The transition between active and de-activated forms of NADH:ubiquinone oxidoreductase (Complex I) in the mitochondrial membrane of *Neurospora crassa*

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The mammalian mitochondrial NADH:ubiquinone oxidoreductase (Complex I) has been shown to exist in two kinetically and structurally distinct slowly interconvertible forms, active (A) and de-activated (D) [Vinogradov and Grivennikova (2001) IUBMB Life **52**, 129–134]. This work was undertaken to investigate the putative Complex I A–D transition in the mitochondrial membrane of the lower eukaryote *Neurospora crassa* and in plasma membrane of the prokaryote *Paracoccus denitrificans*, organisms that are eligible for molecular genetic manipulations. The potential interconversion between A and D forms was assessed by examination of the initial and steady-state rates of NADH oxidation catalysed by inside-out submitochondrial (*N. crassa*) and sub-bacterial (*P. denitrificans*) particles and their sensitivities to *N*-ethylmaleimide and Mg^{2+}. All diagnostic tests provide evidence that slow temperature- and turnover-dependent A–D transition is an explicit feature of eukaryotic *N. crassa* Complex I, whereas the phenomenon is not seen in the membranes of the prokaryote *P. denitrificans*. Significantly lower activation energy for A-to-D transition characterizes the *N. crassa* enzyme compared with that determined previously for the mammalian Complex I. Either a lag or a burst in the onset of the NADH oxidase assayed in the presence of Mg^{2+} is seen when the reaction is initiated by the thermally de-activated or NADH-activated particles, whereas the delayed final activities of both preparations are the same. We conclude that continuous slow cycling between A and D forms occurs during the steady-state operation of Complex I in *N. crassa* mitochondria.

Key words: energy transduction, enzyme hysteresis, respiratory chain.

INTRODUCTION

Three well-defined functions of crucial importance for cell physiology are carried out by the respiratory proton-pumping NADH:quinone oxidoreductase, usually called Complex I in eukaryotes and NDH-1 in prokaryotes. (i) The enzyme reoxidizes NADH, thus providing certain steady-state NAD^+/NADH ratios required for continuous operation of the oxidative metabolic pathways. (ii) It serves as the major electron entry point to the respiratory chain for further energy transduction. (iii) The enzyme itself is a reversibly operating energy-transducing device and thus significantly contributes to formation of $\Delta\tilde{\mu}_{H^+}$ (the electrochemical potential difference for protons between two phases separated by the coupling membrane). Complex I in mammalian mitochondria is the only enzyme which fulfils all three functions, whereas several other membrane-bound NADH:quinone oxidoreductases in addition to Complex I operate in plant and fungal mitochondria or in plasma membranes of most prokaryotes to carry out functions (i) and (ii).

The mammalian enzyme, as exemplified by the best-studied bovine heart mitochondrial Complex I [1], is of enormous structural complexity [2]. It composed of at least 43 different subunits encoded in both nuclear (at least 35 polypeptides) and mitochondrial (7 polypeptides) genomes with a total molecular mass of about 10^6 Da. Mitochondrial Complexes I with apparent molecular masses of 700–800 kDa or more have been isolated from several plant species [3]. The simpler, albeit not significantly (at least 35 different subunits), Complex I-type enzymes are present in the inner mitochondrial membranes of the Ascomycete *Neurospora crassa*, a eukaryotic organism that is a member of the fungal class related to yeasts [4,5], and in yeast (*Yarrovia lipolytica*) mitochondria [6]. The operons encoding prokaryotic NDH-1-type enzymes of plasma membranes of *Escherichia coli* [7], *Paracoccus denitrificans* [8] and *Rhodobacter capsulatus* [9] contain only 14 genes and all the subunits of prokaryotic NDH-1 have their homologous counterparts in fungal, plant and mammalian enzymes.

Only five to seven out of 14 (prokaryotes), 35 (fungi) or 43 (mammals) subunits have been assigned functional roles, such as binding of NADH or quinone and holding multiple redox components (FMN and up to eight distinct iron–sulphur clusters) [10,11]. The specific functions of 'extra' subunits in Complex I and NDH-1 remain unknown, although somewhat unexpected features have been recognized for several conserved subunits of the mammalian and fungal enzymes. The SDAP bovine heart enzyme subunit [12] and its *N. crassa* homologue [13] have been identified as acyl carrier proteins of the mitochondrial fatty acid biosynthetic pathway [note that the subunits of Complex I are designated either by their N-terminal four amino acids or by their apparent molecular mass and separation after resolution of bovine heart Complex I (e.g. IP15)]. The 39 kDa subunit of the mammalian enzyme (40 kDa in *N. crassa*) contains a pyridine nucleotide-binding motif and is related to members of the reductase/isomerase family [14,15]. The AQDQ subunit (21 kDa homologue in *N. crassa*) is phosphorylated by cAMP-dependent protein kinase [16,17]. The MWFE mammalian enzyme subunit

Abbreviations used: B-SMP, bovine heart SMP; d-NADH, deamino-NADH; NEM, *N*-ethylmaleimide; N-SMP, *Neurospora crassa* SMP; Q_1, 2,3-dimethoxy-5-methyl-6-isoprenyl-1,4-benzoquinone; SBP, *Paracoccus denitrificans* sub-bacterial particles; SMP, submitochondrial particles.

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(9.8 kDa homologue in *N. crassa*) has been reported to be absolutely required for the catalytic activity [18]. Very recently, GRIM-19, a 16 kDa protein (which stands for genes associated with retinal-interferon-β-induced mortality), has been identified as an intrinsic subunit of mammalian Complex I [19]. The presence of multiple subunits, which can hardly be directly involved in electron-transfer and/or proton-translocation mechanisms, strongly suggests that Complex I is under fine short- and long-term control when operating within the cell.

The catalytic properties of the best-studied mammalian enzyme are also far from trivial [20–22]. It has been recognized that most, if not all, preparations of the enzyme are heterogeneous mixtures of two slowly equilibrating forms, A (active) and D (de-activated). The presence of the D form is seen by a as more or less pronounced lag phase in the onset of the catalytic activities assayed in forward (NADH oxidation) or reverse ($\Delta\tilde{\mu}_{H^+}$-dependent NAD^+ reduction by ubiquinol, reverse electron transfer) reactions [23]. The lag phase is completely eliminated after preincubation of the enzyme preparation with NADH under conditions that permit enzyme turnover [23]. The rate of turnover-dependent D-to-A transition increases at acidic pH and decreases at alkaline pH and/or in the presence of Ca^{2+} or Mg^{2+}, whereas the catalytic activity itself as performed by the A form depends on neither pH nor the presence of bivalent cations [24]. The A form is spontaneously converted into the D form in extremely temperature-dependent reaction (activation energy, 270 kJ/mol) and an A/D ratio of $\approx 1:10$ is reached at equilibrium in the absence of substrates [24,25]. The A and D forms differ in their affinity for the specific Complex I inhibitors rotenone and piericidin [25] and in their accessibility to inhibitory thiol group reagents [24]. The small 15 kDa subunit of bovine heart enzyme (most likely IP15) has been identified as the polypeptide which shows a dramatic difference in its thiol-group reactivity with the A and D forms [26]. The mitochondrial Complex I A–D transition has been demonstrated in preparations of different degrees of resolution, such as purified Complex I [27], submitochondrial particles (SMP) [23], intact mitochondria [28] and very recently in *ex vivo* studies of perfused rat hearts [29].

The molecular mechanisms involved in the A–D transition remain unknown. All studies on the phenomenon reported so far have been carried out with mammalian Complex I, which is not suitable for mutational analysis. We therefore extended our studies to include the organisms eligible for the molecular genetic manipulation. We report here that the A–D transition is a characteristic feature of *N. crassa* Complex I whereas the phenomenon is not seen for in the prokaryotic *P. denitrificans* enzyme.

EXPERIMENTAL

Materials

Bovine heart SMP (B-SMP) were prepared and stored as described in [23]. Tightly coupled mitochondria were isolated from *N. crassa* (wild-type RL3-8A, FGSC 2218; Fungal Genetics Stock Center, University of Kansas, Kansas City, KS, U.S.A.) as described in [30] and used for preparation of *N. crassa* SMP (N-SMP) as follows. All operations were carried out at 0 °C. Mitochondria (75 mg/ml) suspended in 10 mM Tris/HCl buffer, pH 7.2, 0.4 M mannitol and 0.4 % BSA were diluted in 0.15 M sucrose/1 mM K-EDTA to a final protein content of 12 mg/ml. The suspension (about 10 ml) was saturated with argon for 10 min and pH was adjusted to 8.6 by adding 1 M NH_4OH. Anaerobic suspension was subjected to ultrasonic treatment under continuous flow of argon (five times for 30 s with 1 min intervals between each irradiation; MSE Soniprep 150) and centrifuged at 30000 ***g*** for 15 min. The precipitated material was discarded and supernatant was centrifuged at 120000 ***g*** for 1 h. The sediments were suspended in 10 ml of 0.25 M sucrose/50 mM Tris/HCl/0.2 mM EDTA, pH 8.0 and centrifuged at 30000 ***g*** for 1 h. The precipitated material was suspended in 2 ml of sucrose/Tris/EDTA buffer with 1 mg/ml BSA and stored in liquid nitrogen in small vials (0.2 ml). The samples were thawed and stored in ice during the experiments.

Sub-bacterial particles (SBP) from an anaerobic culture of *P. denitrificans* (strain Pd 1222) were prepared according to John and Whatley [31] and modified as in [32]. The final preparations of particles (20 mg/ml) were subjected to brief low-speed centrifugation to remove contaminating white polymeric material and stored at 0 °C.

NADH, NAD^+, deamino-NADH (d-NADH), deamino-NAD^+, EDTA, Tris, BSA, 2,3-dimethoxy-5-methyl-6-isoprenyl-1,4-benzoquinone (Q_1), alamethicin, rotenone and *N*-ethylmaleimide (NEM) were from Sigma. Piericidin A was a kind gift from Dr Alexander Kotlyar (Tel-Aviv University, Tel-Aviv, Israel).

Assays

NADH oxidation was measured photometrically ($\epsilon^{340}_{mM} = 6.22$) in the standard assay mixture comprising 0.25 M sucrose, 50 mM Tris/HCl and 0.2 mM EDTA, pH 8.0. When NADH:Q_1 reductase was assayed, 2 mM KCN and 50–100 μM Q_1 were added to the standard reaction mixture. When the activities of SBP were determined 1 mM $MgCl_2$ was added to the standard mixture. We found that the rate of NADH oxidation by SBP when measured in the absence of bivalent cations (Mg^{2+} or Ca^{2+}) rapidly declines during the assay. The reason for specific stabilization of the membrane-bound NADH:quinone reductase in SBP by bivalent cations is not understood and its phenomenology will be reported elsewhere. The experimental details are given in the Figure and Table legends. Protein content was determined by the biuret assay [33].

RESULTS

NADH oxidation catalysed by N-SMP

Despite several reports on *N. crassa* Complex I in current and older literature [34–38], we experienced significant difficulties in finding quantitative data on the standard catalytic properties of purified protein or inside-out SMP derived from this organism. Thus several NADH oxidation activities and their sensitivities to the specific inhibitors as documented in Table 1 were determined before addressing the question of whether the Complex I A–D transition phenomenon exists. N-SMP catalysed the rotenone- and piericidin-sensitive NADH oxidase reaction with a specific activity (on the basis of protein content) of about one-third of that routinely determined for B-SMP. It is worth noting that despite the well-documented presence of the alternative NADH:quinone reductases in *N. crassa* mitochondria [5,39], the NADH oxidase activity of N-SMP was almost completely blocked (≈ 90 %) by the specific Complex I inhibitors rotenone and piericidin, suggesting that the alternative NADH-oxidizing enzymes do not contribute significantly to the overall electron flow from NADH to downstream components of the respiratory chain.

When NADH oxidation was assayed with the exogenous water-soluble homologue of ubiquinone, Q_1, less than half of the activity was rotenone- and/or piericidin-sensitive. This finding suggests that the overall NADH:Q_1 reductase is due to either non-specific interaction of exogenous quinone with Complex I at

Table 1 Catalytic activities of Complex I in N-SMP

The activities were measured at 25 °C as described in the Experimental section. The data for B-SMP are taken for comparison from [21]. All activities are the initial rates of NADH or d-NADH oxidation (100 μM) measured after the particles oxidized 25 μM NADH to eliminate the lag in the onset of their catalytic capacity. Data from representative experiments where the rates were measured with the experimental accuracy of $\pm$10% are shown. Variations in the specific activities of N-SMP prepared from six different batches of mitochondria are indicated in parentheses. For NADH:Q_1 reductase and d-NADH:Q_1 reductase, 50 μM Q_1 was added to the standard assay mixture supplemented with 2 mM KCN.

	Activity (μmol of NADH oxidized/min per mg of protein)	
Assay system	N-SMP	B-SMP
NADH oxidase	0.27 (0.18–0.40)	1.00
+ BSA (1 mg/ml)	0.37	
+ Rotenone (15 μM)	0.03 (89%)*	0.05 (95%)*
+ Piericidin (0.1 μM)	0.03 (89%)*	
d-NADH oxidase	0.21 (0.15–0.25)	
+ Rotenone (15 μM)	0.02 (90%)*	
NADH:Q_1 reductase	0.30 (0.22–0.47)	1.00
+ Gramicidin D (0.2 μg/ml)	0.40	
+ Alamethicin (40 μg/ml)	0.34	
+ Rotenone (15 μM)	0.14 (53%)*	0.1 (90%)*
+ Piericidin (0.1 μM)	0.14 (53%)*	
d-NADH:Q_1 reductase	0.21 (0.13–0.28)	
+ Alamethicin (40 μg/ml)	0.21	
+ Rotenone (15 μM)	0.03 (86%)*	
+ Piericidin (0.1 μM)	0.03 (86%)*	

* Percentage inhibition is indicated in parentheses.

some 'non-physiological' site or to simultaneous operation of Complex I and other NADH:quinone reductase(s). Remarkably, alamethicin, which makes the mitochondrial membrane permeable to NADH [28], did not stimulate NADH:Q_1 reductase activity, thus indicating that N-SMP as prepared are inside-out and uncoupled. To measure the specific catalytic activity of Complex I, d-NADH oxidation was determined [40]. The specific activities of d-NADH oxidase or d-NADH:Q_1 reductase reactions were slightly lower than those measured with NADH and were almost completely sensitive to rotenone and piericidin.

Further evidence for simultaneous operation of at least two NADH-dehydrogenating enzymes in reduction of the externally added quinone were obtained when the effect of Triton X-100, a specific inhibitor of mammalian Complex I [41,42], was examined. Non-linear dependence was seen in plots of $1/v$ against inhibitor concentration for NADH:Q_1 reductase, and simple hyperbolic inhibition with apparent K_i values of 50 μM was evident for d-NADH:Q_1 reductase (Figure 1A, lines 2 and 1, respectively). The inhibition of the reaction with NADH and d-NADH was almost indistinguishable when only the rotenone-sensitive fractions of overall quinone reduction were used to construct the plots, as shown in Figure 1(B).

The rotenone-sensitive d-NADH:Q_1 reductase activities were the same at pH 7.0, 8.0 and 9.0. The apparent K_m values for NADH and d-NADH were 15 and 6 μM, respectively (at 100 μM Q_1). The additions of NAD^+ or deamino-NAD^+ did not affect the reaction rates.

NEM sensitivity of Complex I in membranes of different organisms

The most prominent feature of the mammalian Complex I A–D transition is the dramatic difference between the sensitivities of A and D forms to thiol reagents [24,26]. To assess possible transition in the membranes of eukaryotes (*N. crassa*, *Bos taurus*) and a prokaryote (*P. denitrificans*), the sensitivities of their catalytic activities to NEM under the diagnostic conditions previously established for the mammalian enzyme [24] were examined (Table 2). The NADH:Q_1 reductase activities of N-SMP, B-SMP and SBP were insensitive to 2 mM NEM if particles were aerobically pre-pulsed with NADH before addition of the inhibitor. The activities of N-SMP and B-SMP were strongly inhibited by NEM if active pre-pulsed particles were preincubated at an elevated

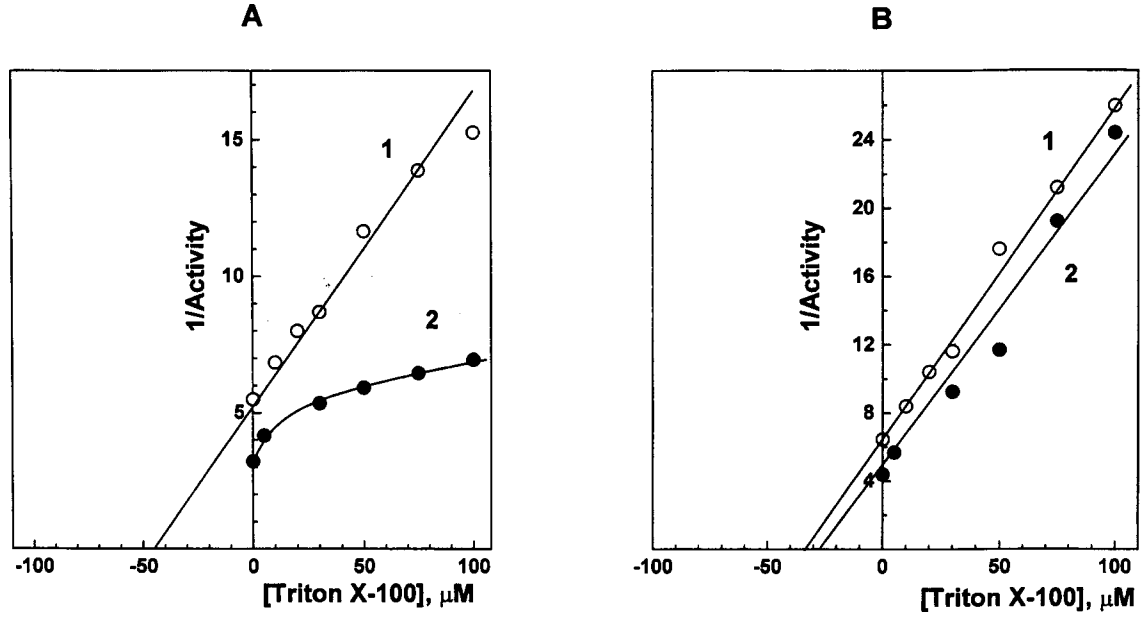

Figure 1 Inhibitory effect of Triton X-100 on *N. crassa* Complex I

(**A**) NADH (●) or d-NADH (○) was used as the substrate for Q_1 reductase reactions, catalysed by NADH-pre-pulsed N-SMP. (**B**) The rotenone-sensitive fractions of the total activities shown in (**A**) were plotted. Activity is expressed in μmoles of NADH oxidized per min/mg of protein.

Table 2 The effect of NEM on the catalytic activities of Complex I in membranous preparations from different species

For the activation treatment B-SMP and SBP (50 μg/ml) were aerobically prepulsed with 25 μM NADH. After complete oxidation of NADH, 2 mM KCN and 100 μM Q_1 were added and the reaction was initiated by the addition of 100 μM NADH. N-SMP (0.1 mg/ml) were prepulsed with 200 μM NADH and after complete oxidation of NADH were placed in ice. The reaction was initiated by the addition of prepulsed N-SMP (10 μg/ml) into the standard reaction mixture containing 100 μM d-NADH, 100 μM Q_1 and 2 mM KCN. For thermal de-activation particles were incubated for 15 min at 37 °C before further treatment by NEM. The final rates reached after a more or less prolonged lag phase were measured to calculate the specific activities. For preparations that were treated with NEM, 2 mM NEM was added and incubation was continued for 1 min at 25 °C (B-SMP and SBP) or for 10 min at 0 °C (N-SMP) before the reaction was initiated.

	NADH oxidation rate (μmol/min per mg of protein)					
Preparation	*Bos taurus* (B-SMP)		*N. crassa* (N-SMP)		*P. denitrificans* (SBP)	
NEM treatment ...	−	+	−	+	−	+
Activated	0.63 (100%)	0.55 (87%)	0.21 (100%)	0.17 (80%)	0.22 (100%)	0.23 (100%)
Thermally de-activated	0.63 (100%)	0.02 (3%)	0.19 (100%)	0.03 (16%)	0.23 (100%)	0.23 (100%)

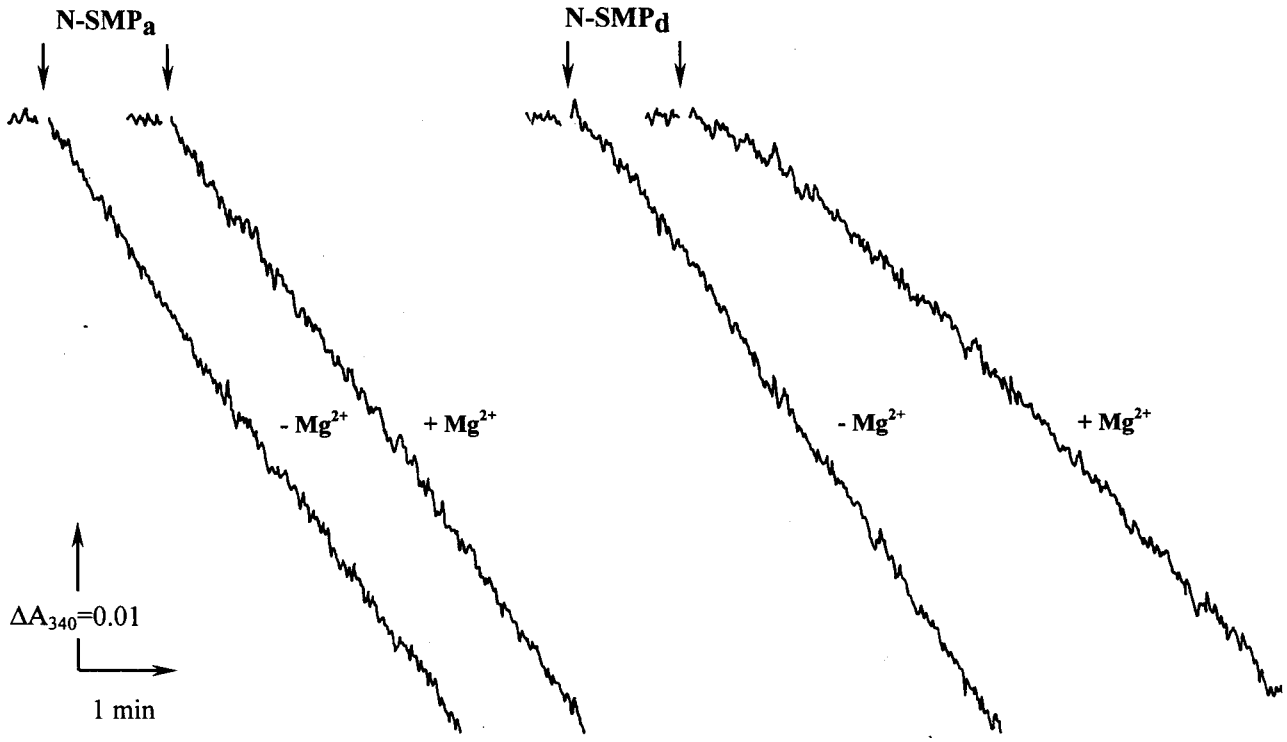

Figure 2 Time course of NADH oxidase reaction catalysed by N-SMP

N-SMP (85 μg/ml) were aerobically prepulsed with 200 μM NADH at 25 °C (N-SMP_a) and placed in ice. N-SMP_d indicates particles (85 μg/ml) that were thermally de-activated by incubation at 37 °C for 15 min. The reactions were started by the addition of particles (8.5 μg/ml) to the standard reaction mixture adjusted to pH 8.5. 50 μM $MgCl_2$ was present where indicated.

temperature (37 °C) before further treatment with the inhibitor. The NADH:Q_1 reductase activity of SBP was insensitive to NEM under all the conditions tested. Also, in accord with the data previously reported by Kotlyar et al. [43] the *P. denitrificans* enzyme never showed any deviation from the linear zero-order time course of its catalytic activity. Thus we have concluded that no slow A–D transition exists in prokaryotic Complex I (NDH-1) and only the *N. crassa* enzyme was examined further.

Kinetics of *N. crassa* Complex I A–D transition

Figure 2 depicts the actual tracings of the NADH oxidase reaction catalysed by N-SMP. The time course of NADH oxidation was dependent on the 'history' of the preparations. Particles prepulsed with 200 μM NADH showed zero-order NADH oxidation, whereas a lag phase was seen in the onset of the reaction catalysed by the sample preincubated at an elevated temperature. The lag phase was considerably prolonged when 50 μM Mg^{2+} was present in the standard assay mixture, whereas Mg^{2+} did not affect the initial rate of NADH oxidation catalysed by the pre-pulsed particles. An increase in Mg^{2+} concentration up to 5 mM did not affect the initial rates of NADH oxidation by the pre-pulsed particles, although the lag phase of the reaction catalysed by thermally de-activated N-SMP increased progressively (see below). Thus qualitatively the pattern of NADH oxidation by N-SMP was closely reminiscent of that described previously for B-SMP [23,24]. The thermally induced de-activation of Complex I in N-SMP was also evident when the actual tracings of d-NADH oxidation in the quinone reductase reaction were followed for the preparations treated with NEM as described in Table 2 (Figure 3). Strong inhibition by NEM was seen for the thermally de-activated particles and the inhibition

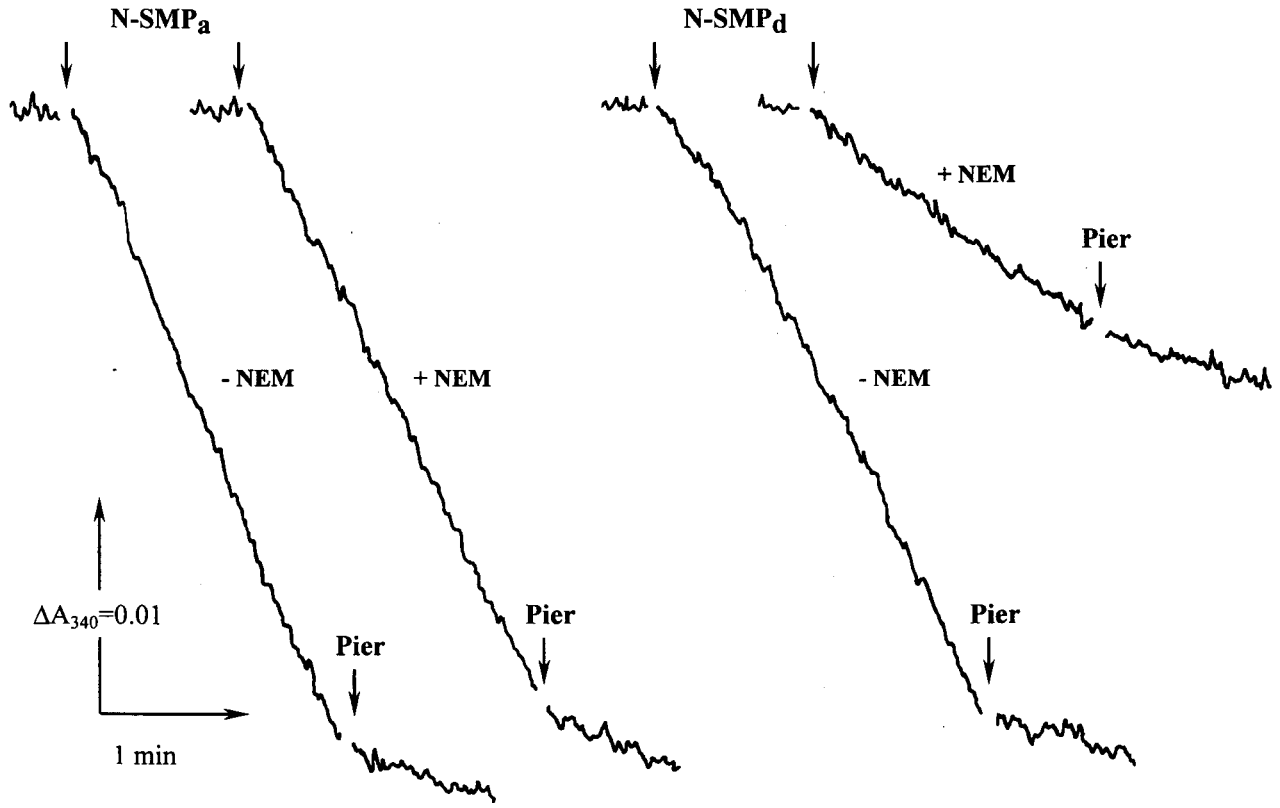

Figure 3 Inhibition of d-NADH:Q_1 reductase by NEM

N-SMP (0.1 mg/ml) were aerobically prepulsed with 200 μM NADH at 25 °C (N-SMP$_a$) and placed in ice; 2 mM NEM was then added and incubation was continued for 10 min. The reaction was started by the addition of particles to the standard assay mixture. N-SMP$_d$, N-SMP were treated exactly as N-SMP$_a$, except that the particles were thermally de-activated by incubation at 37 °C for 15 min before the addition of NEM. 0.1 μM piericidin (Pier) was added where indicated.

was completely prevented by oxidation of a small amount of NADH prior to the addition of NEM.

Next we compared the rates of spontaneous de-activation of the mammalian and fungal Complexes I. This was examined by measuring the initial rates of d-NADH:Q_1 reductase and NADH:Q_1 reductase reactions for N-SMP and B-SMP, respectively, assayed in the presence of Mg^{2+}, and their dependence on preincubation of the samples for different times at different temperatures (Figure 4). Note that since the turnover-induced activation is strongly prevented by Mg^{2+} (Figure 2), the initial rates seen in the presence of Mg^{2+} correspond to the fraction of the A form in the heterogeneous mixture of A and D forms at any given time during preincubation. The data depicted in Figure 4 show that pre-activated Complex I in both N-SMP and B-SMP undergoes slow (in the turnover timescale) temperature-dependent de-activation, with the apparent first-order rate constants of 0.21 and 0.02 min^{-1} at 30 °C for N-SMP and B-SMP, respectively. Although an Arrhenius plot for the deactivation process was not constructed due to technical difficulties with measuring the deactivation rate for *N. crassa* enzyme in a wide temperature range (very rapid deactivation at temperature higher than 30 °C), a significantly lower activation energy for A-to-D transition was evident for the fungal enzyme as compared with the mammalian Complex I.

An apparent affinity for an Mg^{2+} inhibitory effect on the turnover-induced activation was measured in the experiments where the first-order rate constants for D-to-A transition were plotted as a function of Mg^{2+} concentration in the assay (Figure 5). A value of 30 μM at pH 8.5 (24 °C) was determined, which is considerably lower than that reported previously for the mammalian Complex I (0.6 mM) [24]. The first-order rate constant for the turnover-dependent D-to-A transition, as obtained by extrapolation to an Mg^{2+} concentration of zero, was 1.2 min^{-1} at pH 8.5. This value is close to that measured previously by the stop-flow technique for the mammalian enzyme [24].

Relatively rapid de-activation of the enzyme at a moderate temperature (Figure 4) and rapid turnover-dependent D-to-A transition suggest that some fraction of Complex I could be present in the D form under the steady-state NADH oxidation conditions, and hence the steady-state rate is expected to be sensitive to Mg^{2+}, because of its inhibitory effect on D-to-A transition. Indeed, we observed that NADH oxidation in the presence of Mg^{2+} was biphasic when traced for a long time ($\approx$10 min). The initial burst of NADH oxidation was followed by the lower constant zero-order rate when the reaction was started by prepulsed active particles. On the other hand, the same final zero-order rate was reached after a prominent lag phase if the reaction was started by the de-activated particles (results not shown). Thus we conclude that continuous cycling of the A and D forms occurs during the steady-state operation of Complex I.

DISCUSSION

The present results demonstrate that SMP derived from *N. crassa* mitochondria catalyse the NADH oxidase and NADH:Q_1 reductase reactions at about the same rates (Table 1), thus

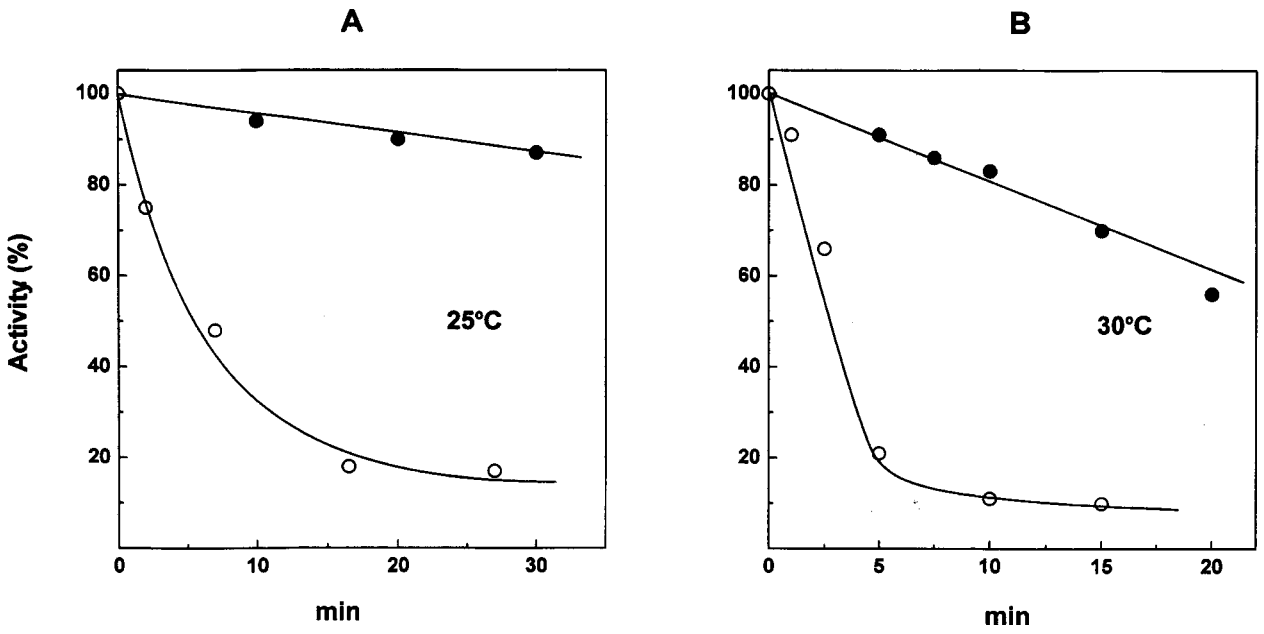

Figure 4 De-activation of Complex I in N-SMP at different temperatures

50 μg/ml N-SMP (○) and B-SMP (●) were pre-pulsed aerobically with 25 μM NADH in a spectrophotometric cuvette at 25 or 30 °C. After complete oxidation of NADH the incubation was continued for the times indicated on the abscissa and the initial rates of rotenone-sensitive NADH:Q_1 reductase (B-SMP) or d-NADH:Q_1 reductase (N-SMP) were determined in the presence of 10 or 40 mM $MgCl_2$ at 25 or 30 °C, respectively. 2 mM KCN and 100 μM Q_1 were added and the reaction was initiated by the addition of 100 μM nucleotide.

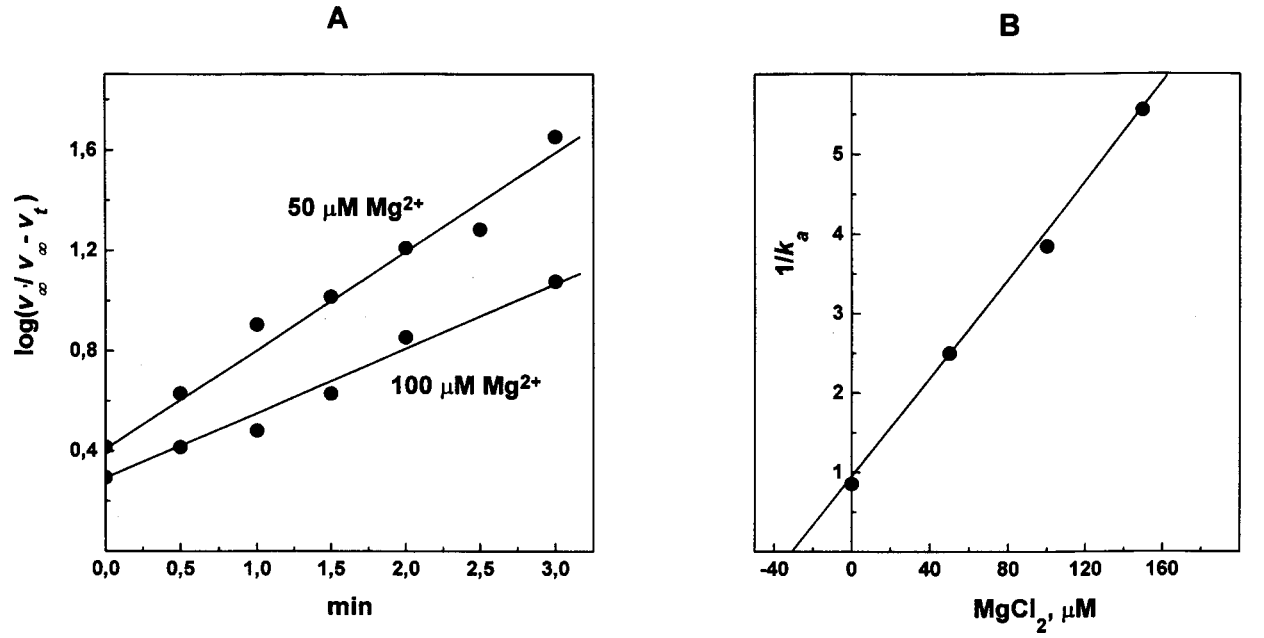

Figure 5 Quantification of the inhibitory effect of Mg^{2+} on the turnover-dependent activation of Complex I

(**A**) The time course of NADH oxidation by thermally de-activated N-SMP was followed as described in Figure 2 at different Mg^{2+} concentrations in the standard assay mixture at pH 8.5 and semi-logarithmic plots were constructed to determine the apparent first-order rate constants. (**B**) Dependence of the apparent first-order rate constant (k_a) for the turnover-induced activation of Complex I on Mg^{2+} concentration. The initial rates of NADH oxidase catalysed by fully activated particles (0.21 μmol/min per mg of protein) were used as v_∞ values in the semi-logarithmic plots. v_t is the instant rate of NADH oxidase at time t.

suggesting that, similar to B-SMP [21], the downstream ubiquinol oxidase activity of the respiratory chain does not limit the overall NADH oxidation. The absolute rate of NADH oxidase catalysed by N-SMP (on a protein-content basis) is about three-times higher than the respiratory activity of parent mitochondria in State 3 with pyruvate plus malate as substrates and is about the same as their NADH oxidase activity [30]. It appears that alternative NADH dehydrogenases facing both matrix and the

intramembranous space significantly contribute to the overall electron flow from NADH to oxygen in intact mitochondria where only 50–60 % of the pyruvate-plus-malate oxidation rate is sensitive to rotenone [30], whereas Complex I is the major contributor to NADH oxidase in inside-out N-SMP, as is evident from its 90 % sensitivity to rotenone or piericidin (Table 1). A significantly higher rotenone-insensitive fraction of the NADH oxidation (about 50 %) is seen when exogenous Q_1 is used as an electron acceptor, and the rotenone sensitivity reappears if d-NADH, the substrate which is believed to be specific only for Complex I [36,40], is used in a Q_1 reductase assay. The data on different patterns of the inhibitory effect of Triton X-100 with NADH and d-NADH in the quinone reductase assay (Figure 1) also show that Complex I and alternative NADH dehydrogenase operate simultaneously when NADH is oxidized by Q_1. It worth noting that the strong inhibitory effect of Triton X-100 on Complex I ([41,42] and this study) seems to be the most likely reason for relatively low specific activity of purified Complex I isolated from *N. crassa* [36] because Triton X-100 was used as a detergent for solubilization and further purification of the enzyme. At present the reason(s) for different rotenone sensitivity of NADH and d-NADH oxidation in oxidase and Q_1 reductase assays cannot be explained unequivocally. One possibility is that Complex I gains priority in competition with alternative NADH dehydrogenase for endogenous ubiquinone. This would be expected if alternative NADH dehydrogenase is more sensitive than Complex I to feasible inhibition by ubiquinol. Another possible explanation is that the quinone-binding site of the alternative NADH dehydrogenase is more readily accessible for exogenous quinone than that of Complex I. Whatever explanation is correct it is safe to conclude that NADH oxidase reaction is a reliable measure of Complex I activity whereas d-NADH is the substrate of choice when Complex I catalytic capacity is to be measured with external quinone as an electron acceptor.

The central finding reported here is that structurally simpler Complex I of lower eukaryote *N. crassa* demonstrates qualitatively the same A–D transition as it has been well documented for the mammalian enzyme [21–23,29]. The 'simple' prokaryotic enzyme in *P. denitrificans* membrane shows no any sign of this phenomenon ([43] and this study). Whether the transition is characteristic of other Complex I-type enzymes remains unknown although close inspection of the actual NADH oxidation tracings catalysed by the yeast *Y. lipolytica* enzyme (see Figure 4 in [44]) suggests that the transition does exist in the respiratory chain of this organism. We are inclined to believe that the A–D transition phenomenon is exclusive to eukaryotes although further studies on the catalytic activity of plant Complex I are needed to strengthen this statement.

The molecular mechanism of A–D transition is not known. The only structural data presently available show that one small subunit of the mammalian enzyme in its de-activated form, most likely IP15, is specifically labelled by a fluorescent NEM derivative [26]. Whether this subunit plays an essential role in the enzyme's catalytic mechanism, or whether the dramatic change in its thiol group's reactivity is a secondary event resulting from gross structural rearrangement of Complex I, remains unknown. The IP15 subunit is not listed in current reviews among those that are conserved in eukaryotic Complex I from different species including *N. crassa* [2,5,45]. It would be of great interest to study the catalytic and other properties of the enzyme in mutants of *N. crassa* specifically lacking or substituted for a defective copy of the IP15 homologous subunit, if present.

An important issue relevant to the present studies is the physiological significance of the A–D transition. It was mentioned in the Introduction that the phenomenon has been demonstrated for purified bovine heart Complex I [27], B-SMP [24], rat heart mitochondria [28] and mitochondria isolated from rat heart during anoxia/reoxygenation perfusion [29]. Because of the extremely high activation barrier for the mammalian enzyme the A-to-D transition can be significant only at 'physiological' temperature (> 35 °C) [23]. The *N. crassa* enzyme is rapidly de-activated at a much lower temperature (Figure 4), thus indicating that certain A/D ratios may exist in this organism under 'physiological' conditions. Taking into account the extreme complexity of eukaryotic Complex I as manifested by the presence of many multifunctional 'extra' subunits and its crucial importance for cell metabolism the enzyme is expected to be precisely regulated under various physiological conditions. Apparent K_m for NADH reported for the mammalian and fungal enzymes [20,36] are much lower than NADH concentration in the mitochondrial matrix and NAD^+ does not inhibit NADH oxidation [20]. Thus it seems unlikely that the enzyme is under direct control of the intramitochondrial $NADH/NAD^+$ ratio. On the other hand, the activity can be finely tuned via regulation of certain A/D ratio under different physiological or pathological conditions. No factors other than temperature are known at present to control the rate and equilibrium of the A-to-D transition, whereas the turnover-dependent reverse process is strongly affected by pH and the presence of bivalent cations ([22] and data reported here). Further work is needed to search for natural compounds that may influence the enzyme's A–D transition. Also, an intriguing possibility exists that the A–D transition affects some properties of the enzyme that are carried out by a number of 'extra' subunits and which are not directly related to the NADH:quinone reductase activity (see the Introduction).

Note added in proof (received 3 December 2002)

After submission of this manuscript, new findings relevant to the content of this paper have been reported. Maklashina et al. [46] found that *N. crassa* and *Y. lipoytica* Complex I show A–D transition with much lower activation barriers for the transition compared with the bovine enzyme. Also, a new 14.7 kDa nuclear encoded subunit of bovine Complex I has been identified [47]. This subunit contains four cysteine residues and is homologous with the *N. crassa* subunit 21.3 and with Tim17, Tim22 and Tim23 proteins that are involved in polypeptide translocation across the inner mitochondrial membrane (J. Hirst, personal communication). This finding suggests that a subunit with an apparent molecular mass of 15 kDa, which is responsible (or involved) in A–D transition, can either be IP15, as we have proposed previously [26], or the new 14.7 kDa subunit.

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