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Slow active/inactive transition of the mitochondrial NADH-ubiquinone reductase

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NADH–ubiquinone reductase of bovine heart submitochondrial particles as prepared is unable to catalyze either the direct or reverse electron transfer from NADH to ubiquinone. The deactivated state of the enzyme in coupled particles was revealed as: (i) the absence of the rotenone-sensitive, $\Delta\tilde{\mu}_{H^+}$-dependent succinate-ferricyanide reductase activity; (ii) a prominent lag in the aerobic succinate-supported, $\Delta\tilde{\mu}_{H^+}$-dependent NAD^+ reduction; and (iii) a lag in the rotenone-sensitive NADH–ubiquinone reductase or NADH oxidase activities. Being inactive as NADH–ubiquinone reductase (direct or reverse), the enzyme is fully active as rotenone-insensitive NADH–ferricyanide reductase. The enzyme can be activated by preincubation with substrates (NADH or NADPH) only under the conditions where the turnover of the NADH–ubiquinone reductase reaction (but not in the NADH–ferricyanide reductase) occurs. Partial activation of the enzyme was observed when the particles were preincubated with rotenone. Neither NADH under the conditions when the ubiquinone pool was reduced nor succinate plus $\Delta\tilde{\mu}_{H^+}$ or dithionite were able to activate the enzyme. Once activated, the enzyme remains in the active state for quite a long time (more than 5 h at 0 °C). The deactivation rate is extremely temperature-dependent, being insensitive to NAD^+, ferricyanide or succinate. A comparison of the enzyme activation/deactivation kinetics showed that the same mechanism is involved in the slow activation of the direct and reverse electron transfer from NADH to ubiquinone. Activated particles catalyze the aerobic $\Delta\tilde{\mu}_{H^+}$-dependent succinate-supported reverse electron transfer in the absence of ATP at a rate comparable with that of NADH–ubiquinone reductase.

Introduction

NADH–ubiquinone oxidoreductase (EC 1.6.99.3), usually termed Complex I, when isolated [1] is the most complicated and the least understood protonmotive device of the mitochondrial respiratory chain. Numerous experimental data on the polypeptide composition [2,3], the nature and properties of the prostetic groups (FMN, iron-sulfur centers) [1,4,5] and some molecular genetic aspects of the enzyme [6] have been accumulated. Despite valuable information about the midpoint reddox potentials [7] and spatial arrangement of the enzyme redox components within the inner mitochondrial membrane [8–10], several working hypotheses describing the mechanism of the enzyme operation [11–15] remain rather speculative because neither the sequence of the electron transfer from NADH to ubiquinone nor the nature of proton translocating groups (if any) is known.

Most of the current studies on the isolated Complex I or NADH–ubiquinone reductase of mitochondria and submitochondrial particles are focused on the molecular aspects of the enzyme. Less recent information is available on the steady-state kinetics of the overall reactions catalyzed by NADH–ubiquinone reductase, although this aspect has been the subject of numerous reports in the earlier literature [16–19].

Several assay systems are available for the NADH–ubiquinone reductase region of the respiratory chain operating in 'direct' or 'reverse' directions. All preparations of Complex I and its fragments (NADH dehydrogenases of different degree of resolution) catalyze the

Abbreviations: Q_n, homologs of ubiquinone having n isoprenoid units in position 6 of the quinone ring; DB, 2,3-dimethoxy-5-methyl-6-decylbenzoquinone; DMSO, dimethyl sulfoxide; DCIP, 2,6-dichlorophenolindophenol; BSA, bovine serum albumin; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

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NADH–ferricyanide reductase reaction [1,20], although the site of electron acception may depend on the type of preparation [16–18]. This reaction is rotenone-insensitive and evidently not coupled with energy transduction step(s). The NADH-quinone (Q_0, Q_1, Q_2, DB, duroquinone) reductase activity of Complex I is partially sensitive to rotenone, and the reaction is coupled with energy accumulation when the enzyme operates in submitochondrial particles [21,22] or liposomes [23–24]. It is generally believed that the reduction of rotenone-sensitive ubiquinone homologs represents a 'physiological' pathway of the electron flow from NADH to ubiquinone. Two reactions catalyzed by Complex I in intact mitochondria or in coupled submitochondrial particles represent an electron flow from ubiquinol to some low midpoint potential component of the enzyme, i.e., the reverse reactions which need free energy to cover a gap between the redox potentials of the acceptor ($NAD^+/NADH$) and donor (Q/QH_2) pairs. The first one, operationally called as reverse electron transfer, is succinate-supported, rotenone- and uncoupler-sensitive NAD^+ reduction originally discovered in intact mitochondria [25,26] and later demonstrated in submitochondrial particles as an ATP-dependent reaction sensitive to inhibitors of succinate dehydrogenase (EC 1.3.99.1), Complex I and F_0F_1-ATPase [27,28]. Another 'reverse' reaction is the rotenone- and uncoupler-sensitive ATP-dependent succinate-ferricyanide reductase catalyzed by the coupled submitochondrial particles [29].

In 1964, Minakami et al. [30] reported that a considerable lag in the appearance of the NADH oxidase and NADH–cytochrome *c* reductase activities was observed in some preparations of submitochondrial particles. This lag was not seen in NADH–ferricyanide reductase or when the second portion of NADH was oxidized after complete oxidation of a small amount of the substrate [30]. This phenomenon has not been clearly understood, although Tyler et al. [31] have proposed that the reduction of some component in the NADH–cytochrome *c* region of the respiratory chain is a prerequisit for activation of NADH oxidation.

In this work further investigation of the phenomenon originally described by Minakami et al. has been undertaken. Some parameters of a turnover induced activation of the NADH–ubiquinone reductase region of the respiratory chain operating in both directions suggest that the slow formation of the specific site for catalytically competent ubiquinone species (most probably ubisemiquinone [32]) in the terminal region of the enzyme is responsible for the activation. In addition, the energy-dependent succinate-NAD^+ (or ferricyanide) reductase activity of coupled submitochondrial particles in the absence of ATP has been demonstrated. Some parts of the present study have been published in a preliminary form elsewhere [33,34].

Materials and Methods

Submitochondrial particles

The preparations used throughout this study were AS-submitochondrial particles prepared essentially as described by Racker and Horstman [35]. As will be shown in this report, several parameters of NADH–-ubiquinone reductase depend on the 'history' of a particular preparation. That is why the procedure used for preparation of 'coupled' AS-particles is described in detail.

Heavy bovine heart mitochondria were prepared as described [36] and stored for 3 days at −15 °C (approx. 1 μg of protein in 30 ml of 0.25 M sucrose). The suspension was thawed, diluted with cold water to about 20 mg/ml and 1 mM neutralized K-EDTA (final concentration) was added. The suspension was saturated with argon for 20 min and the pH was adjusted to 9.1–9.2 by adding 1 M NH_4OH. The suspension was sonicated ('Soniprep-150', MSE) five times for 0.5 min with 1 min intervals at 0 °C and centrifuged at 26 000 × *g* (15 min, 0 °C). The supernatant was centrifuged at 120 000 × *g* (45 min, 0 °C). The sedimented particles (approx. 200 mg) were suspended at 25 °C in 75 mM sucrose, 0.25 M KCl, 2 mM EDTA, 30 mM Tris-SO_4 (pH 8.0), and applied on the column (70 × 2.5 cm) with Sephadex G-50 (coarse) equilibrated with the same solution at 25 °C. The particles were slowly (approx. 1 h) passed through the column at 25 °C and collected by centrifugation at 120 000 × *g* for 45 min (25 °C). The sediments were suspended in 0.25 M sucrose and centrifuged at 120 000 × *g* for 60 min at 25 °C. The pellets were suspended in 0.25 M sucrose (20–30 mg protein per ml) and stored in liquid nitrogen. Before the experiments, the particles were thawed and incubated (10 mg/ml) 2–3 h at 26 °C in the mixture containing 0.25 M sucrose, 1 mg/ml of BSA, 1 mM $MgCl_2$, oligomycin (0.15 or 0.4 μg/mg, as indicated in the legends to the figures and tables), 0.2 mM EDTA, 2 mM malonate, 0.02 M phosphate (potassium salts, pH 8.0). During the experiment the particles were stored at room temperature in the same mixture. All the activities presented in Table I were without significant changes during more than 6 h storage of the suspension at room temperature.

Enzymatic activities

(i) ATP-dependent reverse electron transfer. The reaction was followed at 340 nm (NADH)or 420 nm (ferricyanide) in 'Hitachi-557' spectrophotometer at 26 °C in 2 ml of a mixture containing 0.25 M sucrose, 1 mg/ml of BSA 5 mM NAD^+, 3 mM ATP-Mg, 1 mM $MgCl_2$, 0.2 mM EDTA, 20 mM succinate, 1 mM CN and 0.02 M phosphate (potassium salts, pH 8.0). The reactions were started by the addition of particles (5 μl) or a mixture of the substrates (NAD^+ plus succinate)

TABLE I

Reactions catalyzed by the coupled AS particles

(26 °C, potassium phosphate, pH 8.0.) Particles were treated with oligomycin: c, d and e, 0.15; and a, b, g and f, 0.4 μg per mg of protein, respectively.

Reaction	Rate (2-electron-equiv./min per mg protein) $\times 10^{-6}$	
	without CCCP	+10 μM CCCP
NADH → O_2 [a]	0.27	1.05
Succinate → O_2 [b]	0.43	0.94
Succinate → NAD^+ [c]		
+ ATP (0.2 mM CN^- is present)	0.25	0.00
− ATP (aerobic conditions)	0.25	0.00
Succinate → ferricyanide [d]		
+ ATP (0.2 mM CN^- is present)	0.27	0.00
− ATP (aerobic conditions)	0.27	0.00
ATP → ADP + P_i [e]	0.76	1.52
NADH → ferricyanide [f]	6.50	6.50
NAD → Q_0 [g]		
− rotenone	0.67	1.13
+ rotenone	0.07	0.07

[a] 100 μM NADH.
[b] 50 mM succinate, measured with oxygen electrode.
[c] 5 mM NAD^+.
[d] 100 μM ferricyanide, rotenone-sensitive fraction.
[e] 3 mM ATP, in the presence of ATP regenerating system [41], the reaction is completely sensitive to oligomycin.
[f] 100 μM NADH, 1 mM ferricyanide.
[g] 100 μM NADH, 0.4 mM Q_0.

1–2 min after addition of the particles. When the rotenone-sensitive ferricyanide reductase reaction was assayed, 0.1 mM ferricyanide was added instead of NAD^+ and KCN was substituted for antimycin A (5 μg/ml).

(ii) ATP-independent reverse electron transfer. This was assayed exactly as before, except that ATP, cyanide and antimycin A were omitted.

(iii) NADH–oxidase and NADH–acceptor reductase. The reactions were followed in 'Hitachi-557' spectrophotometer using a stopped-flow accessory. Syringe A contained: particles (0.2 mg/ml) in 0.25 M sucrose, 1 mg/ml of BSA, 40 μM CCCP, 1 mM $MgCl_2$, 0.2 mM EDTA, 2 mM CN^- (when acceptor reductase reactions were assayed) and 20 mM phosphate (potassium salts, pH 8.0). Syringe B contained the same mixture (without particles) supplemented with NADH and either 0.8 mM Q_0 or 2 mM ferricyanide. The registration was started 6 ms ('dead-time' of the instrument) after the mixing of equal volumes of the solutions. The oxidation of NADH was followed as an absorbance change at 340 nm minus 420 nm and the reduction of ferricyanide at 420 minus 500 nm.

Protein content was determined with a biuret reagent [37]. EDTA, BSA and Q_0 were obtained from Sigma (U.S.A.); potassium-phosphate and $MgCl_2$ were from Merck (U.S.A.); NAD^+, ATP were from Reanal (Hungary); CCCP was from Fluka (Switzerland); rotenone was from Ferak (Berlin, F.R.G.); oligomycin and antimycin A were from Serva (F.R.G.). Other chemicals were of the highest quality commercially available.

Results

Among many different preparations of the inner mitochondrial membrane [38] AS particles [35] routinely used in our laboratory have several advantages. Being treated with substoichiometric amount of oligomycin [29], they catalyze all the reactions involved in the overall oxidative phosphorylation being essentially free of the natural ATPase inhibitor protein (IF_1) [35,40] and adenylase kinase (EC 2.7.4.3) (our unpublished observation) which simplifies the studies of the ATP(ADP)-dependent reactions.

Table I demonstrates several enzymatic activities of the particles relevant to the present study. The strong stimulation of succinate oxidase, NADH oxidase and ATPase by the uncoupler indicates that the particles are well coupled. The preparation catalyzes the $\Delta\tilde{\mu}_{H^+}$-dependent rotenone-sensitive reverse electron flow from ubiquinol to the proper electron acceptor (NAD^+ or ferricyanide). Table I demonstrates that the particles catalyzes the $\Delta\tilde{\mu}_{H^+}$-dependent reverse electron flow without added ATP at the expense of the energy generated by oxidation of succinate in the terminal region of the respiratory chain. Utilization of reduced TMPD as the substrate for production of energy in succinate-dependent NAD^+ reduction in submitochondrial particles has been well documented [42]. In contrast to intact mitochondria, this reaction with succinate as a reductant and the substrate for energy production in submitochondrial particles has, to our knowledge, never been demonstrated. Two factors were found to be critical for experimental demonstration of this reaction. The first one is the activation of succinate dehydrogenase by malonate [43,44] to provide succinate oxidase rate sufficient to maintain the proper steady-state level of $\Delta\tilde{\mu}_{H^+}$. The second factor is activation of NADH–ubiquinone reductase, a phenomenon which will be described in details.

The time course of the aerobic succinate-NAD^+ reductase reaction (reverse electron transfer) is shown in Fig. 1. Considerable lag in appearance of the full catalytic activity was observed when the particles were preincubated under aerobic conditions in the presence of succinate and the reaction was started by the addition of NAD^+ (curve 1). No difference in the time-course was noted when the order of the additions was changed or the reaction was initiated by the addition of the particles. The lag-phase was completely eliminated

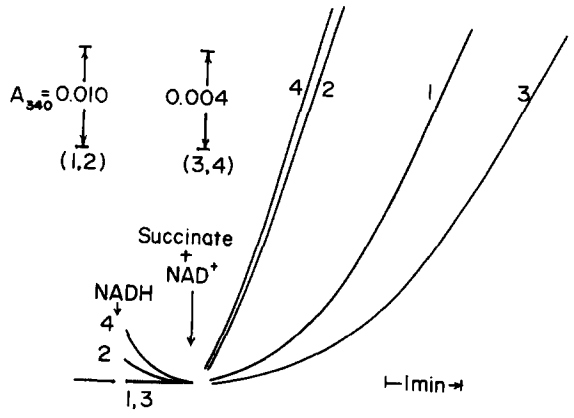

Fig. 1. Time-course of the succinate-supported NAD^+ reduction. AS-particles were treated with oligomycin (0.4 μg/mg) and assayed as described in Materials and Methods. The amounts of particles added were 25 μg/ml (curves 1 and 2) and 10 μg/ml (curves 3 and 4). $5 \cdot 10^{-7}$ M NADH was added where indicated to eliminate a lag-phase (curves 2 and 4).

TABLE II

Activation of aerobic rotenone-sensitive succinate-ferricyanide reductase activity by NADH

Samples and order of additions	Activity (2 electron-equiv/min per mg protein) $\times 10^{-6}$	
	without rotenone	+10 μM rotenone
1 Particles as prepared [a]	0.03	0.03
2 1 μM NADH (1 min), succinate + ferricyanide	0.28	0.02
3 The same as (2), 20 μM CCCP was present	0.03	0.03
4 Ferricyanide, 1 μM NADH (1 min), succinate	0.04	0.03

[a] Particles treated with 0.4 μg of oligomycin per mg of protein were placed in the cuvette (25 μg/ml) containing 0.25 M sucrose, 1 mg/ml BSA, 1 mM $MgCl_2$, 0.1 mM EDTA, 20 mM phosphate (potassium salts (pH 8.0)). The reaction was initiated by the simultaneous addition of 100 μM ferricyanide and 20 mM succinate.

after addition and subsequent complete oxidation of small amount of NADH ($5 \cdot 10^{-7}$ M) before the reverse electron transfer was initiated by the addition of any of the substrates (Fig. 1, curve 2). The results shown in Fig. 1 straongly suggest that the lag in the succinate-supported NAD^+ reduction is due to the autocatalytic process in which NADH newly formed increases the reaction rate. This conclusion was further supported by measuring of the reaction using different amount of the particles. Indeed, as expected, the duration of the lag was increased when a smaller amount of the particles was added (Fig. 1, curve 3). The activating effect of NADH on the Complex I, operating in the reverse electron transfer was further demonstrated using rotenone-sensitive $\Delta\tilde{\mu}_H$+-dependent succinate-ferricyanide assay [29]. In agreement with the data shown in Fig. 1, Table II demonstrates that pretreatment of particles with 10^{-6} M NADH results in the appearance of an activity, which is sensitive to the uncoupler. It also shows that pretreatment of the particles with NADH in the presence of ferricyanide does not result in the activation of the reaction. This observation suggests that the rotenone-insensitive NADH–ferricyanide enzyme turnover gives no activating effect.

It was of interest to examine the activity effect of NADH in the assay system where Complex I operates in the direction of ubiquinone reduction. Fig. 2 demonstrates the time-course of NADH–ubiquinone reductase as revealed by the stopped-flow technique. Oxidation of NADH in the rotenone-sensitive ubiquinone reductase reaction occurs with a lag-phase and the enzyme activation follows first-order kinetics. The apparent lag seen in NADH–quinone reductase assay ($t_{1/2}$ approx. 10 s) is much shorter than that observed in $\Delta\tilde{\mu}_H$+-dependent succinate-NAd$^+$ reductase assay ($t_{1/2}$ approx. 2 min; Fig. 1). An obvious reason for this difference is the presence of high concentration of NAD^+ competing with NADH for the substrate binding site in the reverse electron transfer assay. This conclusion was supported by the data presented in Fig. 3, where the time-courses of NADH–ubiquinone reductase and $\Delta\tilde{\mu}_H$+-dependent rotenone-sensitive succinate-NAD^+ reductase were traced under the same conditions. As seen from Fig. 3, both activities are increased in parallel within the time resolution limits. The results presented above are in agreement with the earlier data [30,31] indicating that NADH, besides being a substrate for Complex I, has a prominent activating effect on the enzyme. It might be proposed that Complex I possesses some NADH-specific high-affinity regulatory site that are distinct from the catalytic site and that the enzyme is active only when the regulatory site is occupied by NADH. To

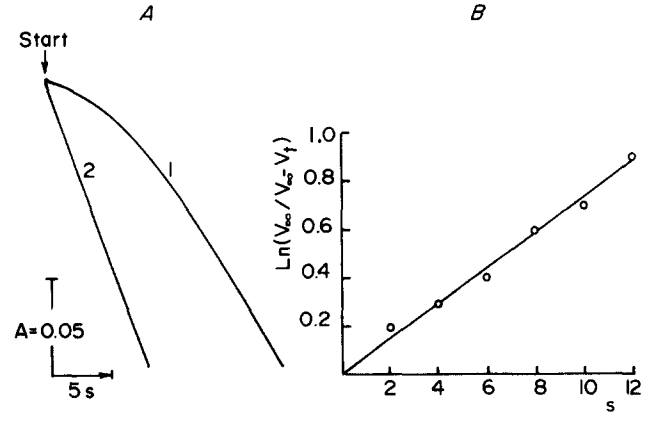

Fig. 2. (A) Kinetics of the rotenone-sensitive NADH-Q_0 reductase reaction. Curve 1, particles as prepared; curve 2, 50 μM NADH was oxidized aerobically before the reaction was measured in the presence of cyanide. (B) Linear anamorphose of curve 1; the activity traced by curve 2 was taken as V_∞.

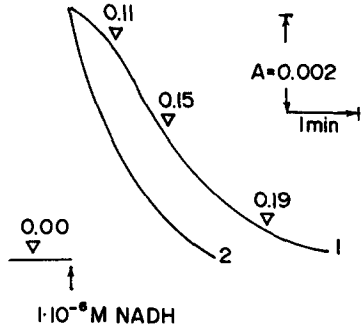

Fig. 3. Comparison of time-dependent activation of NADH oxidase (continuous curves) and reverse electron transfer (figures above the triangles). Curve 1, oxidation of 1 μM NADH in the presence of 5 mM NAD^+ by the particles (100 μg/ml) was traced as described in Materials and Methods (particles were treated with 0.4 μg of oligomycin per mg of protein and CCCP was omitted). The initial rates of NAD^+ (5 mM) reduction started by the addition of 50 mM succinate at the time indicated by the triangles were measured under aerobic conditions (12 μg of the particles per ml). Curve 2, oxidation of 1 μM NADH after 2 μM NADH was completely oxidized before the assay.

examine this possibility, the particles with inactive Complex I were incubated in the presence of a reductant (ethanol), alcohol dehydrogenase (EC 1.1.1.1) and NAD^+ added in an amount which is substantially lower (approx. 10 times) than that of Complex I. The data shown in Fig. 4 demonstrate that the complete activation of Complex I assayed as $\Delta\tilde{\mu}_H$+-dependent rotenone-sensitive succinate-ferricyanide reductase occurs in the presence of substoichiometric amounts of NADH. Thus, the presence of the NADH-specific regulatory site(s) in Complex I was excluded. Another possible explanation for activating effect of NADH, which has been offered by Tyler et al. [31] is that the reduction of some component (e.g., disulfide bond) in Complex I which does not participate in the enzyme turnover is a

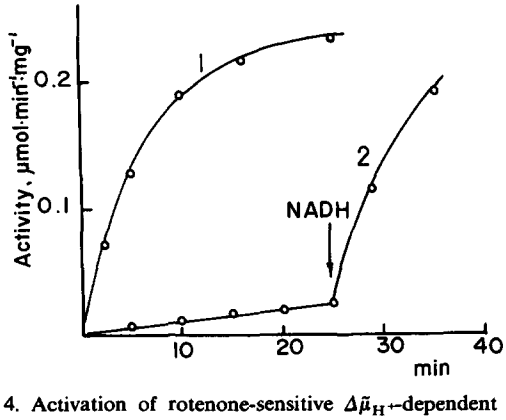

Fig. 4. Activation of rotenone-sensitive $\Delta\tilde{\mu}_H$+-dependent succinate-ferricyanide reductase by substoichiometric amount of NADH. Curve 1, 0.17 M ethanol, alcohol dehydrogenase (0.1 mg/ml), 30 mM semicarbazide and $7.5 \cdot 10^{-8}$ M NADH were added at zero time to the suspension of the particles (10 mg/ml) and incubated at 25 °C in a mixture described in Materials and Methods. Rotenone-sensitive succinate-ferricyanide reductase was assayed. Curve 2, NADH was originally omitted and added where indicated.

TABLE III

Activation of ATP-dependent succinate-NAD^+ reductase under different conditions

Treatments [a]	Initial rate (2 electron-equiv./min per mg protein) $\times 10^{-6}$
1 Cyanide, Mg-ATP, NAD^+ + succinate	0.01
2 NADH (1 min), cyanide, Mg-ATP, NAD^+ + succinate	0.26
3 NADPH (5 min), cyanide, Mg-ATP, NAD^+ + succinate	0.22
4 Succinate, cyanide, NADH (1 min), Mg-ATP, NAD^+	0.02
5 Succinate, cyanide, NADH (1 min), Mg-ATP, Q_1 (1 min), NAD^+	0.17
6 Succinate, cyanide, NADH (1 min), Mg-ATP, fumarate (1 min), NAD^+	0.24

[a] Particles (25 μg/ml) treated with oligomycin (0.4 μg/mg) were preincubated in the cuvette containing: 0.25 M sucrose; 1 mg/ml BSA; 0.1 mM EDTA; 20 mM Hepes (potassium salts, pH 8.0), for 1 min at 30 °C. Further additions were made in the order indicated. The final concentrations were: NAD^+, 1 mM; Mg-ATP, 3 mM; potassium cyanide, 1 mM; succinate, 20 mM; NADH, 1 μM; NADPH, 100 μM; fumarate, 100 μM; and Q_1, 5 μM.

prerequisite for the catalytic activity. This possibility contradicts the following observations (Table III). When the particles were pulsed with NADH in the presence of cyanide and succinate (under the conditions where the ubiquinone pool is reduced) no significant activation of Complex I was observed. The enzyme become activated by further addition of exogenous quinone or fumarate which permitted several NADH oxidizing turnovers. Since neither NADH in the presence of succinate and cyanide (Table III), nor other reducing agents such as $SO_2O_4^{-2}$ or DTT (the data are not shown) were able to activate the enzyme, the 'reductive' mechanism of

TABLE IV

Reversible inactivation/activation of the NADH–ubiquinone reductase

Treatment [a]	Initial rate (2 electron-equiv./min per mg protein) $\times 10^{-6}$	
	NAD oxidase	succinate-NAD^+ reductase
1 As prepared	0.00	0.00
2 Treated with 50 μM NADH for 1 min	0.87	0.24
3 As (2), further incubated for 10 min at 35 °C	0.00	0.00
4 As (3), further treated with 50 μM NADH for 1 min	0.87	0.22

[a] AS-particles treated with 0.4 μg of oligomycin per mg of protein were adjusted to the protein content 0.2 mg/ml and stored in a standard mixture (see Materials and Methods) containing 40 μM malonate.

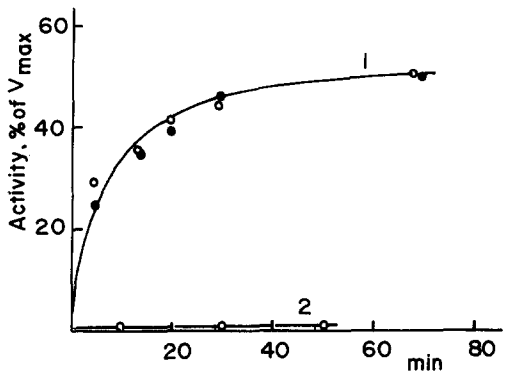

Fig. 5. Actiation of reverse electron transfer by rotenone. Curve 1, AS particles (10 mg/ml) treated with oligomycin (0.4 μg/ml) were incubated at 30 °C as described in Materials and Methods except 0.5 mg/ml of BSA, 2 (●) or 20 (○) μM rotenone dissolved in DMSO were present. The proper amount of the suspension was withdrawn and placed in the assay cuvette (rotenone-sensitive succinate-ferricyanide reductase, see Materials and Methods) containing 2.5 mg/ml of human serum albumin. Particles were further incubated for 7 min at 30 °C to remove rotenone [45] and the reaction was initiated by simultaneous addition of succinate (20 mM) and potassium ferricyanide (100 μM). 100% activity (0.6 μmol of ferricyanide reduced per min per mg of protein) corresponds to the rate obtained with the particles pulsed with 1 μM NADH before the assay (see Table III). Curve 2, rotenone but not DMSO, was omitted from preincubation medium.

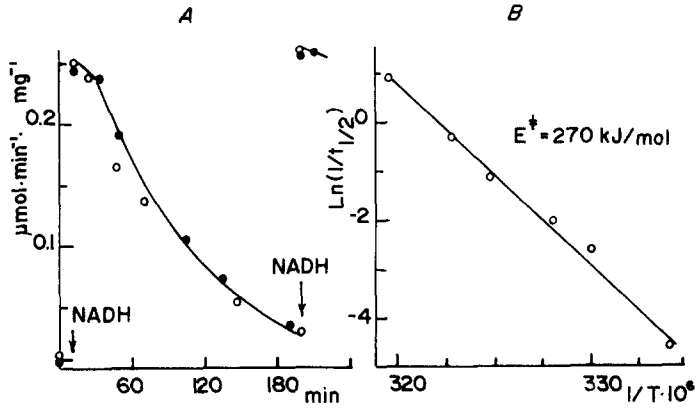

Fig. 6. Activation/deactivation of Complex I insubmitochondrial particles. (A) AS particles (0.5 mg/ml) treated with oligomycin (0.4 μg/mg) were incubated at 26 °C as described in Materials and Methods. The proper amounts of the suspension were withdrawn and rotenone-sensitive succinate-ferricyanide reductase (●) and aerobic succinate-supported NAD^+ reduction (○) (initial rates) were assayed. The pulses of 50 μM NADH are indicated by arrows. (B) the Arrhenius plot for deactivation process measured as shown in (A).

activation seems hardly to be operating. Moreover, the conditions were found where the activation of Complex I proceeds without NADH or any other reducing agents. Fig. 5 shows that rotenone, the specific inhibitor of NADH-ubiquinone reductase [46,47], is also able to activate the enzyme. It should be mentioned that, although the activating effect of rotenone was evident, we were unable to obtain a more than 50% activation of the enzyme as compared to that observed after preincubation with NADH.

Once activated, the enzyme retains its activity for quite a long time. This is illustrated by Fig. 6, where the time-course of enzyme deactivation was followed at different temperatures. An apparent activation energy of 270 kJ/mol was determined from the linear Arrhenius plot within the temperature range of 25–40 °C. The rate of deactivation measured, as shown in Fig. 6, was insensitive to the presence of either ferricyanide or succinate. On the other hand, the deactivation rate was pH-dependent: the half-time of deactivation measured at 33 °C was 2.5, and 7 min at pH 9.0 and 7.0, respectively. Rapid coincident deactivations of the initial NADH oxidase and succinate-NAD^+ reductase activities is illustrated in Table IV. Both enzymatic activities were further restored after short preincubation with NADH.

It is well known that NADH oxidase activity of different types of submitochondrial particle is relatively much more stable than their reverse electron transfer capacity. NADH oxidase (ubiquinone or cytochrome *c* reductase) assays) requires NADH which is a 'rapid' activator of Complex I, whereas no NADH initially present is required for the reverse electron transfer assay. The difference in the stabilities of the direct and reverse reactions catalyzed by Complex I in submitochondrial particles thus seemed to be ambiguous. Indeed, as depicted in Fig. 7, the oligomycin-treated submitochondrial particles completely lost their capacity to catalyze aerobic succinate-supported $\Delta\tilde{\mu}_{H^+}$-dependent NAD^+ reduction after 14 h of a storage at 25 °C being fully capable of NADH oxidase (the data are not shown). However, such preparations exhibited their full capacity to catalyze the reverse electron transfer provided that Complex I had been activated by the NADH pulse before the assay.

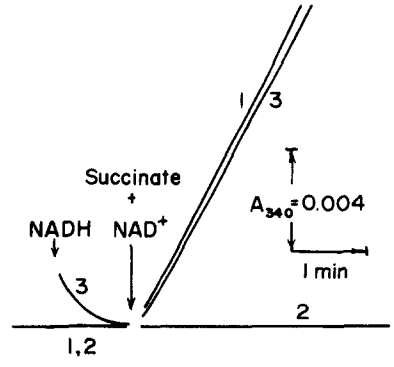

Fig. 7. Activation of the reverse electron transfer after prolonged storage of submitochondrial particles. Curve 1, AS particles (10 mg/ml) treated with oligomycin (0.4 μg/mg) as described in Materials and Methods were pulsed with 1 μM NADH where indicated and the reverse electron transfer was initiated by the addition of succinate (20 mM) and NAD^+ (5 mM). Curve 2, the same as 1 after 14 h of storage at 25 °C. Curve 3, the same as 2, 1 μM NADH was added where indicated before the reaction was initiated.

Discussion

The data presented in this report show that the slow interconversion between active and inactive NADH–ubiquinone reductase exists within the mitochondrial membrane. Neither the reduction of the enzyme, as has been suggested earlier [31], nor the NADH-specific regulatory site is responsible for NADH (NADPH) induced activation of the enzyme. It may be concluded that the oxido-reduction between the enzyme and the ubiquinone pool gives rise to the activated state.

Recently we have shown that rotenone-sensitive ubisemiquinone interacting with some tetranuclear iron-sulfur center of Complex I arises during the steady-state NADH oxidation or energy-linked succinate-supported NAD^+ reduction in the coupled submitochondrial particles [32]. This finding suggest that, as has been proposed [13,14], the $Q \rightarrow QH'$ or $QH' \rightarrow QH_2$ transition (or both) at the specific ubiquinone binding site occurs during the steady-state enzyme turnovers. It may thus be supposed that the slow irreversible reduction of oxidized quinone to semiquinone by some low midpoint redox potential center of the enzyme is coupled with the formation of a quinone-binding site, which further operates as the acceptor active site during the steady-state catalysis. This proposal agrees with the lack of activation under the conditions, when the enzyme is reduced by NADH and the ubiquinone pool is also reduced by succinate in the presence of cyanide (Table III). The 'activating' oxido-reduction cannot be just the initial step of further steady-state overall catalysis, since the first-order rate of activation (k is approx. 5 min^{-1} at pH 8.0) is much slower than the enzyme turnover in either direction.

Several reports in the literature seem to be relevant to the phenomena studied in this report. It has been claimed that non-phosphorylating heart muscle preparations exhibit a pronounced lag upon assay of NADH oxidation activities [48]. This lag was seen with cytochrome c or oxygen as terminal acceptors, but not with DCIP or ferricyanide [48]. A prominent lag attributed to the ATPase in the ATP-dependent succinate-supported NAD^+ reduction catalyzed by phosphorylating submitochondrial particles has been observed by several groups [27,49]. An activation of ATP-dependent succinate-supported NAD^+ reduction after pretreatment of submitochondrial particles with low concentrations of NADH has also been claimed [50]. Very recently it has been reported that the presence of NAD^+ results in a dramatic increase of the ATP-driven O_2^- generation with a concomitant reduction of NAD^+ to NADH [51]. Although this phenomenon has been attributed to the tight binding of NAD^+ in the form of $NAD^\cdot$ [51], no direct evidence for the presence of tightly bound NAD^+ in submitochondrial particles or isolated Complex I has so far been reported. We believe that activating effect of NAD^+ observed by Krishnamoorthy and Hinkle [51] might be due to the formation of NADH and subsequent activation of the enzyme as demonstrated in the present report.

Besides several observations on the slow changes of the enzymatic activities of NADH–ubiquinone reductase [30,52,53], there are some evidence for the NADH-induced rearrangement of enzyme structure. It has been shown that the reactivity of some sulfhydryl groups in Complex I strongly increases after preincubation of the enzyme with NADH [52,54]. On the other hand, in accordance with our data (see Fig. 5), treatment of submitochondrial particles with sulfhydryl reagents results in a considerable potentiation effect on the enzyme inhibition by rotenone, a 'slow' inhibitor [47], which is known to induce dramatic conformational changes of Complex I [55]. An inspection of the data previously reported in the literature [27,30,31,49,50] together with the results obtained in the present study suggests that the same phenomenon, i.e., active/inactive NADH and ubiquinone dependent slow transformation is responsible for several unexplained features of the enzyme.

As shown here, the activity of the NADH–ubiquinone reductase region of the mitochondrial respiratory chain depends on the 'history' of a particular preparation. It should be emphasized that before any conclusions about the structural-functional relations of the enzyme components and ubiquinone based on the kinetics of the iron-surfur centers reduction and reoxidation can be reached, the question as to whether Complex I is in an active or deactivated state, should be answered.

The last point to be discussed is the activity of the reverse electron transfer in submitochondrial particles. Since its original discovery in intact mitochondria [25,26], this reaction has been demonstrated in submitochondrial particles as ATP-dependent succinate-supported reduction of NAD^+ [27,28] or ferricyanide [29], or as ATP-independent succinate-NAD^+ reductase supported by oxidation of ascorbate plus TMPD in the presence of antimycin A [42]. To our knowledge, no reverse electron transfer between ubiquinol and the acceptor site to Complex I operating at the expense of energy generated by succinate oxidation has ever been demonstrated in phosphorylating or non-phosphorylating artificially coupled [39] submitochondrial particles. An obvious explanation for lack of the ATP-independent reaction in submitochondrial particles was that the latter are more leaky for protons compared to intact mitochondria and the reversible F_0F_1-ATPase is a more powerful generator of $\Delta\tilde{\mu}_{H^+}$ than succinate oxidase. The results presented in this report show that this is not the case for oligomycin-treated particles, which catalyze the succinate-supported reverse electron transfer with or without ATP at the same rate. The critical points for

observation of the reaction are activated states of succinate dehydrogenase [43,44] and Complex I, whereas the submitochondrial vesicles themselves show remarkable stability in terms of a proton leakage at ambient temperatures.

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