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Nikolai P. Belevich, Christoph von Ballmoos, and Marina Verkhovskaya

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## Activation of proton translocation by respiratory Complex I

Nikolai Belevich<sup>1</sup>, Christoph von Ballmoos<sup>2</sup>, Marina Verkhovskaya<sup>3\*</sup>

<sup>1,3</sup>Institute of Biotechnology, PO Box 65 (Viikinkaari 1) FIN-00014 University of Helsinki,  
Finland

\* Author for correspondence: e-mail: [Marina.Verkhovskaya@Helsinki.Fi](mailto:Marina.Verkhovskaya@Helsinki.Fi)

<sup>2</sup>Department of Chemistry and Biochemistry, University of Bern, Freiestrasse 3, CH-3012  
Bern,  
Switzerland

## Abstract

Activation of proton pumping by reconstituted and native membrane-bound Complex I was studied using optical electric potential- and pH-sensitive probes. We find, that reconstituted Complex I has a delay in proton translocation, which is significantly longer than the delay in quinone reductase activity, indicating an initially decoupled state of Complex I. Studies of the amount of NADH required for the activation of pumping indicate the prerequisite of multiple turnovers. Proton pumping by Complex I was also activated by NADPH, excluding significant reduction of Complex I or pre-existing  $\Delta\psi$  as activation factors. Co-reconstitution of Complex I and ATPase did not indicate an increased membrane permeability for protons in the uncoupled Complex I state. The delay in Complex I proton pumping activation was also observed in subbacterial vesicles. While it is negligible at room temperature, it strongly increases at a lower temperature. We conclude that Complex I undergoes a conversion from decoupled to coupled state upon the activation. The possible origins and importance of the observed phenomenon are discussed.

## Introduction

NADH:ubiquinone oxidoreductase (Complex I) is the electron input enzyme in the respiratory chain of mitochondria and many bacterial species, which couples electron transfer from NADH to ubiquinone with transmembrane proton translocation. The electron transfer proceeds along the intraprotein redox chain located in the hydrophilic domain and proton translocation is performed by antiporter-like membrane subunits. No redox centers are found in the membrane fragment

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3 where the antiporter-like subunits are disposed in a row so that the energy of the redox reaction  
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5 must be transduced over a distance of 100 Å. Such high spatial separation of electron and proton  
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7 transfers with no obvious coupling site indicates an intricate molecular mechanism of proton  
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9 pumping. Elucidation of this mechanism could be significantly advanced by monitoring the enzyme  
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11 operation upon turnover in real time, which prerequisites the enzyme in its active state. However,  
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13 oxidized mitochondrial Complex I exists in a de-active state (D) which spontaneously converts to  
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15 an active state (A) upon turnover<sup>1-3</sup>, after which the oxidized enzyme slowly relaxes to the D-  
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17 state. The D/A transition is believed to be physiologically relevant since it could serve as a  
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19 protective mechanism preventing the oxidative damage of tissues (see for a review<sup>4</sup>). In contrast  
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21 to mitochondrial complex I, the D/A transition was not observed in bacterial Complex I from  
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23 *Paracoccus denitrificans*<sup>5</sup>, *Thermus thermophilus* and *Escherichia coli*<sup>6</sup> and it was suggested that  
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25 the D/A transition might be a unique property of the former<sup>7, 8</sup>. Subunit composition and  
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27 molecular structures of Complex I from various organisms demonstrate that bacterial enzymes are  
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29 “minimal versions” of the mitochondrial ones, i.e. they exert the exact same molecular function  
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31 with similar rates and stoichiometry<sup>7-10</sup>. Bacterial Complex I is thus a good model for studying the  
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33 coupling mechanism between NADH oxidation and proton translocation, although it might not  
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35 share all properties, such as D/A transition. Upon this transition, mitochondrial Complex I is  
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37 proposed to undergo conformational changes occurring within three subunits at the junction of  
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39 the hydrophilic and membrane fragments of the enzyme<sup>11, 12</sup>. Although the D/A transition has  
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41 been proposed to be a unique property of the mitochondrial enzyme<sup>7, 8</sup>, dynamics and flexibility  
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43 of the region at the hydrophilic/membrane domain<sup>10</sup> introduces some uncertainty in modeling of  
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3 the conformational changes involved and interconversion of these states in bacterial enzymes  
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5 cannot be excluded and must be studied experimentally.  
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9 We have recently shown that *E. coli* Complex I may also exist in two states, resting (R) and active  
10 (A), characterized by a delayed maximal NADH:ubiquinone oxidoreductase activity<sup>13</sup>. In a  
11 subsequent study using fluorescent probes, we obtained results indicating that upon the  
12 activation from state R to state A, Complex I undergoes conformational changes, which can be  
13 mapped to the junction of the hydrophilic and membrane domains in the region of the assumed  
14 acetogenin-binding site<sup>14</sup>. The observed R/A transition was quick (1-2 s)<sup>13</sup>, regardless whether  
15 Complex I was solubilized, reconstituted into liposomes or native membrane-bound and thus  
16 faster than reported for the mitochondrial enzyme ( $\tau_{1/2} \sim 10$  s). The lifetime of A state was too short  
17 to be determined, which is in contrast to an active state of several hours in the mitochondrial  
18 enzyme at 0° C<sup>15</sup>.  
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34 In the previous report, R/A transition with solubilized or reconstituted enzyme was analyzed under  
35 conditions that neglected the proton pumping activity of Complex I<sup>13</sup>. Here, we study the  
36 activation profiles of proton pumping activity using reconstituted and native membrane-bound  
37 Complex I. We find that the activation properties of the two reactions catalyzed by Complex I,  
38 NADH:ubiquinone oxidoreduction and proton translocation across the membrane, are significantly  
39 different.  
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## 53 **Materials and Methods**

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3 **Bacterial growth and purification of Complex I and ATP synthase.** The *E. coli* MWC215 ( $\text{Sm}^{\text{R}}$   
4 *ndh::\text{Cm}^{\text{R}}*) strain was grown in LB medium at 37°C in a 25 L fermenter and harvested at the late  
5  
6 exponential growth phase. The membranes for Complex I purification were prepared by passing  
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8 the cells through an APV Gaulin homogenizer. Then Complex I was purified by two consecutive  
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10 chromatography steps using DEAE-Trisacryl M (Bio-Septra) anion exchanger columns and gel  
11  
12 filtration on Superdex 200 prep grade (GE Healthcare), respectively, as described<sup>16</sup>. ATP synthase  
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14 of *E. coli* was expressed and purified as described previously<sup>17</sup>  
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21 **Reconstitution.** Purified *Complex I* was reconstituted into liposomes as previously described<sup>18</sup>  
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23 except that azolectin was suspended in the buffer containing MOPS-BTP 100 mM, pH 7.0, n-dodecyl  
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25  $\beta$ -D-maltopyranoside (DDM) 0.4%, Na-cholate 0.1%. A ratio of 1:10 (w/w), of the enzyme to  
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27 azolectin was used unless other specified. ATP synthase and ATP synthase/Complex I co-  
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29 reconstitution were performed in the same way.  
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35 **Preparation of subbacterial vesicles.** The vesicles were prepared as described in<sup>19</sup>, but vesicles  
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37 were loaded with 100 mM MOPS-BTP, 7.2, 50 mM KCl and 1 mM  $\text{MgSO}_4$ , frozen and stored at -  
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39 80°C until use.  
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#### 43 **Kinetic measurements.**

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46 Monitoring  $\Delta\psi$  in proteoliposomes was carried out at 24°C using a high-resolution CCD array  
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48 spectrometer (HR2000+, Ocean Optics) combined with a DH-2000-BAL light source (Ocean Optics).  
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50 Upon the reaction, optical spectra in the range of 250-680 nm were collected for 600 s. The  
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52 difference in absorption at 630-590 nm presents  $\Delta\psi$ -sensitive Oxonol VI response. Optical  
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54 changes at 340 nm reflect simultaneous NADH consumption. The assay buffer comprised 100 mM  
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3 MOPS-BTP, 7.0, 1 mM MgSO<sub>4</sub>, 5 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2.5 μM Oxonol VI, 10 nM solubilized bo<sub>3</sub> oxidase,  
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5  
6 decylubiquinone (DQ) 100 μM. Prior to measurements proteoliposomes (protein concentration 7-  
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8 15 μg/ml) were incubated in the assay buffer for 7 min at RT for equilibration of the DQ  
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10 distribution.

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14 Monitoring ΔpH in proteoliposomes were performed using entrapped pH-sensitive probe trisodium  
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16 8-hydroxypyrene-1,3,6-trisulfonate (pyranine) as described in<sup>18</sup>.

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20 Monitoring ΔpH in membrane vesicles was carried out following fluorescence changes of pH-  
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22 sensitive probe acridine orange (AO) with a Hitachi F-7000 fluorescence spectrophotometer, λ<sub>ex</sub> =  
23  
24 493 nm, λ<sub>em</sub> = 530 nm at 24°C unless other specified. The assay buffer comprised 50 mM MOPS-  
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26 BTP, pH 7.2, 100 mM KCl, 1 mM MgCl<sub>2</sub>, 1 μM valinomycin, 100 μM DQ, 1 mM KCN, 3.5 μM AO. The  
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28 final protein concentration was 150-180 μg/ml.  
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### 32 33 ***Fast measurements of reconstituted Complex I NADH:ubiquinone oxidoreductase activity.***

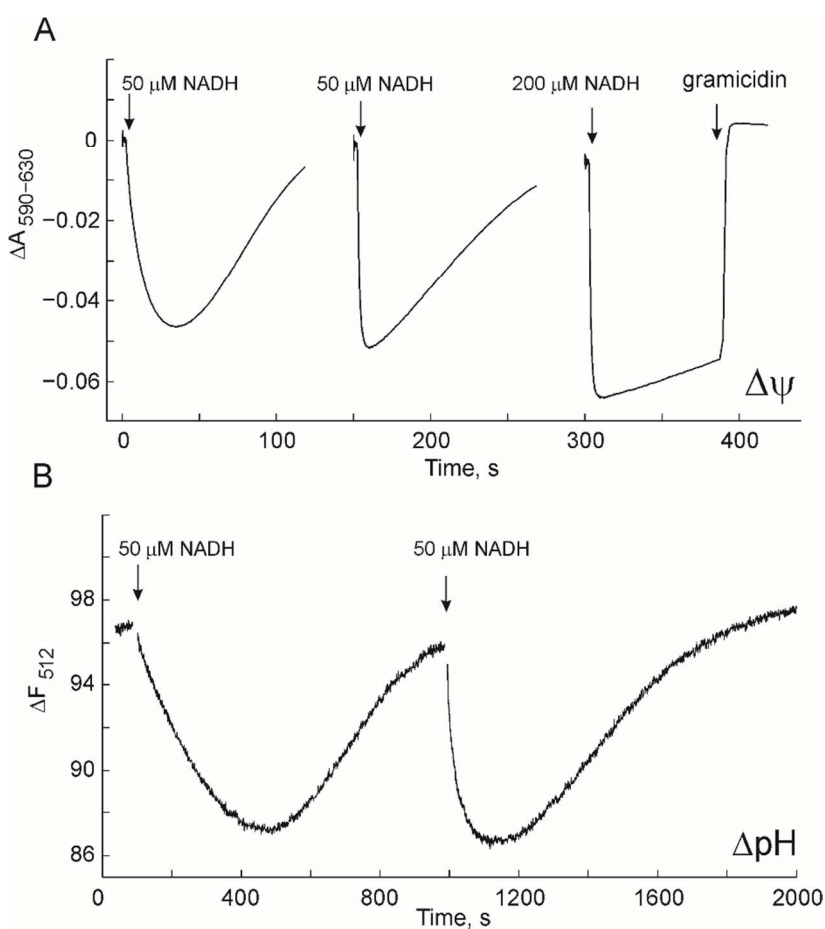
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36 Following the initial stage of NADH oxidation was performed by rapid mixing of equal volumes of  
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38 solubilized Complex I and buffer containing NADH (Unisoku Stopped-flow RSP-2000 apparatus)  
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40 using a high-resolution CCD-array spectrometer (HR2000+, Ocean Optics) combined with a Xenon  
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42 lamp as described in<sup>13</sup>.  
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## 46 47 **Results**

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50 ***Delay of pumping activity in resting Complex I.*** In our previous work, activation of Complex I was  
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52 followed by monitoring NADH oxidation and quinone reduction<sup>13</sup>. In functional Complex I this  
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54 reaction is coupled to electrogenic proton transfer across the membrane that results in generation  
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56 of a Δψ and a ΔpH. In order to monitor transmembrane events, purified Complex I was  
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reconstituted into liposomes and energized by addition of NADH in the presence of DQ, and the generation of  $\Delta\psi$  or  $\Delta\text{pH}$  was monitored (Figure 1). Accumulation of reduced ubiquinone was prevented by addition of a small amount of quinol oxidase  $bo_3$  in detergent micelles, regenerating

oxidized ubiquinone without contributing to transmembrane proton transport.



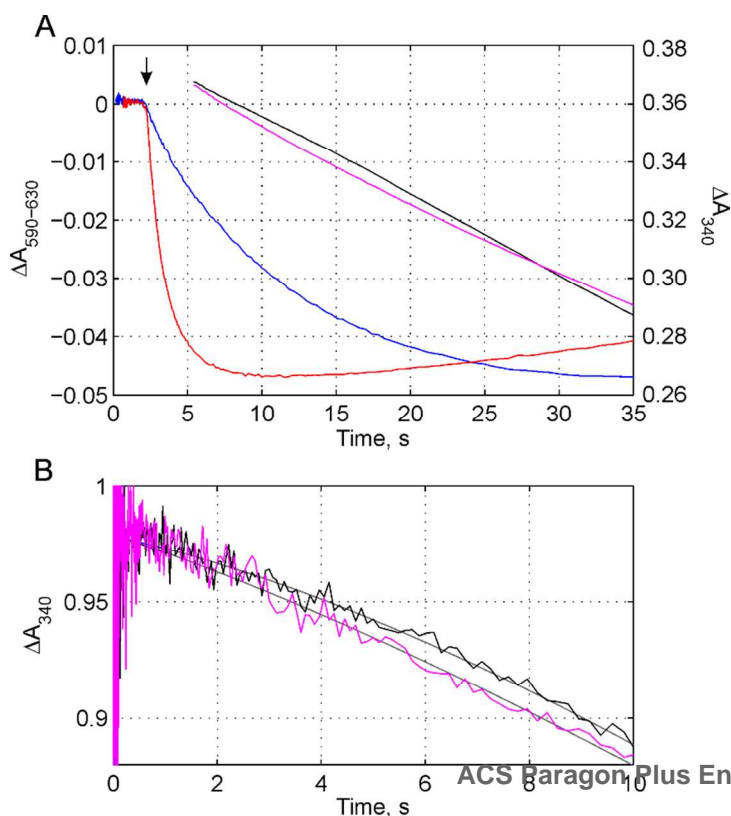
**Figure 1.** Following the  $\Delta\psi$  (A) and  $\Delta\text{pH}$  (B) generation in proteoliposomes by reconstituted Complex I. Following the first additions of NADH were made after full dissipation of  $\Delta\psi$  or  $\Delta\text{pH}$  and complete NADH consumption. 100  $\mu\text{M}$  DQ and  $bo_3$  oxidase was added to proteoliposomes prior to measurements.

Proton pumping initiated by addition of external NADH generates an electric potential (inside positive) across the proteoliposomes membrane that was followed using the  $\Delta\psi$ -sensitive probe,



Oxonol VI. As shown in Figure 1A, initial addition of NADH resulted in  $\Delta\psi$  generation followed by its relaxation to zero level after all NADH was consumed. Notably, subsequent NADH additions caused much faster formation of  $\Delta\psi$ . A similar effect was observed when acidification of the liposome lumen was followed with the pH-sensitive dye pyranine entrapped in the liposomes (Figure 1B). Similar to  $\Delta\psi$  generation, the second addition of NADH caused a faster rate of acidification than the first addition. While the kinetics of the two experiments are different (fewer protons are required to establish a membrane potential than to significantly acidify the buffered liposome interior), the similar trend strongly indicates that initial proton pumping (in the resting enzyme) is much slower than in the activated enzyme.

**Correlation of pumping and NADH:ubiquinone oxidoreductase activity.** In the next series of experiments, activation profiles of both NADH oxidation (as reported in<sup>13</sup>) and  $\Delta\psi$  generation were compared simultaneously (Figure 2A) by two consecutive NADH additions as described above.



**Figure 2. A.** Kinetics of  $\Delta\psi$  generation and NADH consumption by reconstituted Complex I upon the first and the second addition of 50  $\mu\text{M}$  NADH. Upper panel, simultaneous  $\Delta\psi$  and NADH consumption monitoring. NADH addition is indicated by an arrow.  $\Delta\psi$  generation on 1<sup>st</sup> NADH addition, blue, on 2<sup>nd</sup> NADH addition, red. Corresponding NADH consumption tracks, black and magenta.

**B.** Fast measurements of

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3 NADH:ubiquinone oxidoreduction by reconstituted Complex I. At zero time, the solution  
4 containing proteoliposomes equilibrated with 100  $\mu\text{M}$  DQ was mixed 1:1 with the same buffer  
5 containing 100  $\mu\text{M}$  NADH. Non-activated proteoliposomes, black; pre-activated with 30  $\mu\text{M}$  NADH,  
6 magenta. Gray lines, exponential fits of the kinetics.  
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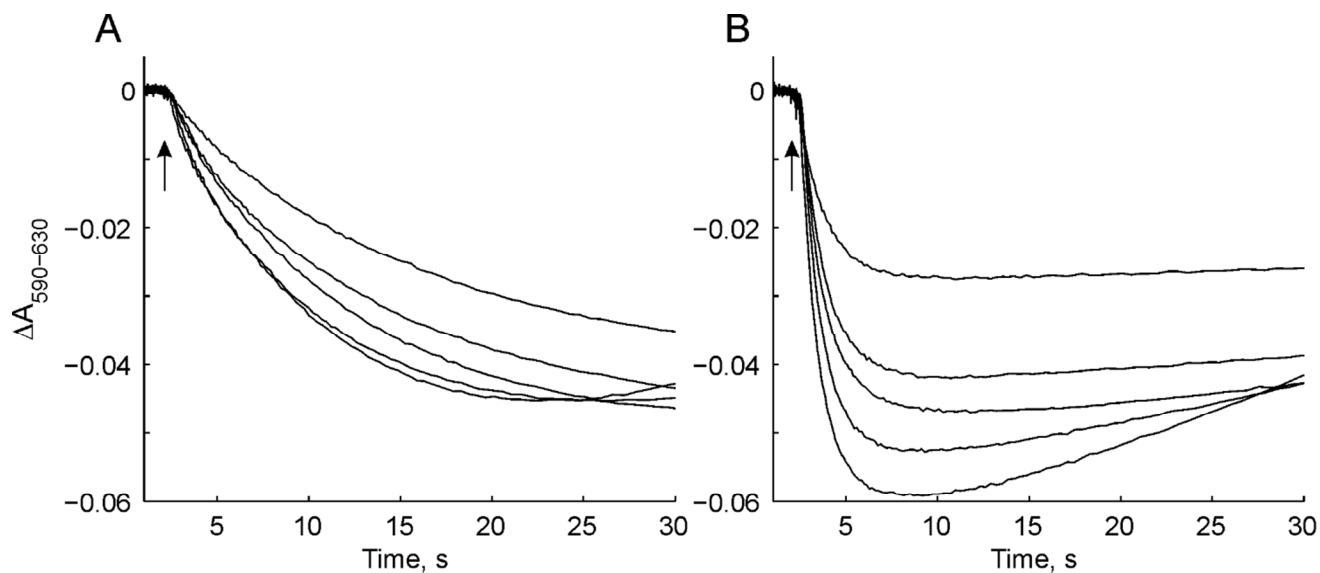
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15 The data in Figure 2A show that the difference in rates between the first and second addition is  
16 much more pronounced for proton pumping than for NADH oxidation. While the NADH  
17 consumption rate in the time interval of 5-10 s at the first NADH addition was  $\sim 70\%$  of the second  
18 addition, the maximal rate of  $\Delta\psi$  response on the first NADH addition was only  $\sim 15\%$  of the  
19 second.  
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28 The discrepancy between the rates of proton and electron transport indicates that Complex I is  
29 partially uncoupled during the first seconds of the measurements, i.e. electron transport is not  
30 tightly coupled to transmembrane proton transport. In the used setup, NADH oxidation could not  
31 be reliably followed in the time interval of 0-5 s because of a strong artificial response at 340 nm  
32 upon NADH dilution. To exclude the possibility that the strong activation of NADH consumption  
33 occurs during this time range, we performed stopped-flow measurements to resolve the initial  
34 time range (Figure 2B). At zero time, suspensions containing proteoliposomes, either untreated or  
35 activated with 30  $\mu\text{M}$  NADH for several minutes, were mixed 1:1 with the same buffer containing  
36 100  $\mu\text{M}$  NADH. The results in Figure 2B show that the difference in the initial kinetics of  
37 NADH:ubiquinone oxidoreductase activities did not exceed 10%.  
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53 ***Dependence the proton pumping activation on the ratio Complex I:azolectin upon reconstitution.***

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56 The energization of proteoliposomes membrane is always a result of a superposition of two  
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3 oppositely directed fluxes, one being active  $H^+$  pumping by the enzyme and the other being  $H^+$  (or  
4 another ion) leakage through the membrane. The signal amplitude of active pumping is thus  
5 expected to increase with the number of Complex I proteins in the liposomes, while the  
6 membrane leakage contribution remains unaffected (Figure 3A).  
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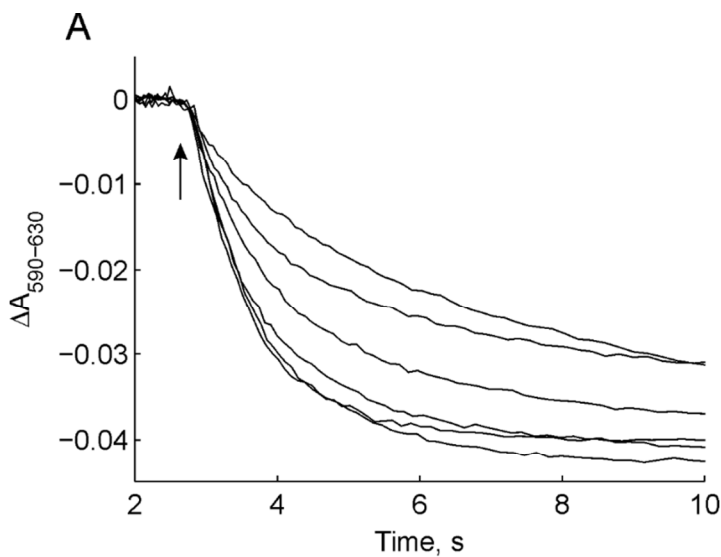


**Figure 3.** Kinetics of  $\Delta\psi$  generation by reconstituted Complex I upon the first (A) and the second (B) addition of NADH at 50  $\mu$ M concentration indicated by arrows. The ratio Complex I/azolectin (w/w) upon reconstitution 1:50, 1:30, 1:20, 1:15, 1:10, from top to bottom. NADH additions are indicated by arrows.

Purified Complex I preparation contains some amount of annular lipids that could be important for the enzyme behavior. Reconstitution into azolectin liposomes may result in a replacement of these lipids dependently on the proportion of the lipid phase, therefore it was important to test whether proteoliposome content affects the observed activation of Complex I. We carried out monitoring

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3  $\Delta\psi$  generation in proteoliposomes with different Complex I:lipid ratios. In Figure 3 the responses  
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6 on 1<sup>st</sup> (A) and 2<sup>nd</sup> (B) NADH addition to proteoliposomes were Complex I was reconstituted in  
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8 ratios of 1:50, 1:30, 1:20, 1:15, 1:10 (w/w) are presented. Although the amplitude of the response  
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10 changed in accordance with protein content, as expected, the proportion between the initial rates  
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12 after 1<sup>st</sup> and 2<sup>nd</sup> NADH additions stayed unaltered. When the ratio Complex I:lipid exceeds 1:10  
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14 (w/w) the signal becomes lower, most probably due to Complex I aggregation and poor  
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16 reconstitution. Taken together, the results show that the activation phenomenon is not affected  
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18 by changes in protein:lipid ratio.  
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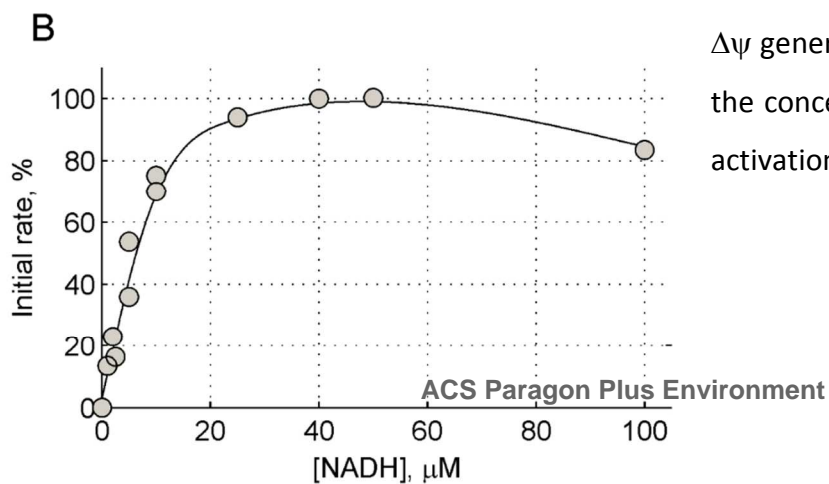
24 **Dependence of Complex I activation on concentration of initially added NADH.** Next, the effect  
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26 of NADH concentrations varying from 2 to 100  $\mu\text{M}$  as the first addition was tested (Figure 4A),  
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28 while the second addition was always 50  $\mu\text{M}$ . The second portion was added only after the  $\Delta\psi$   
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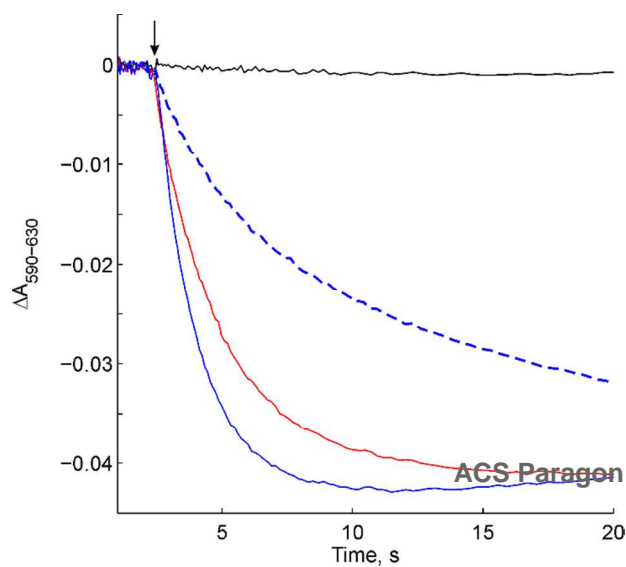
**Figure 4.** Dependence of reconstituted Complex I activation on concentration of initially added NADH. **A.** Generation of  $\Delta\psi$  on the 2<sup>nd</sup> NADH addition at concentration of 50  $\mu\text{M}$  (indicated by an arrow). The first NADH addition was 0, 5, 10, 20, 50, 100  $\mu\text{M}$ , from top to bottom. **B.** Dependence of the initial rate of  $\Delta\psi$  generation by activated Complex I on the concentration of NADH added for the activation.



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16 upon the first addition had completely dissipated. We found that the full activation of proton  
17 pumping required 10-15  $\mu\text{M}$  NADH during the first addition, while further increase in NADH  
18 concentration resulted in the same activation (Figure 4B). Upon the third NADH addition, which is  
19 performed immediately after full NADH consumption of the second addition the profile of  
20  $\Delta\psi$  generation was similar to the second NADH addition.  
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29 **Activation of Complex I pumping activity by NADPH.** The results from the experiments above  
30 indicate that preceding enzyme turnover directly influences the extent of activation. We therefore  
31 tested, if activation is possible with NADPH, which is able to reduce Complex I, showing very slow  
32 turnover. NADPH was found to activate mitochondrial Complex I<sup>15, 20</sup>. As expected, Complex I  
33 incubation with 100  $\mu\text{M}$  NADPH resulted in negligible NADPH:ubiquinone oxidoreductase activity  
34 and almost non-detectable  $\Delta\psi$  generation, since the turnover is so slow that  $\text{H}^+$  pumping cannot  
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47 overcome the membrane leakage (Figure 5, black  
48 trace).



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51 **Figure 5.** Activation of Complex I proton pumping  
52 activity by NADPH. Almost no generation of  
53  $\Delta\psi$  after 100  $\mu\text{M}$  NADPH addition (black line) was  
54 detected, however the enzyme was activated.  
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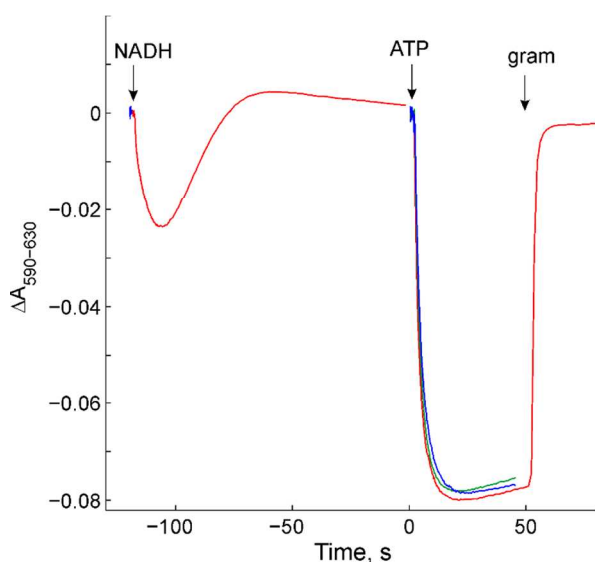
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3 Response to the primary addition of 50  $\mu\text{M}$  NADH after preincubation with NADPH, red.  
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5 Generation of  $\Delta\psi$  without preincubation with NADPH, blue curves: 1<sup>st</sup> 50  $\mu\text{M}$  NADH addition,  
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7 dashed; 2<sup>nd</sup> 50  $\mu\text{M}$  NADH addition, solid. Nucleotides addition is indicated by an arrow.  
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13 However, after an incubation of the proteoliposomes with NADPH for  $\sim 2$  minutes, the addition of  
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15 50  $\mu\text{M}$  NADH shows that Complex I has been significantly (but not completely) activated (Figure 5,  
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17 red trace) if compared with non-activated complex I (blue dotted trace) or fully activated enzyme  
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19 (blue trace). Incubation of the proteoliposomes with 100  $\mu\text{M}$   $\text{NAD}^+$  as a substrate analogue that  
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21 cannot be turned over, did not affect the slow proton pumping on the 1<sup>st</sup> NADH addition (Figure  
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23 S1) and thus not activate Complex I. These data show that the enzyme activation is rather  
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25 dependent on the turnover than on the incubation with a substrate analogue.  
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31 ***Co-reconstitution of ATPase and Complex I.*** As mentioned above, a possible explanation for the  
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33 observed results is that initial NADH oxidation is uncoupled from proton pumping. However,  
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35 another possibility would be that in the inactive state, Complex I confers a higher proton  
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37 permeability to the membrane. The observed effect would then be due to a delayed recovery of  
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39 the low permeability (tightening of the membrane) upon enzyme activation. We tested this  
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41 hypothesis by co-reconstitution of complex I with ATP synthase, a separate proton pump. In these  
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43 experiments, ATP is added and the ATP synthase works in hydrolysis direction as a primary proton  
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45 pump.  
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52 As seen in Figure 6 the amplitude and the kinetics of  $\Delta\psi$  generated by ATP synthase were similar  
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54 regardless of whether it was reconstituted alone (blue trace) or together with Complex I, activated  
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56 (red trace) or non-activated (green trace). The data show that the presence of Complex I in the  
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3 proteoliposomes, either “as prepared” or activated, does not affect the membrane permeability of  
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6 the liposomes upon ATP driven proton pumping. It should be noted that the amplitude of  $\Delta\psi$   
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9 generated by the ATP synthase is higher than that of Complex I, which is explained by the lower  
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11 turnover number of the complex I that is limited by the slow exchange of ubiquinone between  
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13 lipid and water phase<sup>21</sup>.  
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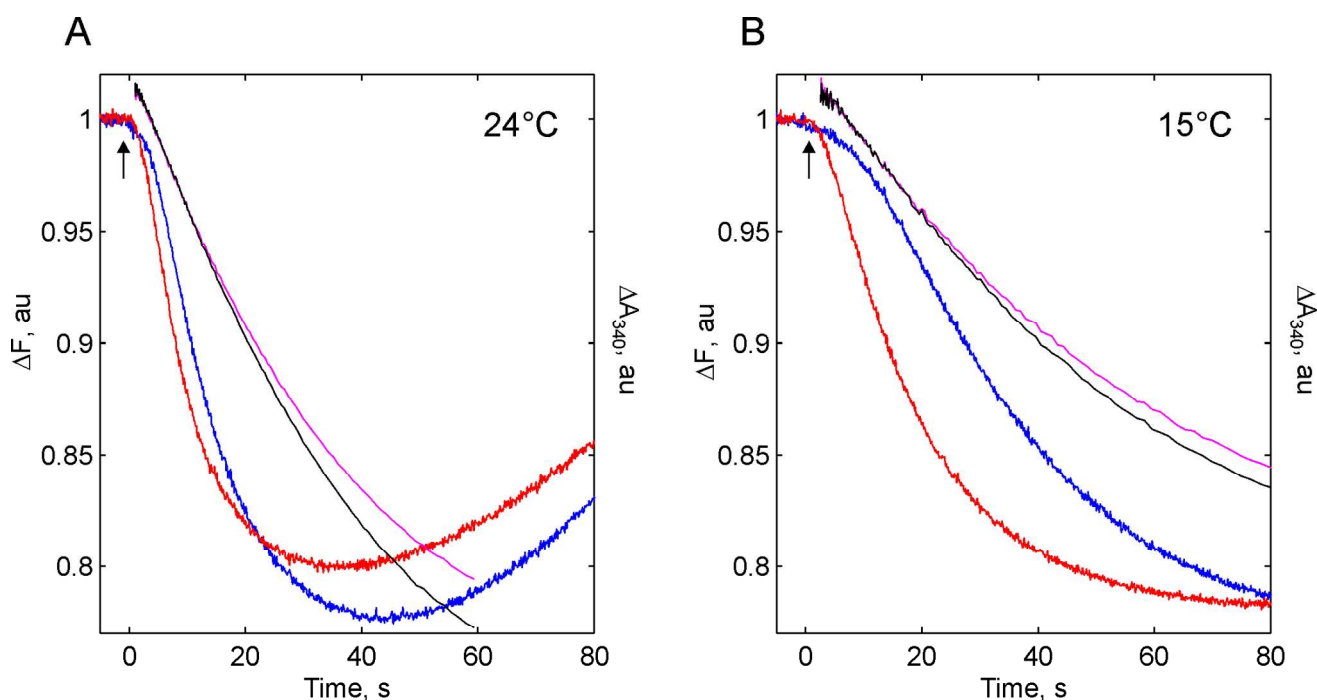


**Figure 6.** Generation of  $\Delta\psi$  by reconstituted ATPase in the presence or absence of Complex I. ATPase reconstituted alone, red curve; ATPase co-reconstituted with Complex I, green curve; ATPase was reconstituted with Complex I, which was activated by 50  $\mu\text{M}$  NADH, blue curve. 0.4 mM ATP was added as indicated. The dissipation of  $\Delta\psi$  was achieved by an addition of 1.5  $\mu\text{g}/\text{ml}$  gramicidin D.

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36 Taken together, these data indicate that the different activation profiles of NADH oxidation and  
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38 proton pumping are indeed due to a lowered proton pumping stoichiometry after the first  
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40 addition.  
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44 **Activation of proton pumping by Complex I in membrane vesicles.** Finally, inverted membranes  
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46 vesicles of *E. coli* were prepared to test whether the different activation profiles can also be  
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48 observed in native membranes. To suppress proton pumping by the  $b_0_3$  oxidase initiated by  
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50 NADH, the medium was supplemented with 1 mM KCN, which specifically blocks  $b_0_3$  oxidase.  
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52 Instead, 100  $\mu\text{M}$  DQ was added as an electron acceptor. In these experiments, only a limited  
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54 amount of NADH could be used, since the reduced ubiquinone, which is accumulated in the  
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3 absence of  $bo_3$  oxidase activity, notably inhibits Complex I, similar to the inhibiting effect of  
4 ubiquinol  $Q_1$  with the mitochondrial Complex I<sup>22</sup>. Due to high density and activity of Complex I in  
5 the membranes, it was impossible to follow the fast development of  $\Delta\psi$ , thus we studied proton  
6 translocation by following the acidification of the vesicle interior by the  $\Delta pH$  sensitive fluorescent  
7 probe acridine orange (AO). To convert  $\Delta\psi$  to  $\Delta pH$ , the assay buffer was supplemented with 1  $\mu M$   
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valinomycin and 100 mM KCl. In parallel, NADH consumption was determined optically in separate samples but under identical conditions. At 24 °C, an insignificant acceleration of  $\Delta pH$  generation by pre-activated Complex I was observed and no reliable difference in NADH consumption by non-activated and activated Complex I was detected as reported previously<sup>13</sup>. However, at lower temperatures the difference in the activity of activated and non-activated Complex I became evident. At 15 °C, the maximal acidification rate is 2-3 times higher if the enzyme was activated and the delay in pumping is much more pronounced (Figure 7).



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6 **Figure 7.** Acidification of the membrane vesicles interior and NADH consumption at 24 °C (A) and  
7 15 °C (B). Before measurements the membranes were equilibrated with AO, 100 μM DQ and 1  
8 mM KCN for 5 min at corresponding temperatures. AO response to: the 1<sup>st</sup> 20 μM NADH addition,  
9 blue; to the 2<sup>nd</sup> 20 μM NADH addition, red. NADH consumption at: 1<sup>st</sup> 20 μM NADH addition,  
10 black; 2<sup>nd</sup> 20 μM NADH addition, magenta. NADH was added at zero time as indicated by arrow.  
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20 NADH consumption as well proceeds with slightly longer delay at lower temperatures, however, it  
21 does not correlate with the delay in acidification. The maximal rate of NADH consumption was  
22 identical in both conditions, whereas there was still a significant difference in the maximal  
23 acidification rates between untreated and activated complex I (Figure 7).  
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## 30 Discussion

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33 The previously found R/A transition of Complex I from *E. coli* is a relatively fast process; it occurs in  
34 the time range of 1-2 s<sup>13</sup>, after which the rate of NADH:ubiquinone oxidoreduction has reached its  
35 steady-state value. Here, we found that in contrast to the full activation of NADH oxidation,  
36 coupled proton translocation in reconstituted Complex I stays significantly depressed much longer  
37 (Figure 2). Since Complex I contains solvent-accessible, low-potential FMN and therefore  
38 produces ROS upon reduction, it could be assumed that at the initial stage of the reaction,  
39 accelerated electron leakage to oxygen occurs. Production of ROS at significant rate could result in  
40 a mismatch between NADH oxidation and proton pumping at this stage. However, previous  
41 measurements of ROS production by Complex I revealed that the rate of ROS production does not  
42 exceed 0.2% of NADH oxidation rate regardless whether Complex I was solubilized or  
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3 reconstituted<sup>23</sup>. Such low electron leakage cannot affect proton pumping. More likely explanations  
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5 of the different activation profiles of electron and proton translocations could be that inactive  
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reconstituted<sup>23</sup>. Such low electron leakage cannot affect proton pumping. More likely explanations of the different activation profiles of electron and proton translocations could be that inactive Complex I either provides higher proton permeability to the membrane, or it undergoes a transition from de-coupled to coupled state (D/C transition) upon turnover. In our experiments, the former possibility was excluded by co-reconstitution of Complex I with ATP synthase, a separate proton pump. Irrespective of the presence or the state of Complex I, proton permeability tested by ATP driven proton pumping was unaffected. Based on our findings, we therefore propose that bacterial Complex I undergoes a transition from decoupled to coupled state upon turnover.

Properties of the system did not let us determine the activation time of proton pumping directly. The rate of NADH consumption may stay at a constant steady state relatively long time if the concentration of substrates is high and of the enzyme is low, allowing the observation of NADH:ubiquinone oxidoreduction activation. In contrast, the membrane energization occurs relatively fast and the final level of  $\Delta\mu_{H^+}$  is approached before the enzyme activation (Figure 1). Therefore, only the comparison of the initial rates of the energization reliably indicates the enzyme state. Some information on activation time can be obtained from data on the dependence of full activation of pumping on the amount of NADH. Complex I concentration in the sample was approximately 15 nM and the addition of 2  $\mu$ M NADH guaranteed over 100 turnovers, yet full activation was not achieved. The obtained data showed that the activation can be reached by multiple turnovers during approximately 80 s required for the consumption of 10  $\mu$ M NADH (Figure 4). The activation of pumping by NADPH also indicates a necessity of multiple turnovers and rules out a requirement of Complex I reduction to the level that can be approached by NADH

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3 addition. In the presence of DQ, the enzyme reduction by NADPH is insignificant due to the much  
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5 lower affinity of the electron donor NADPH as compared to the electron acceptor DQ. These  
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7 findings are in agreement with the data on NADPH effect on A/D transition of mitochondrial  
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9 Complex I: the enzyme reduction by NADH or NADPH in the absence of electron acceptors does  
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11 not initiate its activation, which can be achieved by NADPH only under conditions when electron  
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13 transfer from the substrate to quinone acceptor is permitted<sup>20</sup>. Pre-existing  $\Delta\psi$  maybe also  
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15 excluded as an essential activation factor, as incubation with NADPH did not result in  
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17  $\Delta\psi$  generation due to the slow turnover, yet Complex I was activated.

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24 It is likely that the fast acceleration of NADH:ubiquinone oxidoreduction<sup>13</sup> and simultaneous  
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26 conformational changes<sup>14</sup> are the early stages of an establishment of the fully functional state of  
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28 Complex I. The observed lack of direct correlation between electron and proton translocation  
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30 implies a decoupled state of Complex I that was not detected previously. It is reasonable to raise  
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32 the question whether this feature is an intrinsic property of Complex I or it is acquired due to  
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34 enzyme treatment upon purification and reconstitution. To test this, we studied Complex I  
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36 functioning in native membranes of subbacterial vesicles. Previously we found that  
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38 NADH:ubiquinone oxidoreductase activity of the membrane-bound enzyme has a short (1-2 s)  
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40 delay as well as purified Complex I, and no activation was detected at RT<sup>13</sup>. In this study,  
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42 monitoring of the proton pumping activity showed a small, but highly reproducible acceleration  
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44 after initial activation by NADH. However, lowering the temperature clearly revealed the  
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46 phenomenon of pumping activation (Figure 7). These results imply that the D/C transition is  
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48 indeed a built-in property of Complex I, but further studies are required to manifest this  
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50 statement.  
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3 The proposed transition could be a precursor of active/de-active transition of mitochondrial  
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6 Complex I, which evolved in evolution to ensure the enzyme's regulation in eukaryotes. Testing  
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9 proton pumping by Complex I in the natural environment, in membrane vesicles, indicated that  
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11 D/C transition is an intrinsic property of the enzyme. However, the significantly prolonged  
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13 decoupled state of the reconstituted enzyme could be of artificial nature, i.e. acquired during the  
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15 enzyme isolation and purification and might be caused by a loss of an unidentified factor, which  
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17 provides fast Complex I activation. Nevertheless, it is clear that both native and purified,  
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19 reconstituted Complex I from *E. coli* may exist in resting, non-pumping state, which converts to  
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21 pumping upon slow turnover. Finding of two states of *E. coli* Complex I should be taken into  
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23 account when planning future research, it provides a powerful tool to determine properties of  
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25 coupled and decoupled enzyme and, therefore, opens up opportunities for studying the molecular  
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27 mechanism of Complex I proton translocation.  
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### 45 46 **Supporting Information**

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48 Figure S1 showing that  $\text{NAD}^+$  is incapable to activate proton pumping by Complex I.  
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Activation of proton translocation by respiratory Complex I

Nikolai Belevich, Christoph von Ballmoos, Marina Verkhovskaya

