Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochimica et Biophysica Acta 1606 (2003) 95–103
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
www.bba-direct.com

# Active/de-active transition of respiratory complex I in bacteria, fungi, and animals

Elena Maklashina[a,b,*], Alexander B. Kotlyar[c], Gary Cecchini[a,b,*]

[a] *Molecular Biology Division (151-S), VA Medical Center, San Francisco, CA 94121, USA*
[b] *Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143, USA*
[c] *Department of Biochemistry, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, Israel*

Received 13 May 2003; received in revised form 15 July 2003; accepted 17 July 2003

**Abstract**

Mammalian complex I (NADH:ubiquinone oxidoreductase) exists as a mixture of interconvertible active (A) and de-activated (D) forms. The A-form is capable of NADH:quinone-reductase catalysis, but not the D-form. Complex I from the bacterium *Paracoccus denitrificans*, by contrast, exists only in the A-form. This bacterial complex contains 32 fewer subunits than the mammalian complex. The question arises therefore if the structural complexity of complex I from higher organisms correlates with its ability to undergo the A/D transition. In the present study, it was found that complex I from the bacterium *Escherichia coli* and from non-vertebrate organisms (earthworm, lobster, and cricket) did not show the A/D transitions. Vertebrate organisms (carp, frog, chicken), however, underwent similar A/D transitions to those of the well-characterized bovine complex I. Further studies showed that complex I from the lower eukaryotes, *Neurospora crassa* and *Yarrowia lipolytica*, exhibited very distinct A/D transitions with much lower activation barriers compared to the bovine enzyme. The A/D transitions of complex I as they relate to structure and regulation of enzymatic activity are discussed.
Published by Elsevier B.V.

*Keywords:* NADH:ubiquinone oxidoreductase; Complex I; Active/de-active transition; Mitochondrial respiration

## 1. Introduction

Complex I (NADH:ubiquinone reductase, NDH-1, coupling site I) is found in a variety of prokaryotic and eukaryotic organisms and couples the oxidation of NADH by ubiquinone with formation of a transmembrane proton gradient [1,2]. It was shown that beef heart complex I exists in active and de-activated forms [3,4]. The active (A) form of the enzyme is capable of catalyzing the high turnover rotenone-sensitive NADH-ubiquinone reductase reaction. The de-active (D) form can be fully reduced by NADH and oxidized by artificial electron acceptors, but is unable to transfer electrons to ubiquinone. De-activation, or the A-to-D transition, is spontaneous and characterized by high activation energy. De-activation occurs if complex I is limited due to the absence of one of the substrates of the enzyme (either in the presence of NADH and reduced quinone pool or in the presence of quinone when NADH is limiting). Activation (D-to-A transition) proceeds through activation turnover(s), which includes reduction of the enzyme by NADH, and a slow (at least 100 times slower than enzyme turnover) reoxidation by quinone. The rate of activation decreases at alkaline pH and in the presence of divalent cations [5]. The active form of complex I is not sensitive to sulfhydryl reagents whereas the de-active form is specifically modified and irreversibly inactivated by sulfhydryl reagents [6]. The phenomenon of the A/D transition of complex I has been demonstrated with isolated enzyme [7] and submitochondrial vesicles from bovine heart [3,5], permeablized rat heart mitochondria [8] and isolated perfused rat hearts [9].

Complex I from *Paracoccus denitrificans* is almost identical to its mitochondrial counterpart in terms of composition and thermodynamic properties of its redox centers

*Abbreviations:* SMP, submitochondrial particles; BSA, bovine serum albumin; $Q_1$, homologue of natural ubiquinone; HAR, hexaaminoruthenium; dNADH, deamino NADH; NEM, *N*-ethylmaleimide; A-form, active form of complex I; D-form, de-active form of complex I

* Corresponding authors. Molecular Biology Division (151-S), VA Medical Center, 4150 Clement Street, San Francisco, CA 94121, USA. Tel.: +1-415-752-9676; fax: +1-415-750-6959.
*E-mail addresses:* mclash@itsa.ucsf.edu (E. Maklashina), ceccini@itsa.ucsf.edu (G. Cecchini).

0005-2728/$ - see front matter. Published by Elsevier B.V.
doi:10.1016/S0005-2728(03)00087-2

[10]. All signs of energization of complex I detected by EPR were also observed with bovine and *P. denitrificans* coupled membranes [11], indicating a similar mechanism of energy conservation in the bacterial and mitochondrial enzymes. The only apparent functional difference between the enzymes from the two sources is the ability to undergo the active/de-active transition [12].

Electron microscopy and single particle analysis have shown that complex I from bacteria and mitochondria to be L-shaped molecules with an intrinsic membrane arm extending into the lipid bilayer and a peripheral arm protruding from the membrane [2,13]. However, the subunit composition and size of the enzymes vary from 14 subunits and molecular mass of 530 kDa in a *P. denitrificans* enzyme [14] to 46 subunits and molecular mass of 980 kDa in beef heart complex I [15]. Seven of the subunits from bacterial complex I constitute the peripheral soluble domain harboring all known prosthetic groups: FMN, six to eight iron–sulfur clusters [16–19]. The remaining seven subunits are hydrophobic and form the membrane domain. Homologues to the bacterial complex I subunits are found among evolutionary more advanced eukaryotes. Seven membrane intrinsic subunits are mitochondrially encoded in animals and fungi; all other subunits are nuclear-encoded [20]. Thus, all members of the complex I family contain homologues to a "minimum" *Escherichia coli* enzyme believed to constitute the structural framework for NADH oxidation, proton translocation and quinone reduction [21]. In eukaryotes, the supranumery subunits also share sequence similarities between species as diverse as *Neurospora crassa* and mammalian enzymes [20]. The function of these additional subunits, however, remains unclear.

It was decided to screen complex I from different organisms for their ability to demonstrate the A/D transition. In this study, complex I from a number of organisms, such as bacteria, fungi, non-vertebrate and vertebrate animals, was examined. NADH-oxidase activity sensitive to quinone-site specific complex I inhibitors was assayed in bacterial membranes and submitochondrial particles (SMP). It was found that among organisms tested, only complex I from vertebrate animals and fungi demonstrate the A/D transition phenomenon. Thermodynamic parameters for the transitions were determined and the importance of the phenomenon of the A/D transition for the structure and function of complex I is discussed.

## 2. Materials and methods

### 2.1. Membrane and mitochondrial preparations

*E. coli* (DH5α) and *Yarrowia lipolytica* (9773 ATCC) are standard laboratory strains. *N. crassa* mitochondria were obtained from Dr. G. Turner (University of California Los Angeles). Earthworms (*Lumbricus terrestris*) and crickets (*Acheta domesticus*) were obtained locally. Lobster (*Homerus americanus*), frog (*Rana catesbeiana*), carp (*Cyprinus caprio*) and chicken hearts were freshly harvested at a local market, transported on ice to the laboratory and processed for mitochondrial isolation within 30 min. *E. coli* membranes were isolated by standard procedures [22]. *Y. lipolytica* mitochondrial membranes were isolated as described [23]. Mitochondria from medium-size (4–5 weeks) crickets were isolated according to general procedures used for insects and house fly mitochondria [24]. Mitochondria from lobster, carp and frog skeletal muscles were isolated by similar methods [25]. Mitochondria from frog liver were isolated as previously described [26]. Mitochondria from chicken hearts were isolated according to Ref. [27]. Mitochondria from earthworms were isolated essentially as described in Ref. [25]. Worms were immobilized by cooling to 0–2 °C, cut into small (about 2 mm) pieces, and extensively washed in 0.15 M KCl several times to remove soil. The tissue fragments (about 20 g) were homogenized for 1 min in 0.15 M KCl, 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$ and 1 mM EDTA by using a Waring blender. The homogenate was centrifuged 10 min at 650 × *g* and mitochondria were collected from the supernatant by further centrifugation for 10 min at 8000 × *g*. The mitochondrial pellet was washed once with the same medium.

### 2.2. SMP

Mitochondria were resuspended in 0.125 M sucrose. SMP (except from frog liver) were obtained by sonication (four 15-s pulses with 30-s intervals). The mitochondrial debris was discarded after centrifugation (20 min at 30 000 × *g*) and SMP were collected by centrifugation (1 h at 130 000 × *g*). SMP from frog liver were obtained by a single passage of mitochondria through a cell disrupter at 10 000 psi (Emulsi-Flex-C5, Avestin, Canada). Mitochondrial debris was discarded after centrifugation (20 min at 15 000 × *g*) and SMP were collected from the supernatant (60 min at 130 000 × *g*). All SMP preparations used in this study showed a rotenone-sensitive NADH-$Q_1$ reductase activity.

### 2.3. Enzyme assays

Activities were assayed at 25 °C in 20 mM Tris–HCl (pH 8.5), 0.25 M sucrose, 40 mM KCl, 0.2 mM EDTA, 1 μg/ml gramicidin D with 120 μM NADH (NADH-oxidase), 40–70 μM $Q_1$ and 2 mM KCN (NADH:$Q_1$-reductase). For assay of the *E. coli* and *Y. lipolytica* preparations, deamino NADH (dNADH) was used as a substrate. The reactions were followed by the decrease of NADH absorbance at 340 nm ($\varepsilon^{340} = 6.22\ mM^{-1}\ cm^{-1}$). NADH:hexaaminoruthenium (HAR)-reductase activity was determined with 150 μM NADH and 2 mM HAR in the presence of 1–5 μM rotenone.

The fraction of the active form of complex I in the samples was determined in the presence of 5–10 mM $MgCl_2$ added to the reaction medium before the reaction was started by addition of NADH. The fully activated

enzyme (100% of active form complex I) was achieved by 1-min preincubation of the enzyme in the reaction cuvette with 3 μM NADH before addition of $MgCl_2$.

Stopped-flow kinetics was performed with an OLIS RSM stopped-flow (Olis Inc., Bogart, GA). NADH oxidation by SMP (final concentration 10–25 μg of protein/ml) was monitored in the standard enzyme assay medium and followed at 340 nm.

The protein concentration of samples was determined by the Biuret method.

### 2.4. Materials

NADH, dNADH, rotenone, gramicidin D, Tris, bovine serum albumin (BSA), EDTA, $MgCl_2$, KCl and sucrose were from Sigma. HAR was from Strem Chemicals, Newburyport, MA. $Q_1$ was a gift from Eisai Co, Tokyo, Japan.

## 3. Results

### 3.1. Vertebrate animals

SMP isolated from fish and frog skeletal muscle and from chicken heart show a rotenone-sensitive NADH-oxidase activity and demonstrate the active/de-active transition of complex I similar to bovine SMP [3–5]. Fig. 1 represents the time course of NADH-oxidase activity catalyzed by frog SMP. Complex I became essentially de-active after SMP were incubated at 35 °C for 10 min. The NADH-oxidase activity of such samples is extremely sensitive to the presence of 5 mM $MgCl_2$ (Fig. 1a). This behavior is characteristic for the de-active state of complex I as activation occurs with a lag-phase that becomes very pronounced in the presence of divalent cations [5]. Preincubation of de-activated SMP with 3 μM NADH in the assay cuvette prior to initiation of the NADH-oxidase reaction results in the fully active form of complex I which is insensitive to $Mg^{2+}$ (Fig. 1b). In full agreement with bovine complex I studies, NADH-dehydrogenase activity of the SMP determined with HAR remains unchanged regardless of the SMP pretreatment with NADH (data are not shown).

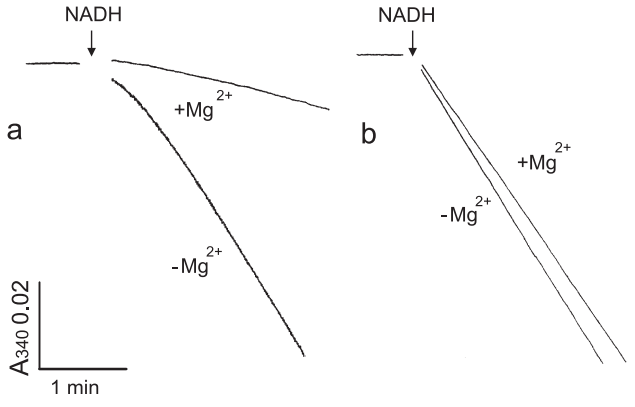


Fig. 1. NADH-oxidase of SMP from frog skeletal muscle. SMP were de-activated by incubation for 10 min at 37 °C. The NADH-oxidase activity of SMP (20 μg/ml in assay cuvette) was measured as described in Materials and methods with or without 5 mM $MgCl_2$ added before the reaction was initiated by addition of 120 μM NADH (indicated by arrows). (a) SMP and (b) SMP were preactivated during oxidation of 3 μM NADH in the reaction cuvette before the addition of $MgCl_2$ and initiation of the reaction.

Table 1
Thermodynamic parameters for activation/de-activation of complex I from different species

| Source | Activation $E$ for de-activation, kJ/mol | Activation $E$ for activation, kJ/mol | References |
|---|---|---|---|
| Carp skeletal muscle | 204 | 67 | this work |
| Frog skeletal muscle | 151 | 66 | this work |
| Bovine heart | 270 | 170 | [3,4] |

The transitions between active and de-active forms of bovine complex I are extremely sensitive to temperature and characterized by high apparent activation barriers (Table 1). The same holds true for the SMP samples tested above. Once activated, the enzymes within SMP remain in their A-forms for hours at 4 °C and rapidly (within minutes) spontaneously convert into the D-forms at temperatures higher than 35 °C. Divalent cations added to the assay provide a reliable and simple method to quantify the amount of complex I A-form. As seen in Fig. 1, only the A-form is catalytically competent in the presence of $Mg^{2+}$. The initial rate of NADH-oxidase or rotenone-sensitive NADH:$Q_1$-reductase activity determined in the presence of divalent cations corresponds to the portion of the A-form of complex I and can be measured with a high degree of accuracy and reliability. Fig. 2 represents an example of thermal de-activation of frog complex I. The initial rate of NADH-oxidase activity determined in the presence of $Mg^{2+}$ decreases upon incubation of SMP at different temperatures and the half-time of de-activation varies from 25 min at 25 °C to just 1 min at 37 °C (Fig. 2a). When complex I becomes substantially de-activated, a brief oxidation of NADH by SMP restores the active form (Fig. 2a, open symbols). The values of the apparent activation energy for the de-activation process for complex I in preparations from frog and carp SMP were calculated from the Arrhenius plots (Fig. 2b and Table 1).

The lag phase in NADH-oxidase activity catalyzed by de-activated frog SMP shows first-order kinetics as revealed by stopped flow measurements (Fig. 3a and b) and is in agreement with data for bovine complex I [3]. The duration of the lag phase depends on the temperature. The Arrhenius plot of $k_a$ determined within the temperature range of 17–35 °C gave the apparent activation energy value of 66 kJ/mol for the D-to-A transition (Fig. 3c) in the frog SMP.

Using the experimental approach described above, the apparent activation energy for the reversible activation and de-activation of complex I from carp was determined

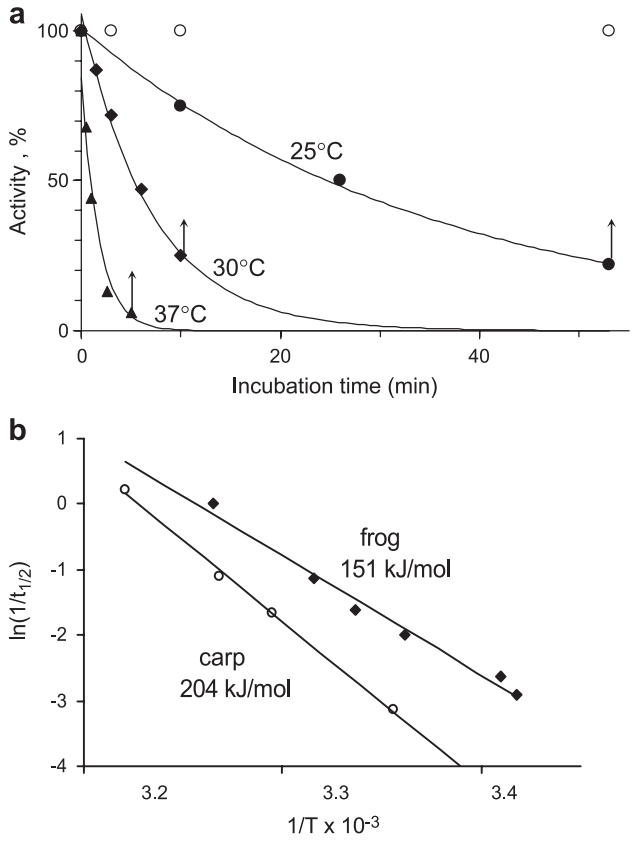


Fig. 2. De-activation of complex I in frog muscle SMP. (a) SMP (0.25 mg/ml) were preincubated with 200 μM NADH at 25 °C, pH 7.5 for 5 min in order to achieve complete activation of complex I and then were incubated for varying time at the temperatures indicated in the figure. An aliquot of suspension (20 μg protein/ml final concentration in the assay) was withdrawn and NADH-oxidase was assayed as described in Materials and methods (closed symbols). The arrows indicate when a second pulse of 200 μM NADH was added to the suspension and the activity was assayed after 2 min (open circles) demonstrating complete restoration of active form of complex I. One-hundred percent of activity corresponds to 600 nmol/min/mg protein. (b) Arrhenius plot of half de-activation time of complex I from frog (closed symbols) and carp (open circles) skeletal muscles.

(Table 1). Comparison of several vertebrate animals showed significant similarities between these species as related to the active/de-active transition of Complex I. In all cases tested, complex I exists as a mixture of two distinct forms: active and de-active. The A/D transitions of complex I in cold-blooded animals (frog and carp), however, are characterized by apparent lower activation barriers compared to that of bovine complex I (Table 1).

### 3.2. Fungal complex I

Oxidation of NADH by mitochondria from fungi is only partially sensitive to the specific complex I quinone-site inhibitors due to the presence of an alternative NADH-dehydrogenase (NDH-2) [28]. NDH-2, in contrast to complex I, is not able to oxidize dNADH. When dNADH is used as a substrate with fungal SMP, the reaction of dNADH oxidation is fully sensitive to rotenone. *Y. lipolytica* mitochondrial membranes, as isolated, show a lag phase in dNADH-oxidation measured at 20 °C that becomes extremely pronounced in the presence of 5 mM $MgCl_2$ (data not shown). When the membranes are pretreated with 5 μM dNADH for 1 min before the reaction, dNADH-oxidation is no longer sensitive to $MgCl_2$. Thus, *Y. lipolytica* complex I shows the active/de-active transition. The rate of activation observed at 20 °C (pH 8.0) increased dramatically with temperature and became undetectable at temperatures higher than 30 °C. However, the D-form of complex I retained its sensitivity to $Mg^{2+}$ and the use of divalent cations allows quantitative determination of the A-form even at elevated temperature as is seen for vertebrate complex I. The dNADH:HAR-reductase activity of *Y. lipolytica* complex I does not change upon preincubation of SMP with dNADH (data are not shown). Examination of *N. crassa* dNADH-oxidase activity also showed kinetic behavior very similar to that of the *Y. lipolytica* enzyme (data not shown) and is in agreement with recent observations of Grivennikova et al. [29].

A significant difference between the reversible A/D transitions in *Y. lipolytica* and vertebrate complex I is a low apparent activation energy barrier for de-activation in the fungal enzyme. This resulted in poor stability of the A-form even at 4 °C. Fig. 4 demonstrates the kinetics of *Y. lipolytica* complex I de-activation at 20 °C. The upper trace shows the oxidation of 5 μM dNADH added to the spectrophotometric cuvette in the presence of SMP. The dashed arrows indicate when 150 μM dNADH was added to the cuvette, and the initial rate of the dNADH-oxidase reaction (lower graph) was determined with 5 mM $MgCl_2$ (filled circles) or without $MgCl_2$ (opened circles). As seen from the figure, SMP are characterized by very low dNADH-oxidase activity when assayed in the presence of 5 mM $MgCl_2$, indicating that complex I is largely in the de-activated form as isolated. Oxidation of 5 μM dNADH for 1 min converted complex I into the active form based upon the high rate of dNADH oxidation measured in the presence of $MgCl_2$. Within 4 min, when about 0.5 μM of dNADH remains in the reaction medium, complex I remains predominately in the active form. However, after almost complete oxidation of dNADH (5 min), a fast de-activation of complex I occurred. A second addition of 5 μM dNADH again completely activated the enzyme within a minute.

### 3.3. N-Ethylmaleimide (NEM)-modification of A and D forms of complex I in frog and Y. lipolytica

The de-activated bovine complex I is irreversibly inhibited by the sulfhydryl modifying agent NEM, while the active form is almost insensitive to the inhibitor [6]. Fig. 5 shows the NEM inhibition of frog and *Y. lipolytica* complex I as a function of the active/de-active transition. Frog complex I showed very similar kinetics to the bovine enzyme for NEM inhibition, i.e. strong inhibition of the

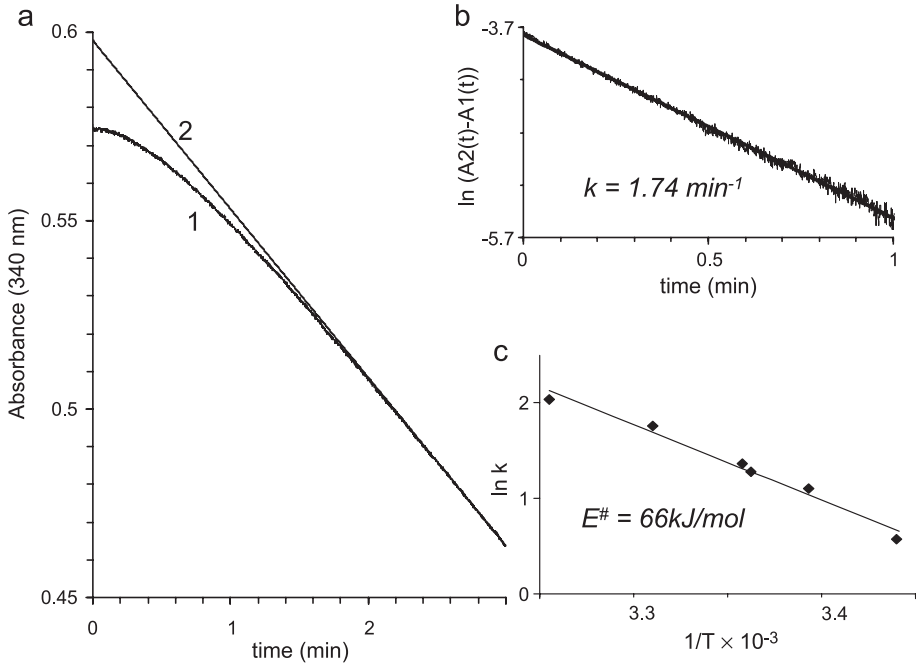


Fig. 3. Activation of frog complex I. Frog muscle SMP (1 mg/ml) were de-activated by incubation for 15 min at 35 °C. (a) The lag-phase in NADH-oxidase reaction during activation of the de-active complex I (trace 1) was recorded with a stopped-flow spectrophotometer as described in Materials and methods (pH 8.0, 17 °C). Final protein concentration was 25 μg/ml. Trace 2 shows the extrapolation to a linear phase of trace 1. (b) Determination of activation constant (*k*). The difference in absorbance between traces 2 and 1 in semi-logarithmic coordinates. (c) Arrhenius plot for activation process measured as shown in (b).

de-active form (Fig. 5a). Due to the poor stability of the active form of *Y. lipolytica* complex I, it is impossible to reproduce the above experiment with the fungal SMP. Instead, *Y. lipolytica* SMP were incubated with NEM directly in the reaction cuvette (Fig. 5b). De-activated SMP (20 μg/ml) were incubated with 0.5 mM NEM at

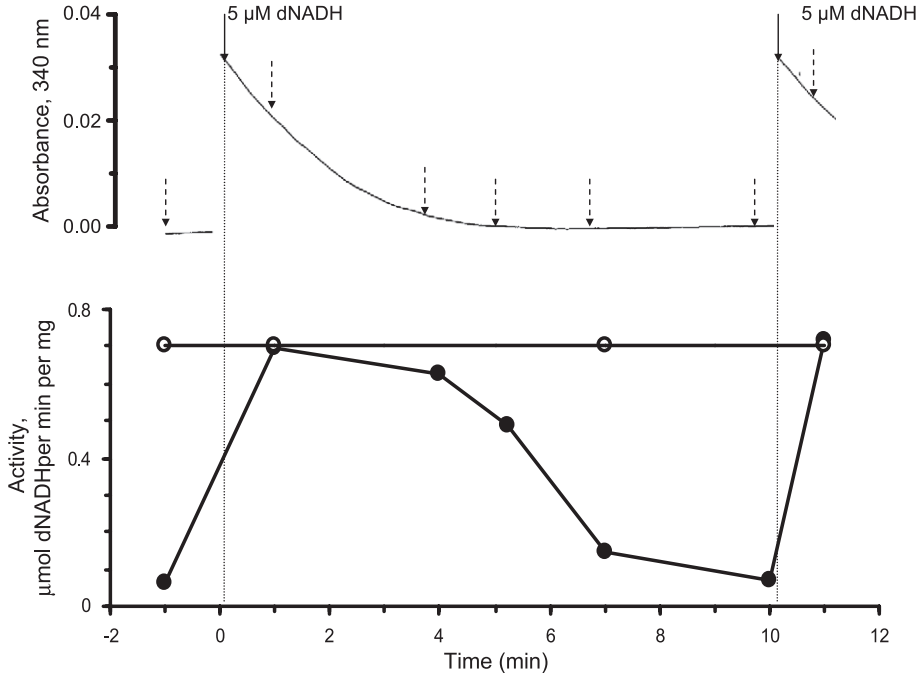


Fig. 4. Active/de-active transitions of *Y. lipolytica* complex I. SMP (12.5 μg/ml) were placed in a 1-ml cuvette (upper trace) containing 50 mM Tris, 0.25 M sucrose, 0.2 mM EDTA and 1 mg/ml BSA (pH 8.4), at 20 °C. At zero time, 5 μM dNADH (solid arrows) was added to the cuvette and upon its oxidation by SMP at the time indicated by dashed arrows, dNADH-oxidase activities of complex I were determined with (closed circles) and without (open circles) 5 mM $MgCl_2$ (lower graph).

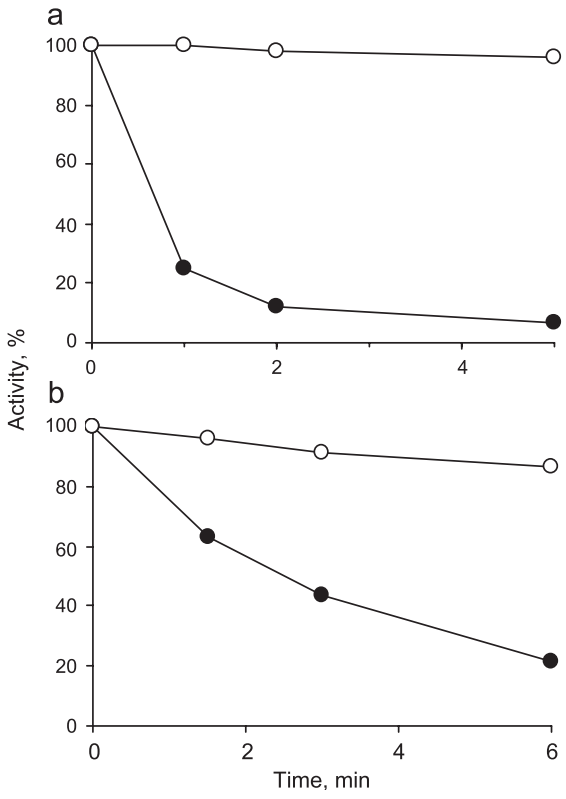


Fig. 5. Irreversible inhibition of complex I in SMP from frog skeletal muscle and *Y. lipolytica* by NEM. (a) Frog SMP (0.5 mg protein/ml) were activated with 100 μM NADH for 5 min at 25 °C (open circles) or de-activated by incubation for 10 min at 35 °C (closed circles). The samples were then incubated with 1 mM NEM at 20 °C for the time indicated in the abscissa. An aliquot of suspension was withdrawn and NADH:$Q_1$-reductase reaction was assayed. (b) De-activated *Y. lipolytica* SMP (25 μg/ml) were added to the spectrophotometric cuvette and incubated with 0.5 mM NEM. Then the dNADH-$Q_1$-reductase reaction was measured at the indicated time (closed circles). To keep SMP in the active form during the experiment, 5 μM dNADH was added to the cuvette with SMP for 1 min prior to addition of 0.5 mM NEM. When the concentration of dNADH dropped to 1 μM, another portion of 5 μM dNADH was added. At the time indicated the dNADH-$Q_1$-reductase reaction was determined (open circles).

20 °C for the period of time indicated on the abscissa and the dNADH-$Q_1$ reductase reaction was initiated by simultaneous addition of KCN, dNADH and $Q_1$. To keep complex I in its active form during the incubation of SMP with NEM, 1–5 μM of dNADH was maintained in the cuvette. In agreement with active/de-active complex I phenomenology, both frog and *Y. lipolytica* SMP show similar patterns of NEM inhibition, where the active form is resistant to inhibition.

### 3.4. Complex I that does not show the A↔D transitions

The third group of organisms tested in this study consisted of a bacterium (*E. coli*) and non-vertebrate animals (earthworm, cricket, and lobster) which did not show the active/de-active transition of complex I. The NADH-oxidase and NADH:$Q_1$-reductase activities of SMP isolated from non-vertebrate animals were completely sensitive to rotenone. Fig. 6 represents a typical experiment on thermal incubation of SMP from non-vertebrate animals. SMP (1–5 mg/ml) were incubated at the indicated temperature and the initial rates of NADH-oxidase activity were determined in the presence (open circles) or absence (closed circles) of $MgCl_2$. The complex I activities in all samples were linear and not sensitive to divalent cations upon prolonged incubation of membranes at elevated temperature. The same pattern of dNADH-oxidation as seen in Fig. 6 is characteristic for *E. coli* membranes (data are not shown). In addition, no difference in NEM inhibition was found in the membranes before and after thermal incubation (data not shown).

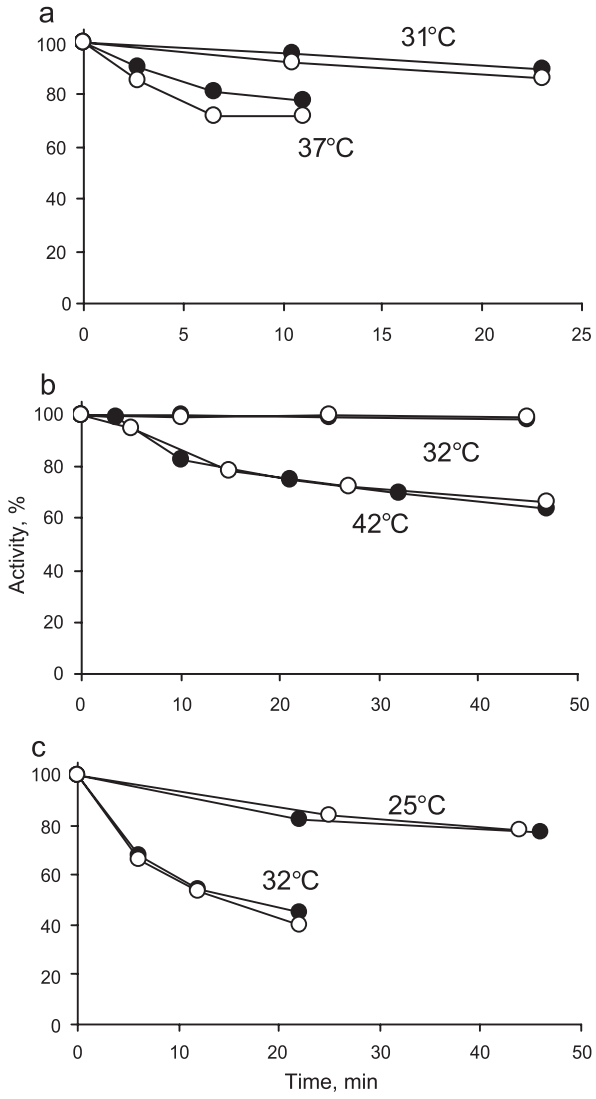


Fig. 6. Thermal incubation of complex I from various organisms. SMP from earthworm (a), cricket (b) and lobster (c) were incubated at a concentration of 1–5 mg protein/ml at the indicated temperature. An aliquot of suspension was withdrawn and NADH oxidase was determined in the presence (closed circles) or absence (open circles) of 5 mM $MgCl_2$ in the reaction medium as described in Materials and methods. One-hundred percent activity corresponds to 260 (a), 700 (b) and 530 (c) nmol of NADH oxidized per minute per milligram of protein.

These data are evidence for the absence of an active/de-active transition in complex I from *E. coli*, cricket, earthworm and lobster.

## 4. Discussion

This work was initiated because of the ability of bovine heart complex I to undergo the active/de-active transition, whereas there is a lack of this phenomenon in the bacterial *P. denitrificans* enzyme. The functional properties of bacterial complex I are almost identical to those of the mitochondrial one; however, the bacterial enzyme is structurally much simpler. The only functional difference between the enzymes is the active/de-active transition. Simple kinetic characteristics for the A/D transition were used in this study: (i) presence of a lag-phase in the NADH-quinone reductase reaction sensitive to divalent cations and corresponding to complex I activation; (ii) strong dependence of de-activation rate on temperature; (iii) selective inhibition of the D-form of complex I by SH-modifying agents. Table 2 summarizes the data on complex I from different species indicating the ability of the enzyme to undergo the active/de-active transition. The organisms from the Table 2 can be divided into three distinct groups. The first group is represented by vertebrate animals in which complex I exists in two thermodynamically stabilized forms and the reversible transitions between the forms satisfy the above criteria; importantly, that A/D equilibrium may be completely shifted towards fully active or de-active forms of the enzymes. It should be noted that the active/de-active phenomenology of complex I is not tissue-specific and is observed in complex I from heart, liver and skeletal muscle. The second group represents bacteria and non-vertebrate animals, organisms where complex I remains in its active form upon prolonged thermal incubation, and none of the above criteria established for active/de-active transition are satisfied. The third group is represented by lower eukaryotic organisms. Although complex I from fungi clearly displays the active/de-active transition phenomenon, the kinetic parameters of the transition are different from those of vertebrates. This is especially true for the *Y. lipolytica* enzyme characterized by the low activation energy of the transition in either direction. The latter leads to fast interconversion between the forms into stabilized de-active form even at temperatures as low as 4 °C.

Table 2
Active/de-active transition in complex I from various organisms

| Source | Active/de-active phenomenology | References |
|---|---|---|
| *Bacteria* | | |
| *E. coli* | – | this work |
| *P. denitrificans* | – | [12] |
| *Th. thermophilus* | – | V. Sled, personal communication |
| *Non-vertebrate* | | |
| Earthworm | – | this work |
| Cricket | – | this work |
| Lobster | – | this work |
| *Vertebrate* | | |
| Carp skeletal muscle | + | this work |
| Frog; skeletal muscle, liver | + | this work |
| Rat, heart | + | [8,9] |
| Beef, heart | + | [3,7] |
| Chicken, heart | + | this work |
| *Fungi* | | |
| *N. crassa* | + | [29] and this work |
| *Y. lipolytica* | + | this work |

+, Shows active/de-active transition.
–, Active/de-active transition is absent in these organisms.

The bacterial enzymes represented in Table 2 contain 14 subunits [10]; in contrast, the fungal enzymes contain more than 35 subunits [23], and mammalian enzymes contain 46 subunits [13]. An attractive explanation for the absence of the A/D transition in the bacterial enzymes would be their simpler structural organization compared to mammalian and fungal complex I. Unfortunately, there is no information available on the composition of complex I from the non-vertebrates used here; however, it can be assumed that the enzyme from these eukaryotes would also contain 'accessory' subunits like those found in the other eukaryotes. Thus, by itself the number of 'accessory' subunits is not sufficient to explain the lack of the A/D transition in the non-vertebrates. However, the difference between active and de-active forms of complex I is not only in the kinetic behavior. Based on selective irreversible modification of the D-form by NEM, it was suggested that A and D forms are also structurally different [4,6]. The similar effect of NEM inactivation on the enzyme from different organisms is indicative of a similar mechanism for the active/de-active transition. It was shown that a subunit of bovine complex I, which has an approximate mass of 15 kDa, is specifically modified by the sulfhydryl reagent in the de-active form of the enzyme [6]. However, the specific subunit has not yet been identified and thus no data on homology of the subunit within various species have been presented. Thus, another possible reason for the lack of the A/D transitions in bacterial and non-vertebrate animals is the absence of a homologue to that of the 15-kDa subunit and/or posttranslational modification of other subunits that may be essential for the enzyme transitions.

The different sensitivity of the active and de-active forms to the specific quinone-site inhibitor rotenone ($K_i$ for rotenone in active and de-active forms is 1 and 80 nM, respectively [30]) indicates that the major perturbation of complex I structure in the active and de-active forms is within the quinone-binding region. Recent data on photoaffinity labeling of complex I with the potent quinone-site inhibitor trifluoromethyl-diazirinyl[$^3$H]pyridaben gave distribution of radioactivity between the ND-1 and PSST subunits of complex I in bovine SMP [31]. Interestingly,

incubation of SMP with NADH increased PSST labeling and decreased ND-1 labeling [32]. In standard preparations of bovine SMP, about 60% of complex I is in the active state [7]. Thus, the above data support the suggestion of structural difference in the Q-binding site of A and D forms in mammalian complex I. In contrast, the photoaffinity probe labeled only the NQO6 subunit of these bacteria, the counterpart of mitochondrial PSST subunit [33]. This is consistent with the data from Table 2 showing that *P. denitrificans* and *Thermus thermophilus* NDH-1 exist only in the active form.

Complex I as a major source for electron input into the respiratory chain is an attractive site to regulate the overall rate of electron transfer. Bacteria often adjust the composition of electron transport chains depending on available energy sources and final electron acceptors. Different organisms may also develop various adaptation mechanisms to control NADH oxidation including alternative NADH-quinone reductases (NDH-2) [34] or enzyme modification such as phosphorylation [35]. Most eukaryotic animals contain a single complex I in the inner mitochondrial membrane. The evidence reported in this manuscript supports a suggestion that regulation of NADH oxidation in the mitochondrial respiratory chain via reversible activation/de-activation of complex I has been achieved in evolution by introducing additional protein subunits. The A/D transitions in warm-blooded animals are characterized by high apparent activation energy. The body temperature of 37–39 °C is high enough to enable interconversion between the forms within minutes. If the activation state of complex I regulates overall NADH oxidation in mitochondria, then the activation energy of the transitions should correlate with body temperature. Indeed the results presented in Table 1 support the above suggestion, with the transition in frog and carp complex I characterized by lower activation barriers compared to mammalian enzyme.

## Acknowledgements

This study was supported in part by the Department of Veterans Affairs and by NIH grants GM61606 and TW06041. We thank Brian A.C. Ackrell for critical reading of the manuscript.

## References

[1] J.E. Walker, The NADH: ubiquinone oxidoreductase (complex I) of respiratory chains, Q. Rev. Biophys. 25 (1992) 253–254.
[2] T. Yagi, A. Matsuno-Yagi, The proton-translocating NADH-quinone oxidoreductase in the respiratory chain: the secret unlocked, Biochemistry 42 (2003) 2266–2274.
[3] A.B. Kotlyar, A.D. Vinogradov, Slow active/inactive transition of the mitochondrial NADH-ubiquinone reductase, Biochim. Biophys. Acta 1019 (1990) 151–158.
[4] A.D. Vinogradov, Catalytic properties of the mitochondrial NADH-ubiquinone oxidoreductase (complex I) and the pseudo-reversible active/inactive enzyme transition, Biochim. Biophys. Acta 1364 (1998) 169–185.
[5] A.B. Kotlyar, V.D. Sled, A.D. Vinogradov, Effect of $Ca^{2+}$ ions on the slow active/inactive transition of the mitochondrial NADH-ubiquinone reductase, Biochim. Biophys. Acta 1098 (1992) 144–150.
[6] E.V. Gavrikova, A.D. Vinogradov, Active/de-active state transition of the mitochondrial complex I as revealed by specific sulfhydryl group labeling, FEBS Lett. 455 (1999) 36–40.
[7] E.O. Maklashina, V.D. Sled, A.D. Vinogradov, Hysteresis behavior of complex I from bovine heart mitochondria: kinetic and thermodynamic parameters of retarded reverse transition from the inactive to active state, Biochemistry/Biokhimiia 59 (1994) 946–957.
[8] V.G. Grivennikova, A.N. Kapustin, A.D. Vinogradov, Catalytic activity of NADH-ubiquinone oxidoreductase (complex I) in intact mitochondria. Evidence for the slow active/inactive transition, J. Biol. Chem. 276 (2001) 9038–9044.
[9] E. Maklashina, Y. Sher, H.-Z. Zhou, M.O. Gray, J.S. Karliner, G. Cecchini, Effect of anoxia/reperfusion on the reversible active/de-active transition of NADH-ubiquinone oxidoreductase (complex I) in rat heart, Biochim. Biophys. Acta 1556 (2002) 6–12.
[10] T. Yagi, T. Yano, S. Di Bernardo, A. Matsuno-Yagi, Prokaryotic complex I (NDH-1), an overview, Biochim. Biophys. Acta 1364 (1998) 125–133.
[11] R. van Belzen, A.B. Kotlyar, N. Moon, W.R. Dunham, S.P.J. Albracht, The iron–sulfur clusters 2 and ubisemiquinone radicals of NADH: ubiquinone oxidoreductase are involved in energy coupling in submitochondrial particles, Biochemistry 36 (1997) 886–893.
[12] A.B. Kotlyar, S.P.J. Albracht, R.J.M. van Spanning, Comparison of energization of complex I in membrane particles from *Paracoccus denitrificans* and bovine heart mitochondria, Biochim. Biophys. Acta 1365 (1998) 53–59.
[13] L.A. Sazanov, J. Carroll, P. Holt, L. Toime, I.M. Fearnley, A role for native lipids in the stabilization and two-dimensional crystallization of the *Escherichia coli* NADH-ubiquinone oxidoreductase (Complex I), J. Biol. Chem. 278 (2003) 19483–19491.
[14] T. Yano, T. Yagi, H(+)-translocating NADH-quinone oxidoreductase (NDH-1) of *Paracoccus denitrificans*. Studies on topology and stoichiometry of the peripheral subunits, J. Biol. Chem. 274 (1999) 28606–28611.
[15] J. Carroll, I.M. Fearnley, R.J. Shannon, J. Hirst, J.E. Walker, Analysis of the subunit composition of complex I from bovine heart mitochondria, Mol. Cell, Proteomics 2 (2003) 117–126.
[16] T. Friedrich, The NADH: ubiquinone oxidoreductase (complex I) from *Escherichia coli*, Biochim. Biophys. Acta 1364 (1998) 134–146.
[17] H. Beinert, S.P.J. Albracht, New insights, ideas and unanswered questions concerning iron–sulfur clusters in mitochondria, Biochim. Biophys. Acta 683 (1982) 245–277.
[18] T. Ohnishi, Iron–sulfur clusters/semiquinones in complex I, Biochim. Biophys. Acta 1364 (1998) 186–206.
[19] S.P.J. Albracht, E. van der Linden, B.W. Farber, Quantitative amino acid analysis of bovine NADH: ubiquinone oxidoreductase (Complex I) and related enzymes. Consequences for the number of prosthetic groups, Biochim. Biophys. Acta 1557 (2003) 41–49.
[20] A. Videira, Complex I from the fungus *Neurospora crassa*, Biochim. Biophys. Acta 1364 (1998) 89–100.
[21] T. Friedrich, Complex I: a chimera of a redox and conformation-driven proton pump, J. Bioenerg. Biomembranes 33 (2001) 169–177.
[22] E. Maklashina, D.A. Berthold, G. Cecchini, Anaerobic expression of *Escherichia coli* succinate dehydrogenase: functional replacement of fumarate reductase in the respiratory chain during anaerobic growth, J. Bacteriol. 180 (1998) 5989–5996.
[23] R. Djafarzadeh, S. Kerscher, K. Zwicker, M. Radermacher, M. Lindahl, H. Schägger, U. Brandt, Biophysical and structural characterization of proton-translocating NADH-dehydrogenase (complex I) from the strictly aerobic yeast *Yarrowia lipolytica*, Biochim. Biophys. Acta 1459 (2000) 230–238.

[24] S.G. Van den Bergh, Insect mitochondria, Methods Enzymol. 10 (1967) 117–122.
[25] L. Ernster, K. Nordenbrand, Skeletal muscle mitochondria, Methods Enzymol. 10 (1967) 86–94.
[26] D. Johnson, H. Lardy, Isolation of liver or kidney mitochondria, Methods Enzymol. 10 (1967) 94–96.
[27] P.V. Blair, The large-scale preparation and properties of heart mitochondria from slaughterhouse material, Methods Enzymol. 10 (1967) 78–81.
[28] S.J. Kerscher, A. Eschemann, P.M. Okun, U. Brandt, External alternative NADH: ubiquinone oxidoreductase redirected to the internal face of the mitochondrial inner membrane rescues complex I deficiency in *Yarrowia lipolytica*, J. Cell Sci. 114 (2001) 3915–3921.
[29] V.G. Grivennikova, D.V. Serebryanaya, E.P. Isakova, T.A. Belozerskaya, A.D. Vinogradov, The transition between active and deactivated forms of NADH: ubiquinone oxidoreductase (Complex I) in the mitochondrial membrane of *Neurospora crassa*, Biochem. J. 369 (2003) 619–626.
[30] V.G. Grivennikova, E.O. Maklashina, E.V. Gavrikova, A.D. Vinogradov, Interaction of the mitochondrial NADH-ubiquinone reductase with rotenone as related to the enzyme active/inactive transition, Biochim. Biophys. Acta 1319 (1997) 223–232.
[31] F. Schuler, T. Yano, S. Di Bernardo, T. Yagi, V. Yankovskaya, T.P. Singer, J.E. Casida, NADH-quinone oxidoreductase: PSST subunit couples electron transfer from iron–sulfur cluster N2 to quinone, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 4149–4153.
[32] F. Schuler, J.E. Casida, Functional coupling of PSST and ND1 subunits in NADH:ubiquinone oxidoreductase established by photoaffinity labeling, Biochim. Biophys. Acta 1506 (2001) 79–87.
[33] E. Nakamaru-Ogiso, K. Sakamoto, A. Matsuno-Yagi, H. Miyoshi, T. Yagi, The ND5 subunit was labeled by a photoaffinity analogue of fenpyroximate in bovine mitochondrial complex I, Biochemistry 42 (2003) 746–754.
[34] S.J. Kerscher, Diversity and origin of alternative NADH: ubiquinone oxidoreductases, Biochim. Biophys. Acta 1459 (2000) 274–283.
[35] S. Papa, The NDUFS4 nuclear gene of complex I of mitochondria and the cAMP cascade, Biochim. Biophys. Acta 1555 (2002) 147–153.