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Reductive inactivation of the mitochondrial three subunit NADH dehydrogenase

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The 3-subunit iron-sulfur flavoprotein (NADH-artificial electron acceptor oxidoreductase) derived from complex I (EC 1.6.5.3) is rapidly and irreversibly inactivated in the presence of NADH. The rate of inactivation increases with a decrease of the enzyme concentration. The activities with ferricyanide, menadione and cytochrome *c* were lost synchronously during preincubation of the enzyme in the presence of NADH or dithionite under either aerobic or anaerobic conditions. The titration of the inactivation rate with the NADH/NAD^+ pair suggests that reduction of a component with $E'_m = -325$ mV ($n = 2$) is a prerequisite for a loss of the enzyme activity. Among the compounds tested only FMN and NAD^+ were able to protect the enzyme against the reductive inactivation. NADH-induced loss of the enzyme activity in diluted solutions is accompanied with the synchronous appearance of a fluorescence characteristic for free FMN. It is concluded that the reduction of flavin leads to a strong decrease of FMN affinity to its specific binding site, and possible implications of the redox-dependent affinity changes in operation of NADH-ubiquinone reductase are discussed.

Introduction

Numerous attempts to resolve the interrelationship of the structure and functions of the mammalian respiratory chain site I energy transduction have resulted in isolation of three major types of NADH dehydrogenase (EC 1.6.5.3) with different properties and activities. The only enzyme preparation which shows all the properties of NADH-ubiquinone oxidoreductase activity in submitochondrial particles, including energy conservation after insertion into phospholipid vesicles, is complex I, capable of catalyzing the rotenone-sensitive NADH oxidation by exogenous quinones [1–3]. The second type is high molecular mass, phospholipid and ubiquinone free, water soluble NADH dehydrogenases (the type I dehydrogenases [4–7]) which catalyze the rotenone-insensitive (with one exeption [5]) reduction of ubiquinone homologues. The type I dehydrogenase is unstable and under a variety of conditions it degradates to the low molecular mass fragments [8–10] which include iron-sulfur flavoprotein (Type II NADH dehydrogenase), capable of oxidizing NADH by a number of artificial electron acceptors [11]. The third type, which is apparently the most pure catalytically competent type II NADH dehydrogenase, is the soluble 3-subunit iron-sulfur flavoprotein (FP fraction) purified after chaotropic resolution of complex I at 38°C under anaerobic conditions [12]. The NADH dehydrogenase thus prepared, when subjected to SDS–gel electrophoresis, shows three polypeptide bands with apparent molecular masses of 51, 24 and 9 kDa, and is considerably enriched in FMN, nonheme iron and acid-labile sulfur (13, 60 and 60 nmol/mg protein respectively [13]) compared with the original complex I preparation (1.4, 26 and 28 nmol/mg protein, respectively [14]). FP catalyzes rapid rotenone-insensitive oxidation of NADH by menadione and ferricyanide (the most effective electron acceptors [11–13]), FMN-mediated [4B-^{3}H] NADH-H_2O exchange [15] and NADH-acetylpyridine adenine dinucleotide transhydrogenation [16]. It is now generally agreed that 3-subunit iron-sulfur flavoprotein (FP) is the minimal catalytically competent unit of complex I still capable of NADH oxidation by artificial electron acceptors. The enzyme bears the NADH-specific binding site on the 51 kDa subunit [17], and two (one binuclear, $E_m =$

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Abbreviations: menadione, 2-methyl-1,4-naphthoquinone; Hepes, 4-(2-hydroxy-ethyl)-1-piperazineethanesulphonic acid; FP, 3-subunit iron-sulfur flavoprotein derived from complex I (NADH-ubiquinone oxidoreductase, EC 1.6.5.3); SDS, sodium dodecyl sulfate.

−456 mV and one tetranuclear, $E_m = -410$ mV) iron-sulfur clusters [18]. FP has been further resolved into FP-I (51 kDa) and FP-(II + III) (24 kDa + 9 kDa) fractions which are catalytically inactive [19]. FMN is lost during dissociation of FP [19], and it is not known to which subunit the flavin is bound. Although the substrate and acceptor specificity of the simplest form of NADH dehydrogenase is evidently altered by relatively drastic procedures applied for its isolation [12], FP may serve as a subject of studies on the initial steps of NADH oxidation.

In this report experiments are presented demonstrating that the reduction of the enzyme (most probably flavin) results in a strong decrease of FMN affinity to the protein.

Materials and Methods

Complex I [14] and NADH dehydrogenase (using 0.5 M $NaClO_4$ for complex I resolution [13]) were prepared according to published procedures. Protein content was estimated by the methods of Lowry et al. [20] or by the biuret procedure [21]. Enzymatic assays were carried out at 26°C using Hitachi 557 spectrophotometer at 340 nm (NADH oxidation, $\epsilon_{mM} = 6.22$), 550 nm (cytochrome *c* reduction, $\epsilon_{mM} = 20$) and 420 nm (ferricyanide reduction, $\epsilon_{mM} = 1.0$) The details of the enzymatic assays are indicated in the legends to the Figures and Tables. Fluorescence measurements were carried out using a Hitachi F3000 spectrofluorometer. The sources of the chemicals used were as follows: NADH, EDTA, Hepes and glucose oxidase (EC 1.1.3.4) were from Sigma (USA); NAD^+, cytochrome *c* and catalase (EC 1.11.1.6) were from Fluka (Switzerland); FMN and riboflavin were from Serva (Germany); menadione was from Calbiochem (USA); $Ru(NH_3)_6Cl_3$ was from Strem Chemicals (USA). Other chemicals were of the highest quality commercially available.

Results

When NADH-artificial electron acceptor oxidoreductases catalyzed by FP were assayed, considerable variations of the specific enzyme activities were observed which depended on the time intervals and order between successive additions of the substrate, acceptor, and enzyme, and on the protein concentrations in the assay mixture. Fig. 1 gives a representative example of the phenomemon for the NADH-menadione oxidoreductase assay. A substantial time-dependent loss of activity occurred when the FP was incubated in the presence of NADH prior the addition of menadione (compare traces 1 and 2). No changes in the NADH-menadione or -ferricyanide reductase activities were observed following incubation with NADH of either complex I or submitochondrial particles (results not shown). The FP inactivation was not due to the possible production of superoxide radical [22,23] which might be deteriorating, since the loss of activity was observed under either aerobic or anaerobic conditions. The NADH-induced inactivation was found to be unspecific for the electron acceptors used for the assays (Fig. 2). The first-order kinetics of the enzyme inactivation was observed for the NADH-menadione, -ferricyanide or -cytochrome *c* reductase activities (Fig. 2), whereas

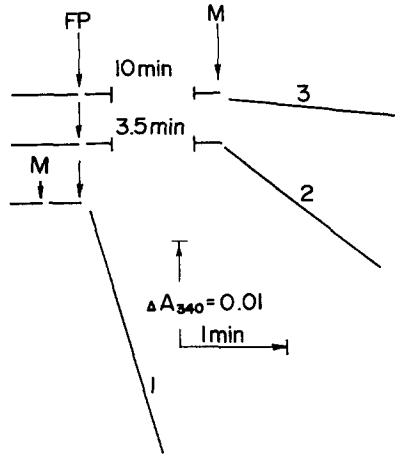


Fig. 1. Effect of NADH on NADH-menadione reductase activity of NADH dehydrogenase. The assay cuvette (2 ml) contained 0.25 M sucrose, 0.2 mM EDTA, 20 mM Hepes (pH 8.0), 10 mM glucose, catalase (5 μg), glucose oxidase (0.4 mg) and 100 μM NADH. 100 μM menadione (M) and 0.06 μg/ml of NADH-dehydrogenase (FP) were added as indicated.

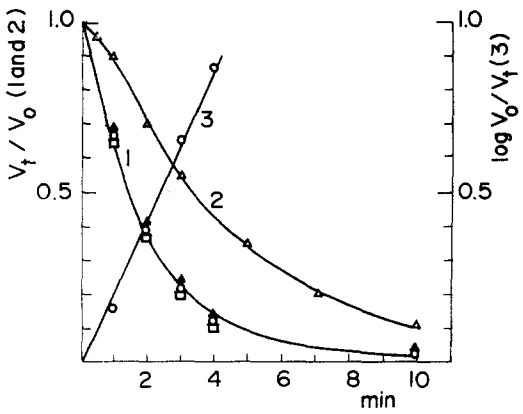


Fig. 2. Kinetics of NADH-induced inactivation of NADH dehydrogenase. The assays of the NADH-artificial electron acceptor reductases were performed aerobically as described in Fig. 1. Curves 1 and 2 (left ordinate), the reactions were started after preincubation of the enzyme (0.05 μg/ml) with 100 μM NADH for the time intervals indicated on abscissa by the additions of: (○), 0.54 mM ferricyanide; (▲), 100 μM menadione; (□), 10 μM cytochrome *c*; (△), 25 μM hexammineruthenium(III). The original specific activities (zero time preincubation) with ferricyanide, menadione, cytochrome *c* and hexammineruthenium(III) were 57, 48, 6.3 and 224 μmol of NADH oxidized/min/mg protein. ν_t and ν_0, the specific enzyme activities after preincubation at time *t* and at zero time respectively. Line 3 (right ordinate), semilogarithmic anamorphose of curve 1 (ferricyanide reductase).

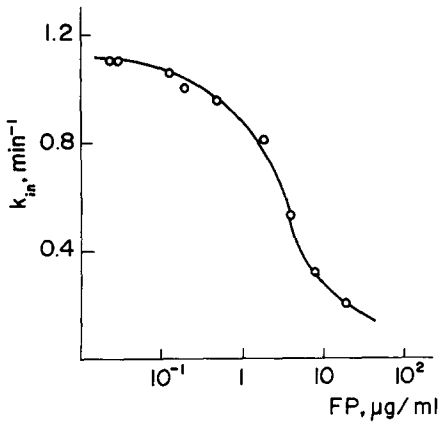


Fig. 3. Apparent first-order rate constant for NADH-induced inactivation of NADH dehydrogenase as a function of the enzyme concentration in preincubation medium. The time dependence of 0.4 mM NADH-induced inactivation was measured as described in Fig. 2 using 100 μM menadione as electron acceptor. The apparent first-order rate constants were estimated from the semilogarithmic plots (see Fig. 2).

more complex time dependence was seen with hexammineruthenium(III), which seems to be the most efficient electron acceptor for FP (Sled, V.D. and Vinogradov, A.D., in preparation).

The apparent first-order reaction rate constant for the NADH-induced inactivation was increased with a decrease of protein concentration in the preincubation mixture (Fig. 3) and reached half-maximal value at about 1.2 μg/ml, which is equivalent to $1.6 \cdot 10^{-9}$ M enzyme concentration (assuming FMN content of 13 nmol/mg [13]).

The data presented above are consistent with the mechanism of inactivation which includes the dissociation of some catalytically active component from the reduced diluted enzyme followed by irreversible denaturation of the protein. FMN noncovalently bound at the enzyme specific site seemed to be the most conceivable candidate for such a component.

If such a mechanism is operating, it might be expected that FMN would specifically protect the enzyme against reduction-induced inactivation. Table I depicts the protective effect of FMN and shows that unspecific reduction of the enzyme by dithionite also results in FMN-protected inactivation. Among the compounds tested, only FMN and NAD^+ were able to prevent the NADH-induced inactivation. The inactivation was irreversible and neither FMN nor NAD^+ was effective in restoration of the activity lost.

More direct evidence for the FMN-dissociative mechanism of NADH-induced enzyme inactivation was obtained from the fluorescence measurements in the diluted NADH dehydrogenase solutions, as shown in Fig. 4. The enzyme as prepared showed negligible fluorescence at 540 nm, characteristic for free FMN at neutral pH. After incubation in the presence of NADH

TABLE I

Reductive inactivation of NADH dehydrogenase

FP (0.5 μg/ml) was preincubated at 25°C in a mixture containing 20 mM Hepes (pH 8.0), 0.25 M sucrose, 0.2 mM EDTA (other additions are indicated) for 4 min and initial rates (v_i) of NADH (90 μM) oxidation by menadione (100 μM) were then determined.

Additions to the preincubation mixture	NADH-menadione reductase (μmol NADH oxidized/min/mg protein)	v_i / v_0 (%)
1. None (v_0)	46	100
2. NADH (90 μM)	4.6	10
3. NADH (90 μM) FMN (0.5 μM)	21	46
4. NADH (90 μM) Riboflavin (0.5 μM)	5.6	12
5. NADH (90 μM) NAD^+ (1 mM)	30 [a]	76 [a]
6. Dithionite (2 mM)	5 [b]	11 [b]
7. Dithionite (2 mM) $FMNH_2$ (50 μM)	32 [b]	70 [b]

[a] The activity measured in the presence of 1 mM NAD^+ (40 mol NADH oxidized/min/mg protein) was taken as the control (v_0).

[b] 2 mM (final concentration) sodium dithionite was added to the anaerobic preincubation mixture containing 0.6 μg/ml FP. After 4 min proper amount of the mixture was transferred to an assay cuvette containing 90 μM NADH and 100 μM menadione.

the inactivated enzyme solution exhibited typical fluorescence with the maximum at 540 nm. A remarkable coincidence between the appearence of fluorescence and the loss of enzyme activity is evident (Fig. 4A).

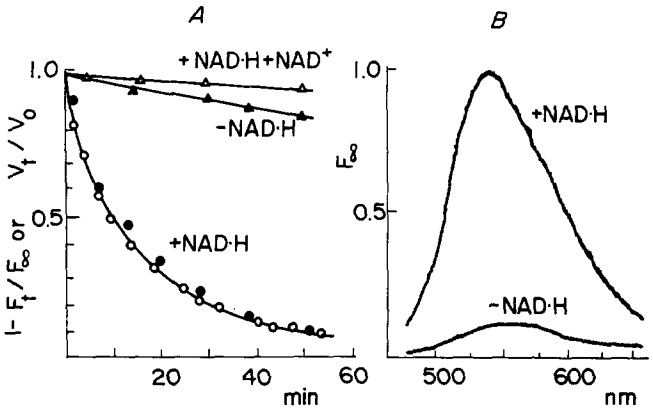


Fig. 4. The flavin fluorescence change during reduction-induced inactivation. (A) The enzyme (15 μg/ml) was incubated in a mixture comprising 50 mM Hepes (pH 8.0), 0.2 mM EDTA and nucleotides (150 μM NADH, 1 mM NAD^+) where indicated. The fluorescence intensity (○, F_t excitation at 450 nM, emission at 540 nm) and the enzyme activity in the presence of 75 μM NADH and 100 μM menadione (●) were measured. The initial fluorescence intensity was negligible (less than 10%) compared to the final level (F_∞) and was subtracted from the values of F_t. Two upper curves, the enzyme activity during preincubation without NADH (▲), or in the presence of NADH and NAD^+ (△). (B) Fluorescence spectrum of the mixture after incubation in the presence of 150 μM NADH for 2 h (F_∞); excitation at 450 nm.

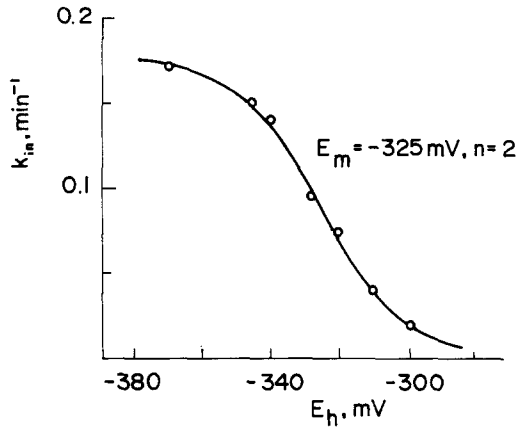


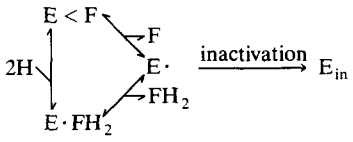
Fig. 5. Anaerobic redox titration of the reduction-induced inactivations of NADH dehydrogenase. The enzyme (0.12 μg/ml) was incubated as described in Fig. 1 in the presence of 100 μM NADH and various concentrations of NAD^+. The first-order rate constants (○) for the inactivation process were estimated from the semilogarithmic plots as described in Fig. 2. The apparent redox potentials (E_h) were calculated assuming E_0' for the couple NADH/NAD^+ equal to −320 mV. Continuous line is the theoretical curve for the titration of a compound with $E_m = -325$ mV, $n = 2$.

Both the fluorescence increase and the enzyme activity decrease were prevented by NAD^+.

The protective effect of NAD^+ on NADH-induced inactivation seemed to be a helpful tool for the estimation of the redox potential of the component, whose dissociation is a prerequisite for the enzyme inactivation (most likely FMN). When the first-order rate constants for the irreversible inactivation process were measured as a function of NADH/NAD^+ ratio, the experimental points obtained closely fit the theoretical redox titration curve with the parameters: $E_m = -325$ mV and $n = 2$ (Fig. 5).

Discussion

The most conceivable scheme describing the process of the reduction-induced inactivation of NADH-dehydrogenase may be formulated as follows:



Scheme I.

where E · stands for the 3-subunit apoflavoprotein, F and FH_2 stand for oxidized and reduced FMN respectively, < and · denote tight and loose binding of FMN respectively, and E_{in} stands for irreversibly inactivated enzyme.

According to Scheme I, the reduction of flavin by the substrate (NADH) or unspecifically by dithionite (see Table I) results in a strong decrease of FMN affinity to the protein and in a subsequent appearance of an unstable rapidly denaturating apoprotein (E ·).

Since no substantional inactivation occurs when the enzyme catalyzes the NADH-acceptor oxidoreductase reactions, it appears that FMN is in oxidized form during the steady-state turnover, and oxidation of NADH is the rate limiting step of the overall reaction. The titration curve shown in Fig. 5 suggests no intermediate FMN → FMN˙(H) and FMN˙(H) → FMN H_2 steps during the reductive inactivation. The possibility of FMN → FMN˙(H) cycling during the steady-state catalysis cannot be ruled out. However, due to technical reasons (high turnover number of the enzyme and relatively low sensitivity of ESR spectroscopy) it would be extremely difficult to register the free radicals during the steady-state NADH oxidation.

The vast majority of the flavoproteins bind flavin cofactors (FMN or FAD) very tightly [24] (in some enzymes FAD is covalently attached to the protein polypeptide chain [25]). This is also true for intact complex I where FMN is apparently located in a cleft which is not readily accessible for the surrounding medium [18]. The resolved NADH dehydrogenase seems to be desheathed as judged by alterations of some kinetic properties of FP compared with the original complex I [11,12,16]. The 3-subunit enzyme still retains FMN tightly bound and no exogenously added flavin is needed for the catalytic activity, although FMN is dissociated and removed when the enzyme is passed through a Florisil column [26]. Our data on irreversible inactivation of the enzyme after $FMNH_2$ dissociation are in agreement with the earlier observation of Kumar et al. [27] on a loss of the enzyme reactivity toward all electron acceptors after complete removal of FMN on DEAE-cellulose and on lack of the restoration the activity by FMN.

The molecular basis for the reduction-induced FMN dissociation remains obscure, since neither the amino-acid residues of apoprotein participating in the cofactor binding nor the subunit location of flavin in FP are known. The significant change in geometry of a butterfly-like structure of the isoalloxazine ring upon reduction [28] may contribute to the strong change in FMN affinity to its binding site.

Two points which seems to be relevant to the mechanism of FMN participation in the reaction mechanism of the NADH-ubiquinone oxidoreductase reaction catalyzed by the native enzyme merit brief discussion in light of the data presented in this report.

Much weaker binding of FMN H_2 compared with that of oxidized FMN (Scheme I) suggests that the midpoint redox potential of the couple FMN H_2/FMN bound to the enzyme is much more negative (the actual value is −325 mV, Fig. 5) compared with free flavins (−219 mV [29]). It is of interest to note that a bell-shaped titration curve with the peak at −330 mV has

been observed for iron-cluster N-3 spin coupled flavin free radical in complex I [30]. Taking the midpoint redox potential value of the enzyme bound $FMNH_2/FMN$ as low as -325 mV, two thermodynamic gaps between $FMNH_2$ and bulk ubiquinone which can provide two energy accumulation steps would be expected in NADH-ubiquinone oxidoreductase: one is between $FMNH_2$ and iron-sulfur center N-2 ($E_m < -100$ mV [31]), and the second one is between center N-2 and bulk ubiquinone. The existence of two coupling subsites in site I energy accumulation in the respiratory chain has been suggested earlier [31].

The second point to be discussed concerns the possible function of FMN as a mobile proton carrier at coupling site I, as was originally proposed by Mitchell [32] and further elaborated by several investigators [3,33,34]. When the flavin-specific binding site(s) is sheathed by other polypeptides in native complex I and dissociation of $FMNH_2$ and its removal to the surrounding medium are protected, the reduction of flavin may result in its dislocation from the FMN-site to the $FMNH_2$-site; these sites may be located on the same or different subunits. Such a redox-dependent dislocation of FMN may provide a structural basis for the functioning of the redox-driven electrogenic direct loop mechanism of proton translocation [32] or indirect proton-pump mechanism [35].

It is not clear at present whether the reductive inactivation of FP described here and NADH-induced fragmentation and subsequent inactivation of the high molecular mass NADH-dehydrogenase [10] are different appearances of the same flavine reduction-dependent mechanism. Should this be true, the latter may be considered as evidence for flavin reduction-dependent strong conformational change of the multisubunit protein. Such a conformational change seems to be a prerequisite for an operation of the redox-driven proton pump mechanism.

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