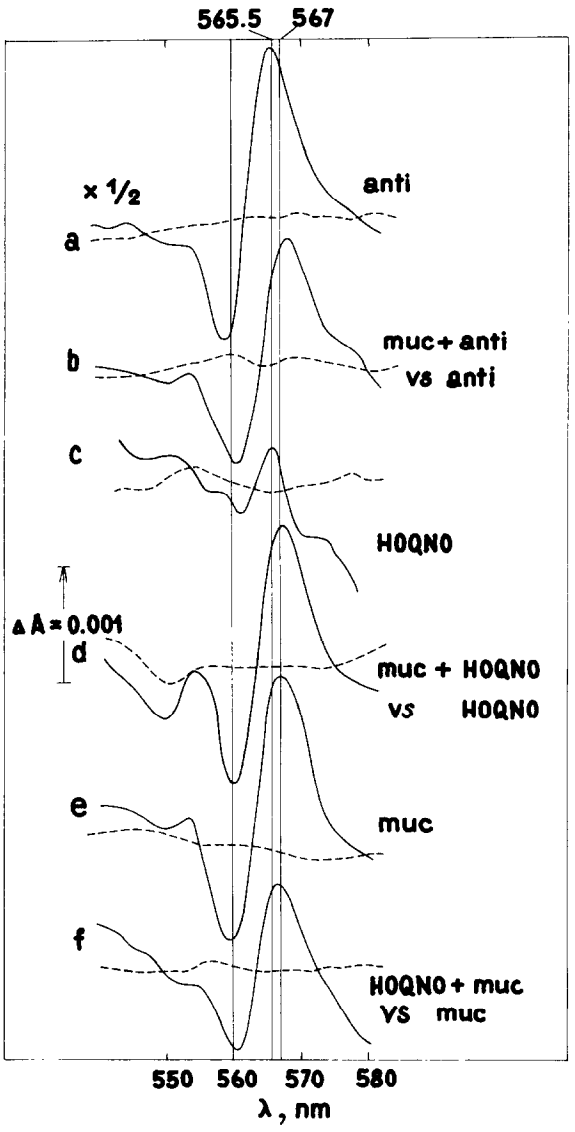

Fig.1. Spectral responses of reduced *b* cytochromes to antimycin, HOQNO and mucidin. Beef heart SMP (2.4 mg protein/ml) in the medium containing 0.29 M sucrose, 20 mM HEPES-KOH (pH 7.5), 0.5 mM EDTA and 4 mM KCN were reduced by solid dithionite (1–2 mg/ml). Additions: antimycin, 1.5 μM; mucidin, 6.5 μM; HOQNO, 5.7 μM. Equal amounts of ethanol were added to the reference (final concentration not more than 0.5%). The dotted baselines correspond to absorption difference between the sample and reference cells in the absence of $b \cdot c_1$ inhibitors (a,c,e), or in the presence of antimycin (b), HOQNO (d) and mucidin (f). Note the changed ordinate scale in the case of the antimycin-induced effect (a).

The spectral perturbation brought about by mucidin with A_{min} at 560 nm, A_{max} at ~567 nm and isobestic point at ~564 nm is clearly different and virtually independent of the shifts induced by antimycin and HOQNO, but closely resembles the effect of myxothiazol (not shown; see also [9]). In [9] the myxothiazol shift was assigned to heme *b*-566. At the same time our preliminary experiments at controlled redox potentials gave some evidence that in isolated complex $b \cdot c_1$, both *b* cytochromes are involved in the spectral effect of mucidin [10]. Notably, in the antimycin-treated SMP, the mucidin-induced difference spectrum appears to be shifted slightly to the red (cf. spectra b, d, e in fig.1), which is consistent with the suggested contribution of ferrocytochrome *b*-562 to the spectral response evoked by mucidin [10].

Relationships between the effects of HOQNO and antimycin are not yet clear. The extremes of the two responses are not appreciably different although the exact lineshape and magnitude of the HOQNO-induced difference spectrum proved somewhat variable at this high sensitivity of the instrument. Anyhow, antimycin prevented the effect of HOQNO (not shown) whereas mucidin did not (fig.1e). As the modes of action of HOQNO and antimycin on electron transfer are much the same and the two inhibitors probably compete for the same specific binding-site [11,12], it is reasonable to assume provisionally that HOQNO, like antimycin, affects the spectrum of ferrocytochrome *b*-562.

We have found that ATP addition to dithionite-reduced SMP results in a perturbation of cytochrome *b* spectrum rather similar to that induced by HOQNO (cf. spectra b and d in fig.2). The effect is characterized by a trough at 560 nm and a peak at 565 nm. Whether there are additional weaker extremes cannot yet be said with certainty. The amplitude of the energy-linked difference spectrum was consistently ~1/3rd of the HOQNO-induced spectrum. However, it has to be mentioned that according to our observations only about 30% of cytochrome chains respond to energization of beef heart SMP by ATP, when energy-linked oxidation–reduction of cytochromes *b*-566, *b*-562 and $c+c_1$ [13], or a spectral shift of ferric cytochrome oxidase [14,15] were measured. Therefore, the specific magnitudes of the spectral effects of ATP and HOQNO on *b* cytochromes may be in fact ra-

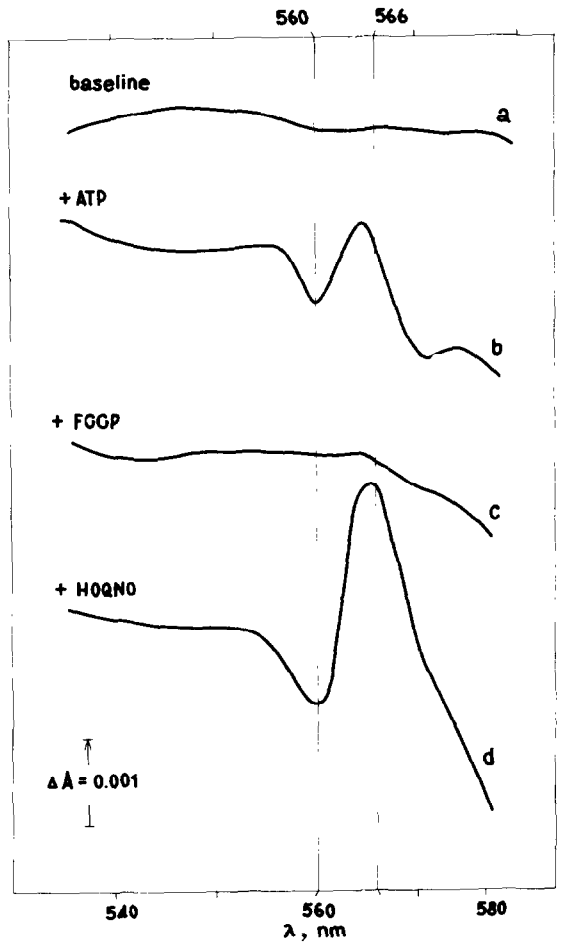

Fig.2. Energy-linked perturbation of ferrocytochrome *b* spectrum. Beef heart SMP (4.8 mg protein/ml) in the medium containing 0.29 M sucrose, 20 mM HEPES-KOH (pH 7.5), 5 mM $MgSO_4$ and 4 mM KCN were reduced by excess dithionite. Baseline recorded (a), 6 mM ATP was added to the sample and the spectrum immediately scanned (b); the response was abolished by 1 μM FCCP (c) and, finally, 7.5 μM HOQNO was added to sample (d). Equal volumes of H_2O (b) or ethanol (c,d) were added each time to the reference.

ther similar. The ATP-induced response of cytochrome(s) *b* is fully reversed by the uncoupler FCCP (spectrum c). Antimycin almost completely prevented the energy-linked spectral changes and HOQNO was at least partially inhibitory, whereas mucidin exerted no significant effect on the energy-linked response (not shown).

4. DISCUSSION

First of all, it has to be emphasized that the absorption changes in the spectrum of reduced SMP brought about by ATP are very small. But many more experiments are required for elucidation of the nature of the energy-dependent response in the vicinity of ferrocytochrome *b* α-absorption band. The following possibilities may be concerned:

4.1. *Oxidation–reduction*

This one is unlikely not only because of the excess dithionite present but also since the shape of the difference spectrum is not consistent with such a possibility (including simultaneous oxidation of *b*-562 and reduction of *b*-566).

4.2. *Electrochromic effect*

The hemes of ferric *b* cytochromes have been reported to be oriented normal to the plane of the mitochondrial membrane [16]. If the same is true for the reduced *b* cytochromes, electrochromic effect(s) associated with the in-plane polarized Q_{ox} and Q_{oy} transitions in the porphyrin ring can be expected upon $\Delta\psi$ generation across the energized membrane (see [17] for a comprehensive discussion), although the magnitude of such effects is difficult to evaluate ab initio.

4.3. *Conformational shift*

This possibility is conceptually appealing since *b* cytochromes are believed to be involved in energy-conservation in site 2. Recently we suggested that the cytochrome couple *b*-566/*b*-562 can exist in 2 alternative conformations $[b]_o$ and $[b]_1$ associated with centres *o* and 1 of the Q-cycle [10]. It is tempting to extend this hypothesis by assuming that:

(i) The 'red-shifted' and 'normal' positions of ferrocytochrome *b*-562 α-band correspond to the above two conformations $[b]_o^{Red}$ and $[b]_1^{Red}$, respectively, the latter being somewhat more stable thermodynamically; ligands, such as antimycin or HOQNO, could stabilize $[b]_o^{Red}$ relative to $[b]_1^{Red}$ to a different extent and thus give rise to a 'red shift' of variable size.

(ii) The thermodynamically favourable transition $[b]_o^{Red} \longrightarrow [b]_1^{Red}$ is coupled to membrane energization, presumably, by virtue of electrogenic proton uptake from the M-phase by reduced *b*-562. Hence the transition can be reversed at

least partially by ATP, resulting in the same 'red shift' as observed with HOQNO.

We have recently confirmed an important finding in [18] that in *Rhodopseudomonas sphaeroides* the antimycin-induced 'red shift' of cytochrome *b*-560 (a counterpart of mitochondrial *b*-562) decays at alkaline pH with a half-transition point at pH ~8 fairly close to pK_r of this hemoprotein. In addition HOQNO has been found to induce a 'red shift' similar to that brought about by antimycin and with roughly the same pH-dependence (experiments in collaboration with Dr Stella Dracheva to be published elsewhere). No such pH-dependence was observed in SMP for spectral changes induced by antimycin, HOQNO or mucidin (not shown, [10]). Accordingly, in beef heart SMP, pK_r of *b*-562 is not reached in the pH range 6–9 [19]. The pH-dependence of the spectral shift of *b*-560 in *R. sphaeroides* nicely fits the hypothesis of the $[b]_o^{Red} \longrightarrow [b]_1^{Red}$ transition linked to proton binding. It can be visualized that above pK_r, reduced cytochrome *b* does not take an H^+ and cannot relax to $[b]_1^{Red}$ state remaining in the 'red-shifted' $[b]_o^{Red}$ conformation. Consequently no additional 'red shift' is brought about by antimycin or HOQNO at alkaline pH.

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