Catalytic Activity of NADH-ubiquinone Oxidoreductase (Complex I) in Intact Mitochondria

EVIDENCE FOR THE SLOW ACTIVE/INACTIVE TRANSITION*

Vera G. Grivennikova, Alexander N. Kapustin, and Andrei D. Vinogradov‡

From the Department of Biochemistry, School of Biology, Moscow State University, Moscow 119899, Russian Federation

The mammalian purified dispersed NADH-ubiquinone oxidoreductase (Complex I) and the enzyme in inside-out submitochondrial particles are known to be the slowly equilibrating mixture of the active and de-activated forms (Vinogradov, A. D. (1998) Biochim. Biophys. Acta 1364, 169–185). We report here the phenomenon of slow active/de-active transition in intact mitochondria where the enzyme is located within its natural environment being exposed to numerous mitochondrial matrix proteins. A simple procedure for permeabilization of intact mitochondria by channel-forming antibiotic alamethicin was worked out for the “in situ” assay of Complex I activity. Alamethicin-treated mitochondria catalyzed the rotenone-sensitive NADH-quinone reductase reaction with exogenously added NADH and quinone acceptor at the rates expected if the enzyme active sites would be freely accessible for the substrates. The matrix proteins were retained in alamethicin-treated mitochondria as judged by their high rotenone-sensitive matrix-cytochrome c reductase activity in the presence of added NADH. The sensitivity of Complex I to N-ethylmaleimide and to the presence of Mg2+ was used as the diagnostic tools to detect the presence of the de-activated enzyme. The NADH-quinone reductase activity of alamethicin-treated mitochondria was sensitive to neither N-ethylmaleimide nor Mg2+. After exposure to elevated temperature (37 °C, the conditions known to induce de-activation of Complex I) the enzyme activity became sensitive to the sulphhydryl reagents and/or Mg2+. The sensitivity to both inhibitors disappeared after brief exposure of the thermally de-activated mitochondria with malate/glutamate, NAD+, and cytochrome c (the conditions known for the turnover-induced reactivation of the enzyme). We conclude that the slow active/de-active Complex I transition is a characteristic feature of the enzyme in intact mitochondria and discuss its possible physiological significance.

In mammalian mitochondria NADH-ubiquinone oxidoreductase (Complex I, coupling Site 1, EC 1.6.99.3) functions as the main entry to the respiratory chain. The enzyme has an extremely complex structure being composed of more than 40 different subunits (1, 2). It contains multiple distinct redox components (FMN, a number of iron-sulfur clusters and tightly bound ubiquinones) operating in unknown sequence of the reactions coupled with vectorial translocation of protons from matrix to intermembrane space. The functions of a vast majority of the enzyme subunits are not known. Most of the recent studies on Complex I and its simpler procaryotic counterparts (Type 1 NADH dehydrogenases) have focused on their structural and functional properties (refs. 7 and 14 for reviews). One form (A) is fully capable of catalyzing the high turnover rotenone-sensitive NADH-ubiquinone reductase reaction. The other, which we operationally call as de-activated form (D) is unable to transfer electrons to the quinone acceptor but is fully capable of the reactions with artificial electron acceptors such as ferricyanide or hexaam-
Some general speculative proposal on the subject has been put forward (14, 18), although they remain speculative because no evidence for the enzyme A → D transition in situ were yet available. To our knowledge there is only one report in the literature which suggests indirectly that Ca\(^{2+}\) sensitivity of the de-activated Complex I was the reason for a decrease of respiration rate with NAD\(^{-}\)-linked substrates seen in intact liver mitochondria after Ca\(^{2+}\) load (19).

It can hardly be overemphasized that an unambiguous demonstration of any regulatory property of Complex I in intact mitochondria is extremely difficult because the inner mitochondrial membrane is not permeable to NADH and respiration of mitochondria in the presence of NAD\(^{-}\)-linked substrates involves, besides Complex I itself, obligatory operation of the dicarboxylate transport system, particular dehydrogenase, and the downstream components of the respiratory chain. It also worth noting that Complex I in intact mitochondria, at least its matrix-protruding part, operates within rich protein environment which may or may not significantly affect the catalytic and/or regulatory properties of the enzyme.

The aim of the studies reported here was 2-fold. First, we searched for a reliable experimental procedure for direct quantitative measurement of the Complex I catalytic activity in sealed mitochondria. We have used the channel-forming antibiotic alamethicin (20, 21) previously employed for unmasking several ATP-dependent enzymatic activities in sealed membrane preparations and to reveal latent NADH oxidase activity in intact mitochondria (22–24). The present article shows that permeabilization of mitochondria by alamethicin provides a valuable tool for measurement of the specific NADH oxidase and/or NADH-quinone reductase activities in mitochondria.

The second problem we have addressed was to find out whether slow pseudo-reversible Complex I A → D transition exist when the enzyme operates in the natural matrix protein environment. Having succeeded in measurement of Complex I activity in situ we were able to show that this unique property is indeed an intrinsic feature of the enzyme in mitochondria.

**MATERIALS AND METHODS**

**Rat Heart Mitochondria**—These were isolated from trypsin-treated heart muscle (two hearts were handled for one preparation) essentially as described by Jacobus and Saks (25). The final precipitate of mitochondria was suspended in 0.3 M sucrose, 10 mM Hepes, 0.2 mM EDTA (potassium salts, pH 7.4), and BSA (1 mg/ml) and stored in ice. The mitochondria oxidized malate/glutamate (5 mM each) in the reaction mixture comprising 0.25 M sucrose, 10 mM Tris/Cl, 0.2 mM EDTA, and 6 mM potassium phosphate (pH 8.0) at the average rate of 14 and 130 respiratory control ratio of about 10) nanomoles of oxygen per min per mg of protein in the presence and absence of 200 \(\mu\)M ADP, respectively, at 22 °C.

**Bovine Heart SMP**—SMP were prepared (15) and their NADH oxidase was activated (26) as described. The uncoupled particles (in the presence of gramicidin D, 0.2 \(\mu\)g/ml) catalyzed the rotenone-sensitive oxidation of NADH (more than 99%) NADH oxidase reaction at the average rate of 1 \(\mu\)mol/min/mg of protein at 22 °C, pH 8.0.

**Complex I**—Complex I was purified according the standard procedure (27). Its activity was determined at 38 °C in the reaction mixture containing: 0.25 M sucrose, 50 mM Tris/Cl (pH 8.0), 0.2 mM EDTA, BSA (1 mg/ml), 2.5 mM MgCl\(_2\), 5 mM NADH, and 100 \(\mu\)M ubiquinone-1 (Q\(_1\)) after preincubation for 20 min with soybean phospholipids (2 mg/mg of Complex I).

**Bovine Heart Mitochondrial Matrix Protein Fraction**—This was prepared from the supernatant left after sonic treatment of bovine heart mitochondria during SMP preparation. The supernatant (15 ml) stored at −20 °C was thawed and diluted 2 times with cold water. 10 ml of 100 mM Tris/Cl (pH 7.5) was added and pH of the mixture was adjusted to 6.0 with acetic acid. The slightly turbid mixture was centrifuged (30,000 × g, 30 min) to remove residual membranes, pH of clear supernatant was adjusted to 8.0 with 1 N KOH and solid ammonium sulfate was added up to 70% saturation. The mixture was left on ice for 20 min, precipitated protein was collected (30,000 × g, 30 min), suspended in 2.5 ml of 10 mM Tris/Cl (pH 8.0), and dialyzed for 24 h against 1 liter of the same solution. The clear soluble protein fraction thus obtained was stored in liquid nitrogen.

**The NADH Oxidase and NADH-quinone Reductase**—The activities were assayed at 30 °C as a decrease of absorption at 340 nm with 200 \(\mu\)M NADH as the substrate (oxidase) or 200 \(\mu\)M NADH and 100 \(\mu\)M ubiquinone-1 (Q\(_1\)) in the presence of 1.5 mM KCN potassium EDTA, and mitochondrial (25 \(\mu\)g of protein/ml). The standard assay mixture contained: 0.25 M sucrose, 50 mM Tris/Cl (pH 8.0), 0.2 mM potassium EDTA, and the enzyme preparation (mitochondria or SMP (~10 \(\mu\)g of protein/ml).

**The Malate-Cytochrome c Reductase**—This was assayed following cytochrome c reduction at 550 nm in the presence of 5 mM malate, 5 mM glutamate, 1.5 mM KCN, and 15 \(\mu\)g cytochrome c. The hypothesis assay mixture contained: 10 mM Tris/Cl (pH 8.0), 0.2 mM potassium EDTA and mitochondria (~25 \(\mu\)g of protein/ml). All the activities throughout the paper are expressed as micromoles of NADH oxidized per min per mg of protein.

**The Malate Dehydrogenase Activity**—This activity was determined as the rate of NADH oxidation in the reaction mixture containing 20 mM potassium phosphate (pH 8.0), 0.2 mM EDTA, 5 \(\mu\)g rotenone, 150 \(\mu\)M NADH, and 20 \(\mu\)M oxaloacetate. The mitochondrial preparations (~20 mg of protein/ml) were solubilized at 0 °C by Triton X-100 (1%, w/v, 20 min) and diluted 10 times in 0.25 M sucrose, 10 mM Hepes, 0.2 mM EDTA (pH 7.4). Small samples (~2 \(\mu\)g of protein/ml) thus treated were added to the reaction mixture and NADH oxidation was started by the addition of oxaloacetate.

**Aspartate-2-oxoglutarate Transaminase Activity**—The aspartate-2-oxoglutarate transaminase activity of the mitochondria solubilized by Triton X-100 was determined as the rate of NADH oxidation in the reaction mixture containing 20 mM potassium phosphate (pH 8.0), 0.2 mM EDTA, 5 \(\mu\)g rotenone, 150 \(\mu\)M NADH, 0.1 mM 2-oxoglutarate, 0.1 mM aspartate and malate dehydrogenase (1 unit/ml). About 50 \(\mu\)g of the mitochondrial protein per ml was added to the assay mixture.

**Permeabilized Mitochondria**—The following procedure based on our experimental findings (see “Results”) was employed to prepare the mitochondrial preparation capable of the rotenone-sensitive oxidation of externally added NADH. Intact mitochondria (10–20 mg/ml) were diluted 20 times with the mixture comprising 0.25 M sucrose, 10 mM Hepes/KOH (pH 7.4), 0.2 mM EDTA, BSA (1 mg/ml), 2.5 mM MgCl\(_2\), and alamethicin (40 \(\mu\)g/ml). The suspension was incubated at 20 °C for 5 min, diluted 2.5 times with the same cold mixture containing no MgCl\(_2\) and alamethicin, and centrifuged at 30,000 × g for 15 min. Precipitated mitochondria were suspended in 0.25 M sucrose, 50 mM Tris/Cl (pH 8.0), 0.2 mM EDTA, and BSA (10 mg/ml), and stored in ice during the experiments.

**Protein Content**—The protein content was determined with biuret reagent (28) using BSA as the standard.

NADH, NADPH, NAD\(^+\), EDTA, Tris, Hepes, BSA, malic acid, glutamic acid, L-aspartate, 2-oxoglutarate, ADP, Q\(_1\) (C-7956, Lot 117H32551), cytochrome c, and NEM were from Sigma. Malate dehydrogenase was from “Reanal” (Hungary). Alamethicin was a kind gift from Dr. S. Kotelevtzev (Laboratory of Physico-chemical membranology, School of Biology, Moscow State University).

**RESULTS**

**Catalytic Activity of Complex I in Alamethicin-permeabilized Mitochondria**—Intact rat heart mitochondria prepared by a mild isolation procedure as compared with other reductase preparations were used to study the effects of alamethicin on permeability of their inner membranes for the respiratory substrates. Table I demonstrates that besides expected uncoupling effect on ΔµH\(_{\text{t}}\)-controlled respiration (state 4), alamethicin drastically potentiated the rotenone-sensitive oxidation of externally added NADH. Remarkably, alamethicin did not affect the NADH-Q\(_{1}\) reductase activities of inside-out SMP, and that otherwise potentiated purified preparation of Complex I. When alamethicin-induced NADH oxidation was followed by spectrophotometric techniques in the standard Mg\(^{2+}\)-free reaction mixture routinely employed to assay NADH oxidase we noted that the stimulatory effect of the antibiotic on NADH oxidation was time-dependent and much less pronounced than that shown in Table I. Further inspection has revealed that the presence of Mg\(^{2+}\) was needed for rapid and effective channel forming activity of alamethicin in mitochondria. At pH 8.0 the stimulatory effect of alamethicin was saturated at Mg\(^{2+}\)-con-
TABLE I

Effect of alamethicin on several oxidoreductase activities catalysed by the mitochondrial preparations different degree of resolution

Oxidase activities of intact rat heart mitochondria were measured as the respiration rates with oxygen-sensitive electrode in the medium containing 0.12 M sucrose, 75 mM KCl, 2.5 mM MgCl₂, 10 mM Hepes, pH 7.4. Oxidation of NADH by SMP and Complex I was measured as described (see “Materials and Methods”) in the standard reaction mixture in the presence of 2.5 mM MgCl₂.

| Preparation and reaction | Specific activity | − Alamethicin | + Alamethicin (30 µg/ml) |
|--------------------------|------------------|--------------|--------------------------|
| Intact rat heart mitochondria | Succinate oxidase | 0.02 | 0.16 |
|                          | Succinate oxidase (+ FCCP, 1 µM) | 0.15 | 0.15 |
|                          | NADH oxidase | 0.02 | 0.62 |
|                          | NADH oxidase (+ FCCP, 1 µM) | 0.02 | 0.02 |
|                          | NADH oxidase (+ rotenone, 5 µM) | 0.03 |
| Bovine heart SMP | NADH-Q₁ reductase (+ FCCP, 1 µM) | 0.60 | 0.58 |
| Lodge-1 | NADH-Q₁ reductase (+ rotenone, 5 µM) | 0.05 | 0.05 |
| Complex I | NADH-Q₁ reductase | 2.34 | 2.42 |

It was of interest to know whether the matrix proteins are retained in alamethicin-treated mitochondria. This was verified by measuring several enzymatic activities which requires the enzymes located in matrix. Table II shows that alamethicin-treated washed mitochondria lost their endogenous NAD⁺ whereas the preparation significantly retained their malate dehydrogenase and transaminase. The specific cytochrome c reductase activity (0.08) in the presence of added NAD⁺ was found to be close to that found in the standard polarographic experiments (0.130) at State 3 (see “Materials and Methods”). It should be noted that the hypotonic reaction mixture was used for the cytochrome c reductase activity assay to provide accessibility of the inner membrane for added cytochrome c; thus quantitative comparison of the NADH oxidase and cytochrome c reductase activities is to be taken only as an approximation. The specific activities of malate dehydrogenase and transaminase were decreased in the alamethicin-treated preparations. This was not unexpected, because alamethicin pore is not specific and permeable for large cations and anions (29). Thus alamethicin induces swelling of mitochondria which can change the permeability of the inner membrane and disruption of the outer membrane (30).

The results presented above showed that alamethicin-treated mitochondria can be used for assay of the specific in situ Complex I activity. It was of interest to compare the kinetic properties of the mitochondrial enzyme which is exposed to a number of matrix proteins with those previously reported for inside-out SMP (7). Fig. 2 shows the concentration dependence of the initial reaction rates on ubiquinone homologue Q₁ and NADH which were essentially the same as those for SMP. Pronounced inhibition of the NADH-Q₁ reductase activity at high concentrations of Q₁ (Fig. 2A) was variable and dependent on the particular sample of the commercially obtained quinone. This phenomenon presumably is due to some unidentified inhibitory contaminants and merits further investigation. It should be noted, however, that with one particular sample of Q₁ the same kinetic behavior was always seen for inside-out SMP and alamethicin-treated mitochondria. The standard kinetic parameters of the rotenone-sensitive NADH-Q₁ reductase activity for alamethicin-treated mitochondria and those for inside-out SMP are summarized in Table III. The catalytic turnover numbers for different preparations were calculated using the values for the enzyme content determined as the minimal amount of piperidin, the specific irreversible inhibitor of Complex I (31, 32), needed to block the activities. The very close turnover numbers and Kₘ values thus obtained for mitochondria and SMP preparations suggest that the enzyme in sealed mitochondria behaves as its active sites would be freely accessible for the substrates and that the matrix located proteins do not affect the catalytic activity of Complex I. Slightly lower enzyme turnover number in rat heart mitochondria may be due to species difference.

Complex I A ↔ D Transition in Mitochondria—The differences in catalytic properties of A and D forms of Complex I summarized in the Introduction provide at least two simple diagnostic criteria for their relative content in any particular preparation: one is the sensitivity of NADH-quinone reductase to the sulfhydryl group reagents; the other is a lag-phase in the catalytic activity which is seen as inhibition of the enzyme by divalent cations at alkaline pH. Both tests were employed in further studies of alamethicin-treated mitochondria. It was of interest to compare the kinetic properties of the mitochondrial enzyme which is exposed to a number of matrix proteins with those previously reported for inside-out SMP (7). Fig. 2 shows the concentration dependence of the initial reaction rates on ubiquinone homologue Q₁ and NADH which were essentially the same as those for SMP. Pronounced inhibition of the NADH-Q₁ reductase activity at high concentrations of Q₁ (Fig. 2A) was variable and dependent on the particular sample of the commercially obtained quinone. This phenomenon presumably is due to some unidentified inhibitory contaminants and merits further investigation. It should be noted, however, that with one particular sample of Q₁ the same kinetic behavior was always seen for inside-out SMP and alamethicin-treated mitochondria. The standard kinetic parameters of the rotenone-sensitive NADH-Q₁ reductase activity for alamethicin-treated mitochondria and those for inside-out SMP are summarized in Table III. The catalytic turnover numbers for different preparations were calculated using the values for the enzyme content determined as the minimal amount of piperidin, the specific irreversible inhibitor of Complex I (31, 32), needed to block the activities. The very close turnover numbers and Kₘ values thus obtained for mitochondria and SMP preparations suggest that the enzyme in sealed mitochondria behaves as its active sites would be freely accessible for the substrates and that the matrix located proteins do not affect the catalytic activity of Complex I. Slightly lower enzyme turnover number in rat heart mitochondria may be due to species difference.

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The Effect of Matrix Proteins on Complex I A ↔ D Transition in SMP—Another approach for modeling of in situ Complex I A ↔ D transition was to see the effect of crude matrix protein fractions on the slow interconversions of the enzyme forms in inside-out SMP. No effects of added matrix fraction (3.3 mg/ml) on the thermally induced de-activation or on NADH-induced reactivation were found. In contrast to NEM, oxidized glutathione (5 mM) had no effect on the D-form of Complex I in SMP (nonenzymatic thiol-disulfide exchange reaction) or in the presence of matrix proteins (possible enzyme-catalyzed exchange reaction, the results are not shown). When the steady-state oxidation of the Krebs cycle substrates was reconstituted in the model system by the addition of malate plus glutamate and NADH, the "turnover conditions" drastically prevented the enzyme de-activation even after incubation of the samples at 37 °C as long as 20 min (Fig. 6).

DISCUSSION

The activity of mitochondrial Complex I is of great importance for cell physiology because the enzyme serves as the main collector of reducing equivalents derived from Krebs cycle substrates and modulation of the enzyme activity is expected to influence the energetic status of any aerobic cell. Despite widespread interest in the functional state of the enzyme at the cellular level the lack of simple and reliable methods for the quantitative determination of its activity in intact mitochondria greatly hampered the progress in several areas of bioenergetics, especially in those of medical importance since a number of diseases are believed to be associated with some defects in Complex I (33, 34). It is a general practice in mitochondriology to correlate respiratory activity in the presence of NADH-dependent dehydrogenase-linked substrates with the catalytic activity of Complex I. Depending on the particular tissue and/or on a number of factors hard to control such as intactness of the mitochondrial membranes, deficiency in nicotinamide nucleotides, specific activities of dicarboxylate translocases and dehydrogenases, such correlation may or may not be judicious. An obvious way to overcome an uncertainty in the specific Complex I activity in mitochondria might be to use a detergent to abolish the permeability barrier for NADH. However, the ubiquinone reductase activity of Complex I has been shown to be extremely sensitive to a number of lipophilic compounds including detergents (35–37), as can be illustrated by strong inhibition of the enzyme by Triton X-100 (36). The permeabilization of intact mitochondria by alamethicin provides a simple procedure for reliable quantitative assay of Complex I without any interference with the substrate translocases and dehydrogenases. Indeed, the kinetic parameters of fully active Complex I in sealed rat heart and bovine heart mitochondria as reported here are very similar to those determined for inside-out SMP (Table III). Another possible important application of alamethicin-induced permeabilization is its use for qualitative and quantitative determination of a heterogeneity of sealed membranous preparations of Complex I such as submitochondrial or sub-bacterial particles. No stimulation of the NADH-Q reductase activity in SMP was found (Table I), thus suggesting that no enzymatically active right-side out particles are present in the preparations routinely used in our laboratory (15). In contrast, considerable stimulation of the uncoupled NADH oxidation reaction by alamethicin was found for the preparations obtained from Paracoccus denitrificans cells which is in accord with the previously reported stimulation by bee venom of NADH oxidation by P. denitrificans subbacterial particles (38).

reaction. However, this was not the case as evident from the experiments where the preparation was first de-activated at 37 °C, and then treated with NEM at 20 °C: rapid inhibition was observed (Fig. 5, closed triangles). Thus, the time dependence of the inhibition by NEM at 37 °C was evidently due to the time dependence of the enzyme de-activation. Moreover, when mitochondria were partially de-activated by incubation at 37 °C for limited periods of time as indicated on the abscissa in Fig. 5 and further treated with NEM at 20 °C for 2 min (the time needed to inhibit completely de-activated enzyme at this temperature), the points (open triangles) corresponding to the residual activity thus revealed perfectly fit the curve characteristic for the time-dependent inhibition by NEM at 37 °C.

The Effect of Matrix Proteins on Complex I A ↔ D Transition

Enzymatic activities of intact and alamethicine-treated rat heart mitochondria

The figures correspond to the specific activities of the representative samples determined as described under "Materials and Methods." No special attempts were made to reveal full catalytic activities by variation of the substrate concentrations, ionic composition, and pH of the assay medium. Thus, the data should be considered as to demonstrate the retention of some matrix located enzymes in alamethicin-treated mitochondria only.

| Enzyme                  | Specific activity | Intact mitochondria | Alamethinin-treated mitochondria |
|-------------------------|-------------------|---------------------|----------------------------------|
| Malate-cytochrome c reductase | 0.30              | <0.01               |
| + NADH (100 μM)         | 0.04              | 0.08                |
| Aspartate-2-oxoglutarate transaminase | 0.6               | 0.34                |
| Malate dehydrogenase    | 27.0              | 17.2                |

FIG. 1. Effect of alamethicin on oxidation of NADH by intact rat heart mitochondria. Mitochondria (30–40 μg of protein/ml) were incubated for 1 min at 30 °C in the standard (3 ml) spectrophotometric cuvette in the mixture containing: 0.25 mM sucrose, 50 mM Tris/Cl (pH 8.0), 0.2 mM EDTA, 2.5 mM MgCl₂, and alamethicin. The NADH oxidation reaction (○) was started by the addition of 200 μM NADH. The NADH-quinone reductase reaction (△) was started by the addition of 200 μM NADH and 100 μM Q₁. 1.5 mM potassium cyanide was present in the NADH-Q₁ reductase assay mixture. 100% of the specific activities correspond to 1.0 and 0.45 μmol of NADH oxidized per min/mg of protein for NADH oxidation and NADH-Q₁ reductase, respectively. NADH oxidase and NADH-Q₁ reductase were 99 and 93% sensitive to 2.5 μM rotenone, respectively.

V. G. Grivennikova, N. V. Zakharova, and A. D. Vinogradov, unpublished observation.
The disadvantage of using alamethicin for the specific assay of Complex I is obvious impossibility of measuring the enzyme activity in energized mitochondria, thus possible $\Delta \mu _{H}^{\circ}$-dependent rapid modulation of Complex I remains to be a “black box.” Also it should be pointed out that partial or complete loss of some small proteins or other unknown substances from the matrix after treatment of mitochondria with alamethicin cannot be ruled out (Table II).

Two diagnostic tests: Mg$^{2+}$ susceptibility at alkaline pH and NEM sensitivity showed that mitochondrial proteins which are

![Graph A](image1)

![Graph B](image2)

**Fig. 2.** Concentration dependence of NADH-Q$_1$ reductase reaction catalyzed by alamethicin-permeabilized rat heart mitochondria. Mitochondria were permeabilized and assayed as described under “Materials and Methods.” 200 μM NADH (A) and 50 μM Q$_1$ (B) were present in the standard assay mixture.

| Preparation       | Complex I content | Turnover number | $K_{m}^{Q_1}$ | $K_{m}^{NADH}$ |
|-------------------|-------------------|-----------------|---------------|---------------|
| Rat heart mitochondria | 0.15              | 40              | 5             | 9             |
| Bovine heart mitochondria | 0.11              | 55              |               |               |
| Bovine heart SMP   | 0.20              | 50              | 10            | 7             |

**TABLE III**

Kinetic parameters of NADH-Q$_1$ reductase reaction catalyzed by Complex I in sealed mitochondria and inside-out submitochondrial particles

Complex I content and the respective turnover numbers were calculated as piericidine “titer.” The latter was determined as the intersection points from the linear graphs: the residual activity versus amount of the inhibitor added; the activities were determined after prolonged (20 min, 30 °C) preincubation of the enzyme preparations with piericidine.
in direct contact with the matrix-exposed part of the enzyme do not protect Complex I against A → D transition and strengthen our hypothesis on the physiological relevance of this phenomenon. An obvious question arises: what are the possible physiological conditions which may lead to the de-activation of Complex I in vivo? It seems unlikely that D-form is present in vivo when Complex I catalyzes the steady-state NADH oxidation (Fig. 6). However, rapid de-activation is expected under strong hypoxic or anoxic conditions when the enzyme turnover is prohibited. An important point for the discussion of such a scenario is that reactivation of the de-activated enzyme is a very slow process in the presence of divalent cations. The millimolar free Mg²⁺ (39) and variable high concentrations of Ca²⁺ (19, 40) are present in the mitochondrial matrix. Thus Complex I is expected to stay in D-form for a long time after reoxygenation. In addition to the adverse effects that result from de-energization of mitochondria during anoxia, further adverse effects are anticipated following re-oxygenation because the D-form is unable to transfer electrons to ubiquinone but can reduce oxygen directly producing large amounts of superoxide. It is well established that D-form by all the parameters studied so far (14) is equivalent to the rotenone-inhibited enzyme and rotenone was reported to activate superoxide generation by Complex I (41, 42). The experiments aimed to prove or disprove our hypothesis on anoxia-induced de-activation of Complex I in Intact Mitochondria
Complex I in intact mitochondria are underway in our laboratory.

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