Unidirectional effect of lauryl sulfate on the reversible NADH:ubiquinone oxidoreductase (Complex I)

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Abstract Lauryl sulfate inhibits the $\Delta\tilde{\mu}_H^+$-dependent reverse electron transfer reactions catalyzed by NADH:ubiquinone oxidoreductase (Complex I) in coupled bovine heart submitochondrial particles and in vesicles derived from *Paracoccus denitrificans*. The inhibitor affects neither NADH oxidase (coupled or uncoupled) nor NADH:ferricyanide reductase and succinate oxidase activities at the concentrations that selectively prevent the succinate-supported, rotenone-sensitive NAD^+ or ferricyanide reduction. Possible uncoupling effects of the inhibitor are ruled out: in contrast to oligomycin and gramicidin, which increases and decreases the rate of the reverse electron transfer, respectively, in parallel with their coupling and uncoupling effects, lauryl sulfate does not affect the respiratory control ratio. A mechanistic model for the unidirectional effect of lauryl sulfate on the Complex I catalyzed oxidoreduction is proposed.

Key words: NADH:quinone oxidoreductase; Complex I; Lauryl sulfate; Unidirectional inhibition; Electrochemical coupling

1. Introduction

The energy-transducing membrane-bound NADH:ubiquinone oxidoreductases, usually termed Complex I for the mitochondrial enzyme or NDH-1 for prokaryotes, catalyze oxidation of NADH coupled with vectorial translocation of four protons per two electrons transferred to the quinone-acceptor [1]. It has been established many years ago that the enzyme in intact mitochondria [2,3] or in coupled submitochondrial particles (SMP) [4] operates reversibly, i.e. when energy is provided by aerobic succinate oxidation or by ATP hydrolysis Complex I catalyzes the rotenone- and/or uncoupler-sensitive ubiquinol:NAD^+ oxidoreduction (reviewed in [5]). Recently, several Complex I-specific inhibitors have been reported to show different efficiency in inhibition of the forward (NADH:quinone reductase) or reverse ($\Delta\tilde{\mu}_{H^+}$-dependent quinol:NAD^+ reductase) reactions [6–9]. ADP-ribose, a competitive inhibitor of NADH oxidation does not affect the reverse reaction, thus suggesting that different binding sites for NADH and NAD^+ participate in these reactions [6]. Direction-specific effects have been also documented for the quinone-junction site-directed inhibitors, rotenone [7,8] and Triton X-100 [9]. The 'unidirectional' effects of the inhibitors cannot be accounted for by possible change of their affinity for the energized or de-energized enzyme since their K_i values are the same for coupled or uncoupled NADH oxidation [10]. The hypothesis has been put forward that different electron transfer pathways operate in the forward and reverse reactions catalyzed by Complex I [11]. If correct, this hypothesis predicts that inhibitors must exist, which specifically affect the reverse reaction. A systematic search for such inhibitors has been undertaken in our laboratories. Here we present data showing that lauryl sulfate (LS) is a specific inhibitor of the reverse electron transfer catalyzed by Complex I in tightly coupled membranous preparations derived from mitochondria and bacteria.

2. Materials and methods

Bovine heart SMP [12] and coupled *Paracoccus denitrificans* vesicles [13] were prepared according to the published procedures. SMP were artificially coupled by preincubation of the stock suspension (5 mg of protein per ml in 0.25 M sucrose, 50 mM Tris–Cl, pH 8.0, 0.2 mM EDTA, 0.1 mM malonate) with oligomycin (0.5 μg per mg of protein) for 30 min at 30°C. A typical preparation catalyzed uncoupled aerobic oxidation of NADH and succinate at rates of 1.0 and 0.5 μmol/min per mg of protein with the respiratory control ratio (RCR) of 6.5 and 2.5, respectively. Uncoupled NADH and succinate oxidase activities of *P. denitrificans* vesicles were 1.0 and 0.4 μmol/min per mg of protein with RCR of 4.5 and 1.8, respectively. Before initiating the catalytic assays Complex I in SMP was activated by aerobic oxidation of a small amount (10–20 μM) of NADH [12]. NADH oxidase, succinate oxidase, NADH:ferricyanide reductase, and succinate-supported reverse electron transfer activities were measured at 25°C in a standard reaction mixture comprised of 0.25 M sucrose, 50 mM Tris–Cl^-, and 0.2 mM EDTA (pH 8.0). Succinate oxidase activity was assayed with an oxygen electrode. NADH/NAD^+ oxidoreduction was measured photometrically at 340 nm ($\varepsilon_{340} = 6.22$). Additions to the standard mixture (substrates and specific inhibitors) and experimental details are indicated in the legends to the figures. Protein content was determined with biuret reagent using bovine serum albumin as the standard. All fine chemicals were from Sigma. Two batches of LS (sodium dodecyl sulfate), one from Serva and the other one from Helicon, were used. The results obtained with either sample were identical.

3. Results

Fig. 1A shows the titration of various enzymatic activities of SMP by of LS. At a given protein content in the assay mixture LS inhibits the succinate-supported $\Delta\tilde{\mu}_{H^+}$-dependent

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Abbreviations: SMP, submitochondrial particles; LS, lauryl sulfate; RCR, respiratory control ratio

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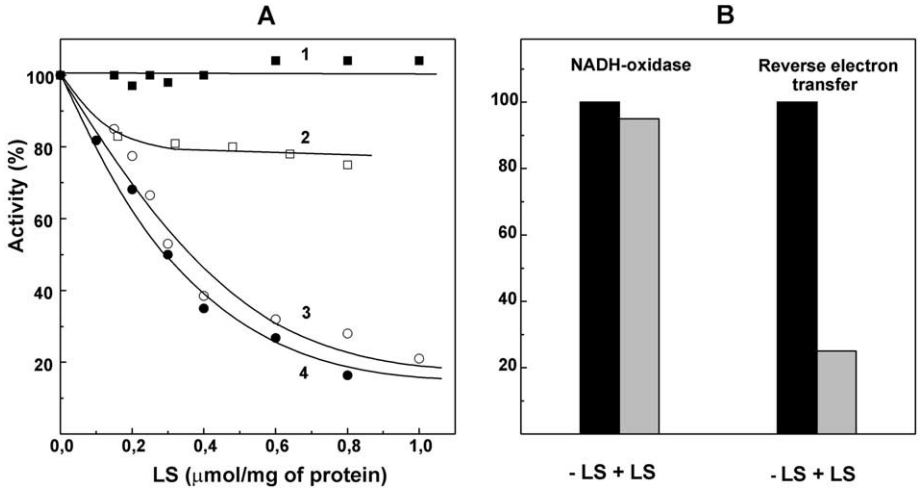

Fig. 1. Effect of LS on various enzymatic activities of bovine heart SMP and *P. denitrificans* vesicles. A: SMP were artificially coupled by treatment with oligomycin as described in Section 2 and their uncoupled succinate oxidase (■, curve 1), uncoupled NADH oxidase (□, curve 2), rotenone-sensitive succinate-supported ferricyanide reduction (○, curve 3), and aerobic succinate-supported NAD^+ reduction (●, curve 4) were assayed. The concentrations of the substrates were: succinate, 10 mM; NADH, 100 μM; NAD^+, 5 mM; ferricyanide, 0.1 mM. Gramicidin D (0.2 μg/ml) was added as an uncoupler. Protein content in the assay mixture was 25 μg/ml for curves 2, 3, 4 and 50 μg/ml for curve 1. 100% activity corresponds to 1.05 μmol of NADH oxidized (curve 2), 0.24 μmol of ferricyanide reduced (curve 3), 0.11 μmol of NAD^+ reduced (curve 4) and 0.46 μmol of succinate oxidized per min per mg protein (curve 1). B: Uncoupled NADH oxidase and aerobic succinate-supported reverse electron transfer activities of *P. denitrificans* vesicles were assayed in the standard reaction mixture containing 1 mM $MgCl_2$ as described in (A). 15 mM ammonium acetate and 0.2 μg/ml of gramicidin were added for uncoupling. Thirty μM LS was present where indicated. Protein content in the assay mixture was 100 μg/ml. Hundred percent activity corresponds to 1.0 μmol of NADH oxidized (or 0.1 μmol of NAD^+ reduced) per min per mg protein.

reduction of NAD^+ or ferricyanide (reverse electron transfer) whereas neither uncoupled succinate oxidase nor uncoupled NADH oxidase activity was significantly altered by the inhibitor within the concentration range employed. The inhibitory effect of LS was protein content-dependent, i.e. the more protein added to the assay mixture the higher the apparent I_{50} observed, although the titration pattern as depicted in Fig. 1A was the same (data are not shown). Slight (about 20%) inhibition of NADH oxidase by LS was persistently observed for SMP preparations (Fig. 1A, curve 2). The most likely reason for this is that small fraction of Complex I remained in the deactivated form even after prepulsing with NADH [11] and LS might affect the slow de-active/active transition. This phenomenon was not further investigated in the present studies. The specific inhibition of Complex I-mediated reverse electron transfer by LS was also evident for coupled *P. denitrificans* vesicles (Fig. 1B). The inhibitory effect of LS on the reverse electron transfer reaction might not seem surprising since any compound which would decrease $\Delta\tilde{\mu}_{H^+}$, e.g. an uncoupler, is expected to result in the effects similar to those depicted in Fig. 1. The ATP/ADP carrier-mediated uncoupling effect of LS (90–160 nmol/mg of protein) on rat heart [14], liver, and skeletal muscle mitochondria [15] has been reported, although other authors found no decrease of the membrane potential by LS in rat liver mitochondria oxidizing malate/glutamate (120 nmol/mg of protein) [16]. Thus, possible uncoupling effects of LS were scrutinized. The experiments presented in Fig. 2 show that LS does not affect coupled respiration with succinate thus leaving RCR constant. Another explanation for the specific effect of LS on the reverse electron transfer is the possibility of a threshold in $\Delta\tilde{\mu}_{H^+}$ required for the reverse electron transfer reaction. It could be expected that a slight decrease of $\Delta\tilde{\mu}_{H^+}$, which is undetectable in the RCR measurement is sufficient for substantial inhibition of the reaction. To test this possibility gradual titration of the initial rate of the reverse electron transfer and RCR was carried out with oligomycin as a 'coupling' effector and with gramicidin as an uncoupler. The titration patterns showed that the stimulating effect of oligomycin on the rate of NAD^+ reduction and the increase of RCR quantitatively coincided (Fig. 3A). The same quantitative correlation between the two parameters was evident for titration by gramicidin (Fig. 3B). These findings are in contrast to the effect of LS (Fig. 2). Thus the specific unidirectional inhibition of the reverse electron trans-

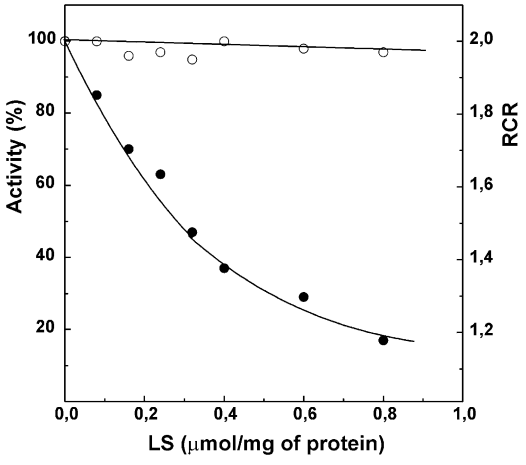

Fig. 2. Titration of the reverse electron transfer activity (●) and RCR (○) by LS. SMP were coupled by oligomycin as described in Section 2 and their aerobic succinate-supported NAD^+ reduction activity was assayed as described in Fig. 1. RCR was determined as the ratio between uncoupled succinate oxidase activity in the presence of gramicidin D (0.2 μg/ml) and coupled succinate oxidase (no gramicidin was added). The protein content in the assay mixture was 50 μg/ml. The specific activities are indicated in Fig. 1.

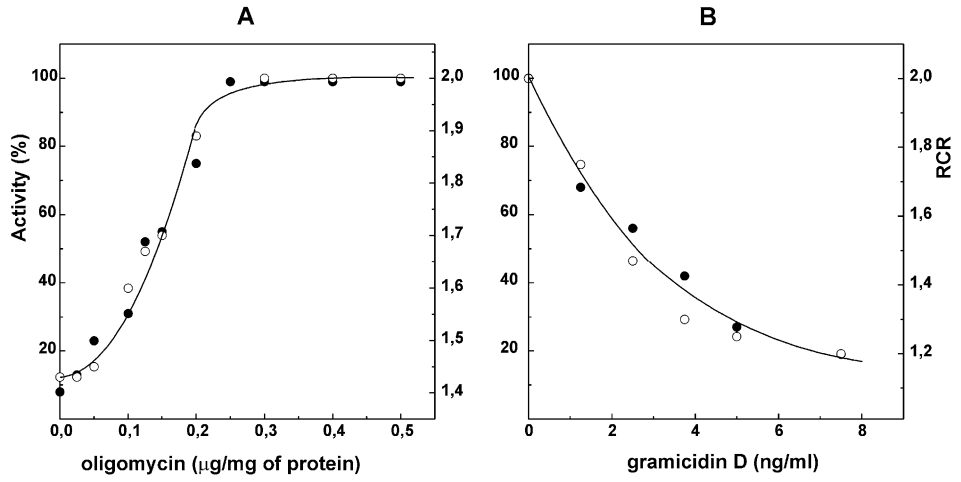

Fig. 3. Effects of coupling (A) and uncoupling (B) on the rate of reverse electron transfer (●) and RCR (○). SMP (5 mg/ml) preincubated with 1 mM malonate and oligomycin (the amount is indicated on abscissa) for 30 min at 30°C. Small aliquots were withdrawn and the initial rates of aerobic succinate-supported NAD^+ reduction (●) and RCR in the succinate oxidase test (○) were determined. Fully coupled SMP (preincubated with 0.5 μg of oligomycin per mg of protein) were assayed in (B). Protein content in the assay mixture was 50 μg/ml.

fer by LS cannot be attributed to its uncoupling effect. The apparent contradiction between our failure to see any uncoupling effect of LS on inside-out particles (Fig. 2) and slight LS-induced, carboxyatractylate-sensitive uncoupling observed in intact mitochondria [14,15] can be attributed to different orientations of the ATP/ADP carrier in different preparations.

4. Discussion

Numerous compounds of great diversity in their chemical structure are known to be more or less specific inhibitors of ubiquinone reduction by Complex I [17–19]. Most of them are either very hydrophobic, such as piericidin [20,21] and rotenone [7], or amphiphilic, such as Triton X-100 [9], *n*-alkyl polyoxyethylenes [22] and fatty acids [23]. Affinity labeling [24,25] and mutational analysis [26–28] suggest that the inhibitors bind to relatively large cavity formed by ND-1 (NUO-H), PSST (NUO-B) and 49 IP (NUO-D) subunits and located at or close to the interface between the membrane and the peripheral part of Complex I. When rotenone or piericidin, the inhibitors most commonly used as a tool to dissect Complex I activity in the respiratory chain, is added in excess over the enzyme content both the forward and reverse reactions are blocked. However, closer quantitative examination has revealed that these inhibitors show directional specificity [7–9]. The simplest explanation for the phenomenon is that different sites for quinone reduction and quinol oxidation operate in the forward and reverse electron transfer reactions [11]. This explanation may also hold for the unidirectional effect of LS as described in this report, although it is hard to reconcile with the very simple chemical structure of the inhibitor. Here we would like to propose a mechanism for the unidirectional inhibition based on asymmetric modification of the membrane surface by LS. We assume that strongly acidic LS does not penetrate the membrane and occupies exclusively the outer leaflet of the phospholipid bilayer. The presence of a negative charge in close proximity of an iron–sulfur cluster (most likely N-2) located at the interface is expected to decrease its midpoint redox potential thus making its $\Delta\tilde{\mu}_{H^+}$-dependent reduction by ubiquinol unfavorable. This would result in inhibition of the reverse electron transfer. It has been reported that iron–sulfur cluster N-2 is about 50% reduced under steady-state aerobic succinate-supported reverse electron transfer, whereas it is almost completely reduced under steady-state NADH oxidation [29,30]. Thus, it is expected that a slight change of the midpoint redox potential of N-2 would dramatically affect the rate of the reverse reaction without a significant effect on the rate of NADH oxidation. A number of testable predictions follow from this model. (i) LS should affect the midpoint redox potential of N-2 in inside-out SMP and bacterial vesicles. (ii) LS is expected to have no inhibitory effect on the reverse electron transfer catalyzed by intact mitochondria. (iii) Positively charged amphiphilic compounds analogous to LS should either stimulate the reverse electron transfer or not affect the reaction. These predictions are currently under investigation in our laboratories. It is worth noting that in light of the findings reported here asymmetric distribution of the acidic phospholipids between the outer and inner leaflets of the coupling membrane may be an important factor controlling the rate of the reverse electron transfer catalyzed by Complex I. Although the physiological significance of this reaction in mammalian mitochondria is not clear, its important role in supplying the reducing equivalents for CO_2 fixation in *Rhodobacter capsulatus* has been documented [31,32].

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