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2-Nitrosofluorene and *N*-hydroxy-2-aminofluorene react with the ubiquinone-reduction center (center N) of the mitochondrial cytochrome bc_1 complex

Peter-Christian Klöhn[a], Ulrich Brandt[b], Hans-Günter Neumann[a,*]

[a]*Institut für Toxikologie, Universität Würzburg, Versbacher Str. 9, 97078 Würzburg, Germany*
[b]*Zentrum der Biologischen Chemie, Universität Frankfurt, Frankfurt, Germany*

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Abstract We determined the sites of artificial electron transfer onto 2-nitrosofluorene (NOF), a metabolite of carcinogenic 2-acetylaminofluorene in mitochondria and isolated cytochrome bc_1 complex. NOF-induced O_2 consumption in mitochondria was sensitive to antimycin A, but insensitive to myxothiazol. In the isolated cytochrome bc_1 complex, NOF induced rapid MOA-stilbene-insensitive reoxidation of cytochrome *b*, whereas in the presence of antimycin A, reoxidation was very slow. The corresponding hydroxylamine, *N*-hydroxy-2-aminofluorene (N-OH-AF), reduced cytochrome *b* specifically through center N of the cytochrome bc_1 complex. We conclude that NOF and N-OH-AF bind to center N of the cytochrome bc_1 complex and act as electron acceptor and donor, respectively. The N-OH-AF/NOF interconversion is considered to be involved in the cytotoxicity of 2-acetylaminofluorene in vivo.

Key words: Cytochrome bc_1 complex; Cytochrome *b*; 2-Nitrosofluorene; *N*-Hydroxy-2-aminofluorene; Mitochondria (rat liver)

1. Introduction

2-Nitrosofluorene (NOF) and *N*-hydroxy-2-aminofluorene (N-OH-AF), metabolites of carcinogenic 2-acetylaminofluorene (AAF), induce cyanide-resistant O_2 consumption in isolated mitochondria [1]. It was concluded that NOF is able to drain electrons directly from the respiratory chain. There are at least two sites where this could occur: (1) at the NADH: ubiquinone oxidoreductase (complex I), since oxygen is consumed in mitochondria inhibited by rotenone which blocks electron transfer at complex I (Klöhn and Neumann, submitted); (2) at the ubihydroquinone:cytochrome *c* oxidoreductase (cytochrome bc_1 complex) or beyond because oxygen is also consumed if respiration is driven by succinate instead of NADH [1]. N-OH-AF also autoxidizes rapidly to NOF in aqueous media in two one-electron reduction steps, thereby reducing molecular oxygen to superoxide anion radicals [1]. Electron drainage from the respiratory chain by NOF induces an uncoupling effect on oxidative phosphorylation (Klöhn and Neumann, submitted). These effects are considered to be related to the chronic toxicity of AAF and thus may explain in part the 'promoting' properties of this carcinogen in rat liver. However, the complex interaction of AAF metabolites with mitochondrial respiration is still poorly understood. In the present paper we analyze the reaction of NOF with isolated cytochrome bc_1 complex and how N-OH-AF interacts with the respiratory chain.

According to the widely accepted protonmotive Q-cycle [2,3], the cytochrome bc_1 complex has two ubiquinone-reactive sites, center P, where ubihydroquinone is oxidized, and center N, where ubiquinone is reduced. By spectroscopically monitoring the redox state of the cytochromes in the presence of specific inhibitors [4,5], the function of the two centers can be studied separately. Center P is inhibited by *E*-β-methoxyacrylate inhibitors like myxothiazol (myxo) and *E*-β-methoxyacrylate stilbene (MOA-stilbene [6]). Center N is inhibited by antimycin A (AA).

2. Materials and methods

2.1. Materials

Succinate, rotenone, bovine serum albumin (essentially fatty acid free), antimycin A, myxothiazol and thenoyltrifluoroacetone were purchased from Sigma (Deisenhofen, Germany). NOF and N-OH-AF were synthesized as described previously [1]. NOF and N-OH-AF were dissolved in DMSO immediately before each experiment. The total concentration of DMSO in the incubation media did not exceed 0.3%. Antimycin A, myxothiazol, rotenone and thenoyltrifluoroacetone were dissolved in ethanol.

2.2. Animals

Male Wistar rats (200–260 g) were obtained from Harlan-Winkelmann GmbH (Borchen, Germany) and had free access to standard diet (Altromin 1324, Altrogge, Lage/Lippe, Germany) and water. They were kept under controlled conditions of temperature and humidity on a 12 h light/12 h dark cycle.

2.3. Isolation of rat liver mitochondria

Rat liver mitochondria were isolated as described previously [1]. Protein concentration was determined according to the method of Lowry et al. [7], with bovine serum albumin as standard.

2.4. Preparation of the cytochrome bc_1 complex and spectroscopic measurements

The cytochrome bc_1 complex was prepared from bovine heart mitochondria according to Engel et al. [8] and stored in 10% glycerol at −80°C. The concentration was determined by the reduced minus oxidized heme *b* spectrum at 562–575 nm ($\varepsilon = 28.5$ mM^{-1} cm^{-1}). The reduction of cytochromes *b* and c_1 was followed at 562–575 and 553–540 nm, respectively, in a Shimadzu UV-300 spectrophotometer as described by Brandt et al. [6]. The enzyme was dissolved in 20 mM K^+/MOPS, 0.05% Triton X-100, 100 mM NaCl, 2 mM NaN_3, pH 7.2.

2.5. Polarographic measurements of oxygen consumption

Polarographic determination of oxygen consumption was per-

*Corresponding author. Fax: (49) (931) 201-3988.

Abbreviations: AA, antimycin A; AAF, 2-acetylaminofluorene; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; Myxo, myxothiazol; MOA, *E*-β-methoxyacrylate stilbene; NBH, nonylubihydroquinone (2,3-dimethoxy-6-methyl-5-nonylbenzohydroquinone); NOF, 2-nitrosofluorene; N-OH-AF, *N*-hydroxy-2-aminofluorene; Succ, succinate; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; TTFA, thenoyltrifluoroacetone

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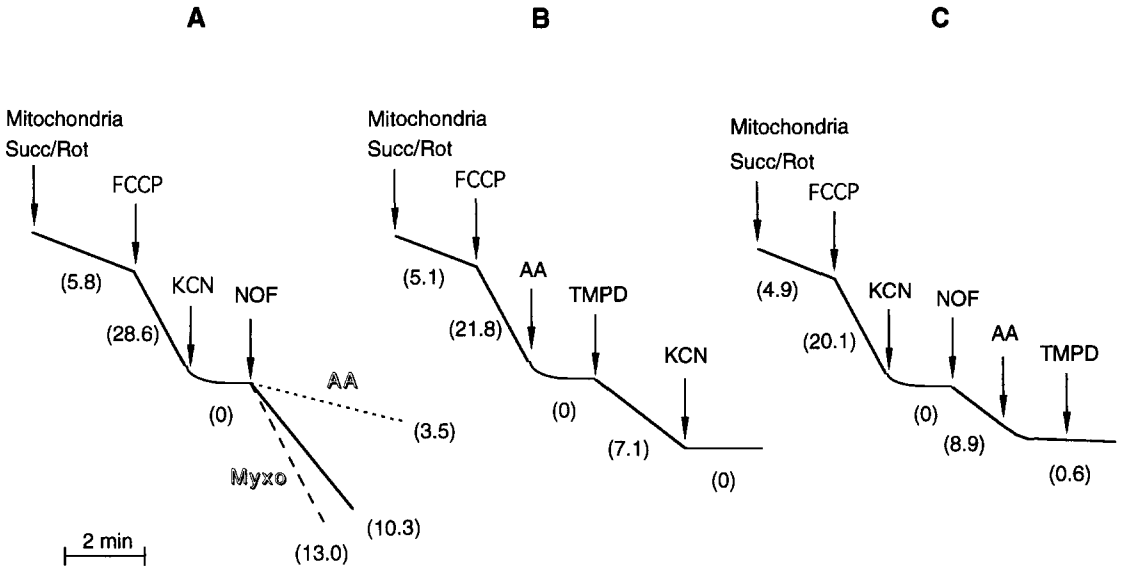

Fig. 1. NOF-induced O_2 consumption in mitochondria in the presence of inhibitors of the bc_1 complex. Mitochondria (2.5 mg/ml) in respiration buffer (pH 7.4), containing 5 mM Succ and 5 μM rotenone, were incubated at 25°C. O_2 consumption was recorded as described in Section 2. (A) 2 μM carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP), 1 mM KCN and 20 nmol/mg NOF were added successively as indicated by arrows (solid line). In addition, mitochondria were preincubated for 4 min with 4 μg/mg antimycin A (AA, dotted line) and 0.4 μg/mg myxothiazol (Myxo, dashed line) prior to the addition of NOF. (B) 2 μM FCCP, 4 μg/mg antimycin A (AA), 50 μM TMPD and 1 mM KCN were added successively as indicated. (C) 2 μM FCCP, 1 mM KCN, 10 nmol/mg NOF, 4 μg/mg AA and 50 μM TMPD were added successively as indicated. Rates of O_2 consumption are given in nmol min^{-1} mg^{-1} mitochondrial protein.

formed with a Clark oxygen electrode as described [1]. Oxygen consumption rates of mitochondrial suspensions were determined in respiration buffer, containing 0.3 M sucrose, 5 mM Na^+/MOPS, 1 mM EGTA, 5 mM KH_2PO_4, 5 mM $MgSO_4$ and 0.1% w/v fatty acid-free bovine serum albumin, pH 7.4. Oxygen consumption in the isolated cytochrome bc_1 complex was measured in incubation medium.

3. Results

3.1. NOF-induced O_2 consumption in mitochondria in the presence of inhibitors of the bc_1 complex

NOF induced cyanide-resistant O_2 consumption in isolated rat liver mitochondria (Fig. 1A), which indicated artificial electron transfer. Two specific inhibitors of the cytochrome bc_1 complex were applied to test whether NOF accepted electrons from this enzyme. In the presence of myxothiazol, an inhibitor of center P, O_2 consumption was slightly increased, while the center N inhibitor antimycin A decreased the rate by more than 60% (Fig. 1A). When both inhibitors were added the rate of O_2 consumption was the same as with antimycin A alone (Table 1). The residual rate was probably due to a leak in the antimycin A block and autoxidation of the reduced quinone formed by succinate dehydrogenase (see below). Preincubation with thenoyltrifluoroacetone (TTFA), a specific inhibitor of succinate dehydrogenase [9], inhibited NOF-induced O_2 consumption almost completely (Table 1).

Table 1
Effects of inhibitors on NOF-induced O_2 consumption in succinate-dependent respiration

Additions				O_2 consumption (nmol min^{-1} mg^{-1})
		KCN	NOF	10.3 ± 1.1
	TTFA	KCN	NOF	10.6 ± 0.3
	Myxo	KCN	NOF	13.0 ± 0.7
	AA	KCN	NOF	3.5 ± 0.3
AA	Myxo	KCN	NOF	3.3 ± 0.2

TTFA was added at a concentration of 100 μM. Other additions were the same as in Fig. 1. The respiratory rates are given in nmol min^{-1} mg^{-1} mitochondrial protein (mean values ± S.D. of four separate experiments).

3.2. Effects of NOF on succinate-dependent respiration in the presence of antimycin A and TMPD in mitochondria

To test for possible reaction sites of NOF downstream of the the cytochrome bc_1 complex, the artificial electron carrier *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) was employed. TMPD allows bypass of the antimycin A block thereby restoring respiration ([10,11], Fig. 1B). O_2 consumption ceased when potassium cyanide (KCN), which blocks O_2binding to cytochrome *c* oxidase, was added. On the other hand, when cyanide-resistant O_2 consumption of NOF was inhibited by antimycin A, addition of TMPD did not increase residual O_2 consumption, indicating that NOF is only reduced at the bc_1 complex (Fig. 1C).

3.3. Reactions of NOF with cytochrome b in isolated cytochrome bc_1 complex

When NOF was added to isolated bc_1 complex that had been reduced by nonylubihydroquinone (NBH) in the presence of MOA-stilbene, which blocks electron transfer through center P [6], rapid oxidation of part of cytochrome *b* was observed (Fig. 2a). Comparable results were obtained in the absence of inhibitors (data not shown). In contrast, in the presence of antimycin A, NOF oxidized cytochrome *b* at a much slower rate (Fig. 2b), which was even slower when both inhibitors were present (Fig. 2c). The residual rate is most likely due to rapid redistribution of antimycin A between the binding sites of the enzyme dimer and the lipids still bound to the isolated complex, respectively [12]. We conclude that NOF can accept electrons from reduced cytochrome *b* via center N of the cytochrome bc_1 complex.

We then determined whether this reaction forms an un-

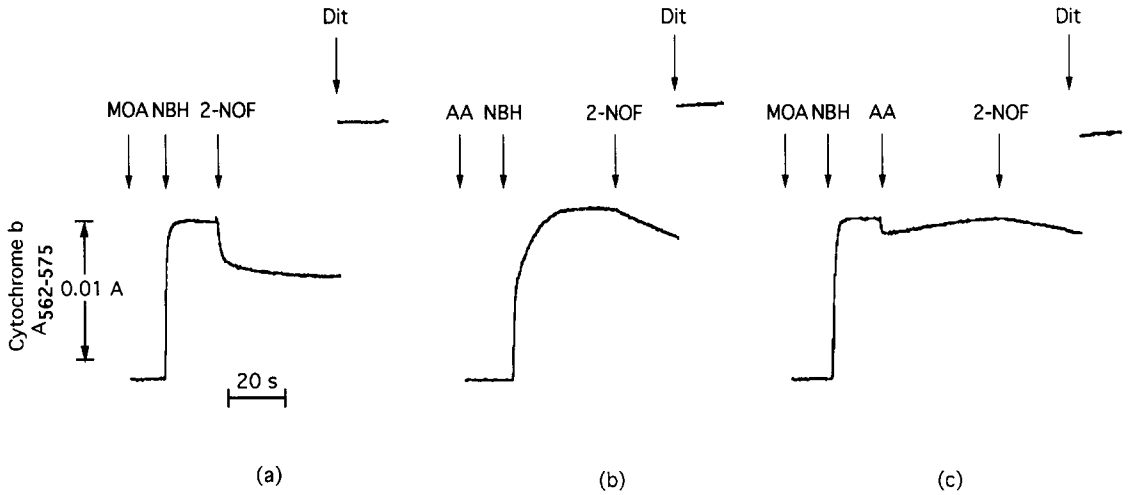

Fig. 2. Reactions of NOF with cytochrome *b* in the isolated cytochrome bc_1 complex. Isolated cytochrome bc_1 complex (1.5 μM) was diluted in incubation medium. 50 μM MOA-stilbene (MOA, trace a) or 50 μM antimycin A (AA, trace b) were added as indicated. After reduction of *b* cytochromes with 15 μM NBH, 10 μM NOF was added. In trace c, 50 μM AA was added prior to NOF. At the end of each experiment the sample was completely reduced by the addition of a few grains of sodium dithionite. The redox state of cytochrome *b* was monitored at 562 minus 575 nm.

stable nitroxyl radical by one-electron reduction of NOF that is expected to autoxidize readily [1,13] and could explain succinate-dependent O_2 consumption in mitochondria (cf. Fig. 1). In the presence of 0.1% bovine serum albumin, MOA-stilbene and excess of the reduced substrate NBH, NOF increased O_2 consumption by isolated cytochrome bc_1 complex in a dose-dependent manner (Table 2). A maximal rate of 22 nmol O_2 min^{-1} mg^{-1} was observed at NOF concentrations of 65 μM and above. The rate was about half-maximal at 15 μM. In the absence of bovine serum albumin O_2 consumption was minimal (data not shown) indicating that it was necessary to prevent aggregation of the quinone. The reaction was inhibited by antimycin A and only a residual rate somewhat higher than in the absence of NOF was observed (Table 2).

3.4. Inhibition of center N of the cytochrome bc_1 complex by NOF

Reduction of NOF at center N could occur through direct binding at the ubiquinone binding site or by electrons being indirectly accepted from the stabilized ubisemiquinone formed during normal catalysis [14]. In the latter case, NOF would not be expected to inhibit cytochrome *b* reduction by NBH via center N. However, increasing concentrations of NOF progressively inhibited the extent of cytochrome *b* reduction (Fig. 3). This inhibition was complete at 300 μM NOF, but already at 5 μM some inhibition was clearly observed. The mixing time of about 1 s in the experiments shown here was too long to decide whether the rate of reduction was also slower in the presence of NOF.

Table 2
Effects of inhibitors on NOF-induced O_2 consumption in isolated cytochrome bc_1 complex

Additions	O_2 consumption (nmol min^{-1} mg^{-1})
(a) MOA, NBH	3.5
(b) MOA, NBH, 8 μM NOF	9.6
(c) MOA, NBH, 15 μM NOF	12.7
(d) MOA, NBH, 30 μM NOF	17.7
(e) MOA, NBH, 65 μM NOF	22.1
(f) MOA, NBH, 150 μM NOF	21.9
(g) MOA, NBH, AA, 150 μM NOF	5.8

Isolated cytochrome bc_1 complex (0.5 mg/ml) in incubation medium, containing 0.1% bovine serum albumin (fatty acid free) was incubated with 50 μM MOA, 40 μM NBH (a) plus increasing concentrations of NOF (b–f) and O_2 consumption was recorded with an O_2 electrode as described in Section 2. In addition, 50 μM AA was added to the incubation mixture prior to the addition of NOF (g). The rates of O_2 consumption are given in nmol min^{-1} mg^{-1} cytochrome bc_1 complex.

3.5. Reduction of cytochrome b by N-OH-AF in isolated cytochrome bc_1 complex

Since NOF was able to drain electrons from cytochrome *b* via center N, the possibility of cytochrome *b* reduction by the corresponding hydroxylamine N-OH-AF was studied. In fact, N-OH-AF reduced cytochrome *b* rapidly in the absence of inhibitors (Fig. 4a) and in the presence of the center P inhibitor MOA-stilbene (Fig. 4b). However, cytochrome *b* reduction was completely inhibited by antimycin A (Fig. 4c) indicating that N-OH-AF also reacted at center N.

N-OH-AF also directly reduced cytochrome c_1 (data not shown).The fact that this reaction was observed even in the presence of inhibitors for both centers of the cytochrome bc_1 complex indicated that it occurred via the cytochrome *c* binding site of cytochrome c_1.

4. Discussion

The present study was designed to analyze the sites of electron transfer to NOF, a metabolite of the carcinogenic AAF in succinate-dependent respiration. Cross-over point analysis using specific inhibitors for the individual enzyme complexes of the mitochondrial respiratory chain revealed that most of the NOF-dependent O_2 consumption was associated with the antimycin A-sensitive center N of the cytochrome bc_1 complex (Table 1).

Using isolated cytochrome bc_1 complex we could show directly that NOF can accept electrons from reduced cytochrome *b* via center N (Fig. 2). We also observed antimycin A-sensitive O_2 consumption with the isolated enzyme and the ubihydroquinone derivative NBH as a substrate with a half-

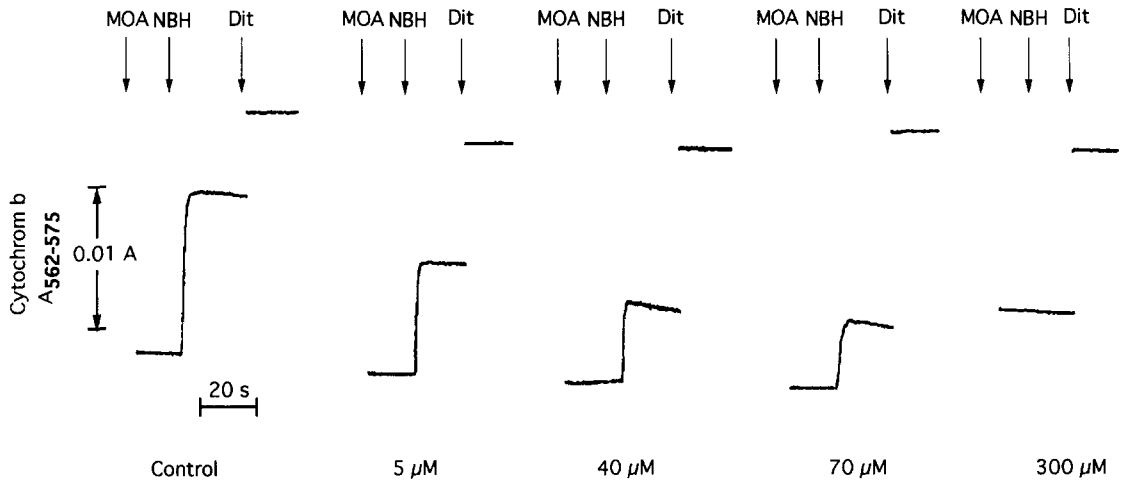

Fig. 3. Inhibition of cytochrome *b* reduction by preincubation with NOF. Isolated cytochrome bc_1 complex (1.5 μM) in incubation medium in the presence of 50 μM MOA-stilbene (MOA) was preincubated for 1 min with increasing concentrations of NOF prior to the addition of 15 μM NBH. At the end of each experiment the sample was completely reduced by the addition of a few grains of sodium dithionite. The reduction of *b* cytochromes was monitored at 562 minus 575 nm.

maximal rate at around 10–20 μM NOF (Table 2). The fact that in the presence of MOA-stilbene increasing concentrations of NOF decreased the extent of cytochrome *b* reduction by NBH could be explained by direct binding of NOF to center N, but as the rate was much less affected an indirect mechanism of electron transfer via a semiquinone cannot be excluded by this observation. However, we observed rapid reduction of cytochrome *b* by the reduced hydroxylamine form of NOF, N-OH-AF. This electron transfer could only be due to direct binding of the compound to center N of the isolated, essentially quinone-free cytochrome bc_1 complex.

Thus, the NOF/N-OH-AF redox couple seems to act as an incomplete substrate capable of only a single electron transfer. The nitroxyl radical then can react with molecular oxygen to form superoxide. It is not clear whether the latter reaction involves the bound nitroxyl radical or whether this species must leave center N to reduce oxygen. It should be noted that NOF/N-OH-AF is the first non-quinoid compound known to be capable of transferring electrons at an ubiquinone binding site and that for the cytochrome bc_1 complex this reaction is specific for center N.

Since we demonstrated recently cyclic interconversion of NOF and N-OH-AF by redox processes in aqueous media [1], N-OH-AF should also be considered a toxic metabolite of 2-acetylaminofluorene. The formation of N-OH-AF in vivo could lead to the formation of superoxide by its oxidation at center N of the cytochrome bc_1 complex and possibly also by cytochrome c/c_1 and/or *c*.

We have recently shown that in isolated mitochondria NOF is reduced NADH-dependently in the presence of rotenone,

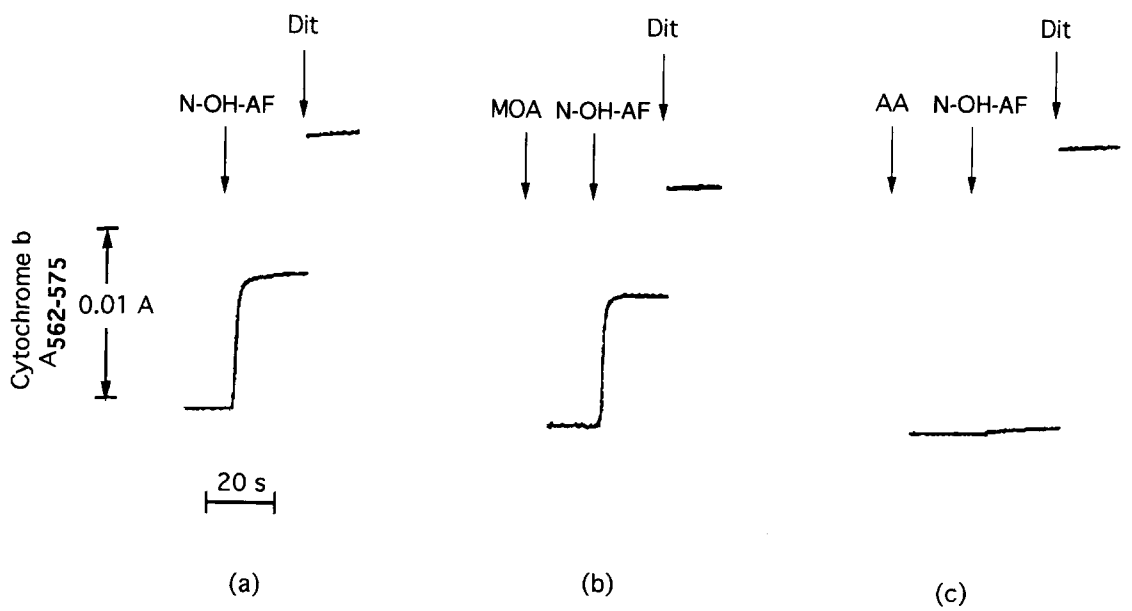

Fig. 4. Reduction of cytochrome *b* by N-OH-AF in isolated cytochrome bc_1 complex. Reduction of cytochrome *b* by N-OH-AF (20 μM) was monitored in incubation medium, containing isolated bc_1 complex (1.5 μM) at 562 minus 575 nm (trace a). In traces b and c, the sample was incubated with 50 μM antimycin A (AA) and 50 μM MOA-stilbene (MOA), respectively, prior to the addition of N-OH-AF. At the end of each experiment the sample was completely reduced by the addition of a few grains of sodium dithionite.

but does not bypass this block by feeding the electrons back into the respiratory chain (Klöhn and Neumann, submitted), which is typical for menadione [15–17]. This could be due to the fact that the free nitroxyl radical formed is not stable enough to establish an alternate electron pathway by diffusion between different redox centers.

The present results may help in gaining a better understanding of the complex mechanisms by which AAF metabolites produce cytotoxicity in rat liver. N-OH-AF is formed in liver cells by deacetylation of *N*-hydroxy-2-acetylaminofluorene [18] and *N*-oxidation of 2-aminofluorene [19,20]. We have shown that N-OH-AF reduces the *b* and *c* cytochromes of the mitochondrial respiratory chain. Oxidation of N-OH-AF generates the more stable and hydrophobic NOF which is 10–15 times more cytotoxic than N-OH-AF in vitro [21]. Although NOF may be detoxified by direct reaction with glutathione [22] it may also diffuse into mitochondrial membranes and drain electrons from complex I and the cytochrome bc_1 complex. We proposed that mitochondria may be a critical target for NOF toxicity, since NOF drains electrons from the respiratory chain (Klöhn and Neumann, submitted) which impairs oxidative phosphorylation and leads to formation of superoxide. The notion that this is also true in vivo is supported by the observations that cellular ATP [23] and mitochondrial but not cytosolic glutathione equivalents [24] decrease in rats kept on an AAF diet.

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