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$\vec{H}^+/2\bar{e}$ Stoichiometry of the NADH:Ubiquinone Reductase Reaction Catalyzed by Submitochondrial Particles

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Abstract—Mitochondrial NADH:ubiquinone-reductase (Complex I) catalyzes proton translocation into inside-out submitochondrial particles. Here we describe a method for determining the stoichiometric ratio $\vec{H}^+/2\bar{e}$ (***n***) for the coupled reaction of NADH oxidation by the quinone acceptors. Comparison of the initial rates of NADH oxidation and alkalinization of the surrounding medium after addition of small amounts of NADH to coupled particles in the presence of Q_1 gives the value of $\boldsymbol{n} = 4$. Thermally induced deactivation of Complex I [1, 2] results in complete inhibition of the NADH oxidase reaction but only partial inhibition of the NADH:Q_1-reductase reaction. N-Ethylmaleimide (NEM) prevents reactivation and thus completely blocks the thermally deactivated enzyme. The residual NADH:Q_1-reductase activity of the deactivated, NEM-treated enzyme is shown to be coupled with the transmembraneous proton translocation ($\boldsymbol{n} = 4$). Thus, thermally induced deactivation of Complex I as well as specific inhibitors of the endogenous ubiquinone reduction (rotenone, piericidin A) do not inhibit the proton translocating activity of the enzyme.

Key words: NADH:ubiquinone reductase, Complex I, energy transduction, respiratory chain, enzyme hysteresis (bovine heart mitochondria)

The mitochondrial proton-translocating NADH: ubiquinone-oxidoreductase (Complex I, EC 1.6.99.3, NADH-dehydrogenase, Coupling Site 1) is the most complex component of the mammalian respiratory chain. The enzyme (molecular mass ~10^6 daltons) is composed of at least 42 different polypeptides [3, 4] and bears several redox components potentially capable of electron and proton transfer: tightly (noncovalently) bound FMN [5], at least 5 iron–sulfur clusters, and at least two tightly bound ubiquinones that interact magnetically with iron–sulfur cluster N-2 when present in their semiquinone forms [6-8]. The enzyme within tightly coupled submitochondrial particles catalyzes oxidation of NADH by ubiquinone (or by its water soluble homologs or analogs) coupled with vectorial proton translocation:

$$NADH + Q + H^+ + nH^+_m \leftrightarrow NAD^+ + QH_2 + nH^+_c, \quad (1)$$

where Q and QH_2 are oxidized and reduced endogenous or exogenous quinone, respectively, H^+_m and H^+_c are protons vectorially translocated from the matrix (m) to the cytoplasm (c), and H^+ is a scalar proton on the left side of Eq. (1) (NADH donates a hydride anion and quinones accept two hydrogen atoms).

The coefficient ***n*** ($\vec{H}^+/2\bar{e}$) is an important parameter of the NADH:ubiquinone reductase reaction because any mechanistic model of the enzyme-catalyzed reaction must take into account the value of ***n***. Experimental determination of the stoichiometric coefficient ***n*** for Complex I is far from being trivial. Experimental approaches for measurement of the stoichiometry in the proton-translocating respiratory chain were developed by P. Mitchell in the 1960s [9, 10]. They are based on the registration of the transitory pH changes in anaerobic slightly buffered suspensions of mitochondria in the presence of the substrates and the K^+ ionophore valinomycin after the addition of a small amount of oxygen (oxygen "pulse" technique). A transitory acidification of the medium (ΔpH increase under conditions where $\Delta\psi$ increase is compensated by free distribution of K^+), which is due to the proton-translocating activity of the complete respiratory chain (1st, 2nd, and 3rd coupling

Abbreviations: SMP) submitochondrial particles; FCCP) carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; $\vec{H}^+/2\bar{e}$) stoichiometric coefficient of proton-translocating NADH: ubiquinone reductase reaction equal to the number of protons vectorially transferred across the membrane on oxidation of one NADH molecule; NEM) N-ethylmaleimide; Q_n) ubiquinone homolog containing *n* isoprenoid residues in the 6th position of quinone ring.

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sites), is observed after an oxygen pulse and is followed by a relatively slow pH equilibration. The latter is due to the spontaneous diffusion of protons across the inner mitochondrial membrane (no scalar protons are involved in the overall stoichiometric equations for oxidation of any substrates by oxygen resulting in H_2O formation). The $\vec{H}^+/2\bar{e}$ ratio in such experiments is then calculated as the ratio between the number of protons that appeared (amplitude of the peak of the pH change) and the amount of oxygen added. Depending on the substrate (succinate or the substrates for NAD^+-dependent dehydrogenases), the total stoichiometric coefficient ***n*** for the 2nd + 3rd or 1st + 2nd + 3rd coupling sites can thus be determined.

The stoichiometry $\vec{H}^+/2\bar{e}$ for the first coupling site (measured or calculated) reported in the literature varies from 2 to 5 [9, 11-16] (see the table in "Discussion"). A number of mechanistic models suggested for the Complex I-catalyzed proton-translocation reaction are explicitly or implicity based on these variable values, and all of them can be considered only as formal speculative schemes [12, 17, 18] (see [19] for a recent review).

Ubiquinone reduction catalyzed by Complex I in the respiratory chain is almost completely (>95%) sensitive to the specific inhibitors of the enzyme piericidin A [20] and rotenone [21]. NADH added in the presence of these inhibitors reduces all iron–sulfur clusters, whereas the electron transfer to ubiquinone is blocked [6].

An interaction between Complex I and artificial water-soluble homologs or analogs of ubiquinone is only partially (50-90% depending on the quinone acceptor and enzyme preparation) piericidin- or rotenone-sensitive [2, 21-24]. Oxidation of NADH by other artificial electron acceptors such as ferricyanide [25] or hexaammine ruthenium (III) [26] is not coupled with proton translocation and insensitive to rotenone or piericidin. Oxidative phosphorylation catalyzed by submitochondrial particles in the presence of NADH and quinone acceptors is inhibited by rotenone [27] and it is generally accepted that the inhibitor (as well as piericidin A) blocks the proton-translocating activity of Complex I; the residual rotenone-insensitive activity is believed to be analogous to uncoupled ferricyanide or hexaammine ruthenium (III) reductase reactions.

The rotenone- or piericidin A-induced inhibition, by all the parameters studied so far, is equivalent to the spontaneous enzyme deactivation, a phenomenon that was rediscovered in our laboratory in the early 1990s [1]. Its major characteristics are briefly summarized below. Mammalian Complex I always appears as a slowly equilibrating mixture of two interconvertible active and deactivated forms[1]. Only the active form can catalyze the rotenone-sensitive and NEM-insensitive forward (NADH oxidase or NADH:Q_1 reductase) and reverse ($\Delta\bar{\mu}_{H^+}$-dependent NAD^+ reduction by ubiquinol) electron transfer reaction at a constant rate (the enzyme turnover number is about $1 \cdot 10^4$ min^{-1} at 25-30°C). At temperature above 30°C in the absence of NADH (or NADPH), the active enzyme is subjected to spontaneous deactivation. The deactivated form is sensitive to NEM and it catalyzes the rotenone-sensitive reactions with a prominent lag phase. Recently, we showed that the sulfhydryl group responsible for NEM-sensitivity is located in a 15-kD subunit, which most likely belongs to the iron–sulfur enriched fraction of the enzyme [28]. The deactivated form becomes active after one (or several) "activating" turnover(s) (oxidation of NADH and slow reduction of ubiquinone). The addition of NADH to the deactivated enzyme results in reduction of all iron–sulfur clusters, whereas rapid electron transfer to ubiquinone does not occurs [29].

We recently showed that active Complex I in SMP catalyzes the NADH:Q_1 reduction coupled with translocation of 4 protons per 2 electrons transferred [30]. Unexpectedly, full proton translocating activity of the reaction was retained in the presence of rotenone. The question of whether the proton translocating activity is retained after the enzyme deactivation remained to be answered. In this paper, we will show that the residual rotenone-insensitive low quinone reductase activity of thermally deactivated enzyme is coupled with translocation of 4 protons per 2 electrons. Detailed description of the method that was used for quantifying the stoichiometry in the NADH:ubiquinone reductase region of the respiratory chain will also be presented.

MATERIALS AND METHODS

Submitochondrial particles were prepared as described [1] and stored in liquid nitrogen. Before experiments, an SMP sample was thawed and diluted to 5 mg protein per ml in 0.25 M sucrose, 0.2 mM EDTA, and 1 mg/ml bovine serum albumin (BSA). Oligomycin (0.5 μg/mg protein) was then slowly added to the well-stirred suspension. Deactivated preparation was obtained by incubation of the samples at 30°C for 30 min. To activate Complex I, NADPH (0.4 mM) was added to the suspension of SMP and the mixture was incubated at 20°C for 25 min [6] with vigorous stirring to provide aeration. Activated particles were kept on ice during the experiments.

When the NADH:quinone reductase reaction was studied, activated SMP (5 mg/ml) were preincubated with myxothiazole (1 nmol/mg protein) at 0°C for 1 h [31].

The NADH:Q_1 reductase reaction and proton translocation were measured in the standard mixture containing 0.25 M sucrose, 3 mM Tris-HCl (pH 8.0), 0.2 mM EDTA, 0.1 mM Q_1, and BSA (1 mg/ml). Phenol Red (30 μM)

[1] We use the term "deactivated" to describe the unusual catalytically inactive form of Complex I [1]. This is somehow equivalent to the widely used term "resting enzyme" and is intended to discriminate between the usual "inactive enzyme" and that which is converted to the catalytically active form under certain conditions.

was added (a non-permeable pH indicator [32]) for quantitative registration of external pH changes. The internal pH changes were qualitatively registered in the presence of 90 μM Neutral Red [33] in highly buffered medium. Rapid changes of optical densities (time constant ≤1 sec) in turbid samples (SMP suspensions) were detected using simple homemade dual wavelength photometer reconstructed from an FEC-60 photocolorimeter produced by Zagorsky (Sergievo-Posadsky) Optico-Mechanical Factory (in the 1970s). Since optical instrumentation for biochemical studies is difficult for most Russian laboratories to afford and our photometer is superior in a number of parameters over expensive imported models (for example, Hitachi-557), we consider it worthwhile to give a brief description of our modification. The diagram of the modified photometer is shown in Fig. 1. The round rotating scale was detached from the original instrument and a cuvette compartment (*1*) made from aluminum plates was attached to the front wall after a rectangular slot was cut out in the original cover. A projecting prism was substituted for two mirrors (*2*) allowing independent projection of two light beams on a single cuvette containing the sample. The cuvette holder (*3*) of the standard colorimeter cuvette (optical path 0.5 cm) (*4*) was equipped with a diaphragm (*7*) (1 × 1.5 cm). The F-22 (350-700 nm region) or F-23 (600-1100 nm region) photocell (*5*) was fitted to the front wall of the cuvette. The sample content was constantly stirred during the experiments by a light plastic rod (*6*) fixed on a vibrating plate (*9*) of an electromagnetic commutator (approximately 90 V, 50 Hz). The tiny tip of a glass tube (*8*) was placed just above the surface of the cuvette and a slight constant flow of argon was maintained to prevent base line drift due to CO_2 absorption from air at low buffer capacity at pH ≥ 6. The stabilized voltage (+280 V) anode feeding of the parent photometer was used for the photocell. Alternating current (modulation of light by a rotating disc at frequency 350 Hz) in the resistor load (the difference between the intensities of two light-induced signals) was recorded after proper amplification and phase-sensitive detection. The different monochromatic light in the two optical channels was selected by a pair of interference filters (optical width ~5 nm) placed in two almost parallel beams. The output voltage (the difference between light absorption at the two different wavelengths) was monitored by a Kipp & Zonen BD 111 recorder. The overall time constant for the measurements determined by the manual addition of dyes to the cuvette from a standard Hamilton syringe was <0.5 sec. The modified photometer as described has been used in our laboratory for more than 10 years for highly sensitive (signal/noise ratio ~20-100 for optical density changes of ~0.01 unit) measurements of reductase activities (succinate and NADH dehydrogenases), Ca^{2+} transport (murexide or Arsenazo as metallochromic indicators), and several ATPase reactions accompanied by H^+-change (pH indicators).

The measuring scales were calibrated by the addition of small known amounts of HCl (when pH was measured) or NADPH (when NADH oxidation was measured) (note that at pH > 6, NADPH oxidation by Complex I is extremely slow). The wavelengths used were: for Phenol Red response, 555-620 nm; for Neutral Red response, 522-702 nm; for NADH, 366-405 nm. A filter with maximal transmission at 366 nm was used in the latter case because of the glass optics and light source (regular tungsten lamp). Although the molar absorption index for NAD(P)H at 366 nm is substantially less than at 340 nm, the signal/noise ratio for the indicated wavelength pair (366-405 nm) was high enough for reliable measurements of 3-10 μM concentrations of the reduced nucleotides.

Protein content was determined with biuret reagent [34].

NADH, NADPH, Q_1, myxothiazol, rotenone, N-ethylmaleimide, oligomycin, valinomycin, gramicidin D,

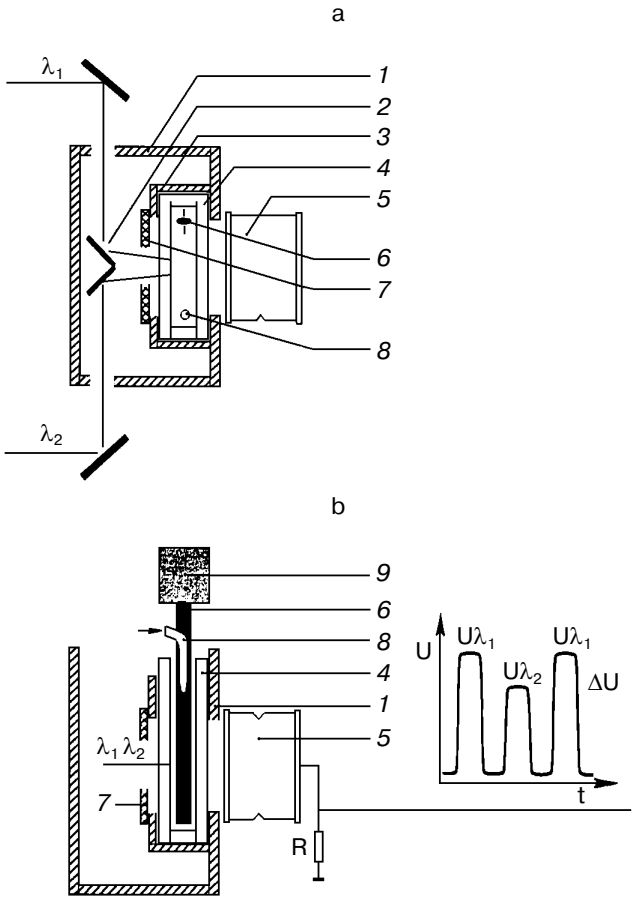

Fig. 1. Schematic diagram of the photometer construction. a) Front view; b) side view. ΔU shown in the voltage–time diagram for the load resistor (R) is proportional to the difference between the light intensities of the particular wavelength pair. See text for the detailed description.

FCCP, BSA, Tris, EDTA, Phenol Red, Neutral Red, and palmitoyl-CoA were from Sigma (USA). Other chemicals were of the purest grade commercially available.

RESULTS

$\vec{H}^+/2\bar{e}$ coefficient for Complex I. SMP routinely prepared in this laboratory show high respiratory control ratio after treatment with oligomycin (0.5 μg/mg protein) in the NADH oxidase (5.0-8.0), succinate oxidase (2.5-3.0), and NADH:Q_1 reductase (2.5-3.5) reactions. The preparations show negligible NADPH oxidase or NADH oxidase activity in the presence of rotenone or myxothiazol (Fig. 2).

The proton-translocating activity of the SMP was monitored by the pH-indicator responses (as described in the previous section). The spectral characteristics of the indicators used are shown in Fig. 3. The absorption changes at the wavelengths pairs used are linearly dependent on the amount of H^+ added within the range of 6.0-8.0 (Neutral Red) and 7.0-9.0 (Phenol Red).

The proton translocating activity measurement requires high affinity of at least one of the substrates to the enzyme under investigation, such as that of oxygen to

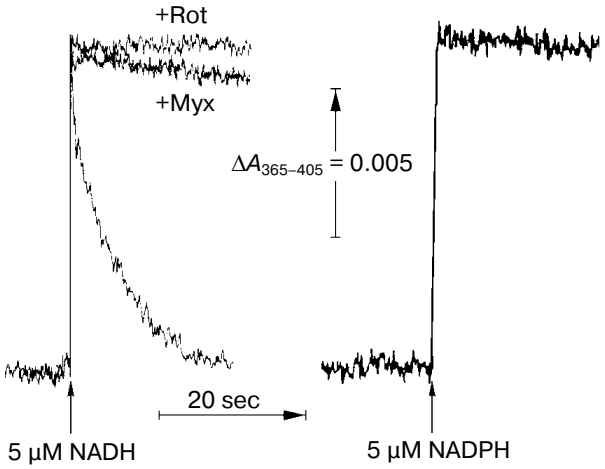

Fig. 2. Oxidation of NADH or NADPH by submitochondrial particles. Oxidation was monitored as described in "Materials and Methods". The reaction mixture contained 0.25 M sucrose, 3 mM Tris-HCl (pH 8.0), 0.2 mM EDTA, bovine serum albumin (1 mg/ml), and 150 μl of stock suspension of SMP (5 mg/ml). The reaction was started by addition of 5 μM NADH or NADPH. Rotenone (Rot, 25 μM) or myxothiazol (Myx) were present where indicated. SMP (5 mg/ml) were preincubated with myxothiazol (1 nmol/mg protein) at 0°C for 1 h.

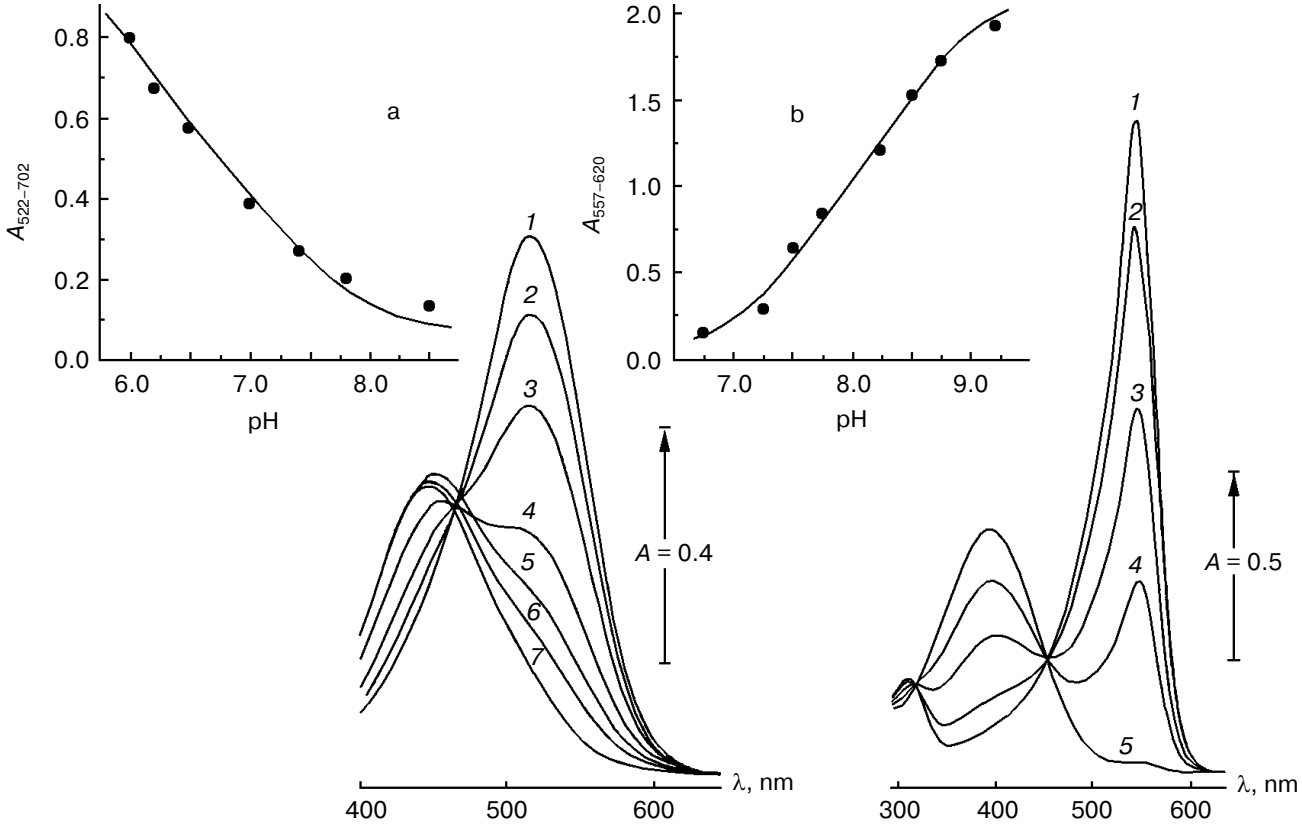

Fig. 3. Optical characteristics of Neutral Red (a) and Phenol Red (b). a) Absorption spectra of 60 μM Neutral Red in 0.25 M sucrose, 50 mM Tris-HCl, 0.2 mM EDTA at pH 6.0 (*1*), 6.2 (*2*), 6.5 (*3*), 7.0 (*4*), 7.4 (*5*), 7.8 (*6*), and 8.6 (*7*). b) Absorption spectra of 30 μM Phenol Red in the same medium at pH 8.8 (*1*), 8.3 (*2*), 7.8 (*3*), 7.3 (*4*), and 6.5 (*5*). Inserts: dependences of absorption differences on pH.

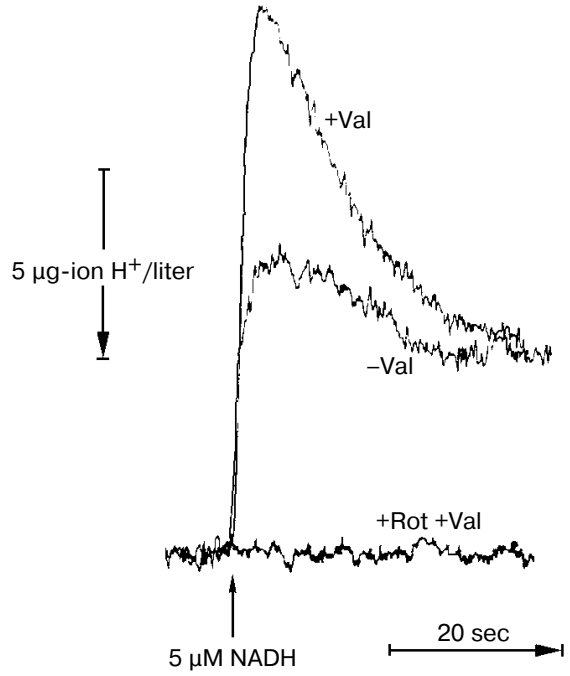

Fig. 4. Proton translocation in the NADH oxidase reaction. SMP were activated as described in "Materials and Methods". Activated SMP (0.5 mg/ml) were placed in a mixture (2 ml) containing 0.25 M sucrose, 3 mM Tris-HCl (pH 8.0), 20 mM KCl, 0.2 mM EDTA, BSA (1 mg/ml), and 30 μM Phenol Red. The reactions were initiated by the addition of 5 μM NADH. Valinomycin (Val, 6 μM) and rotenone (Rot, 25 μM) were added where indicated.

cytochrome *c* oxidase. If this condition does not hold, the low substrate concentration ($\leq K_m$) would not provide sufficient enzyme activity for neglecting the spontaneous conductivity of the coupling membrane for protons. The K_m for NADH in completely activated Complex I is 1-3 μM [35]; this allows use of a "substrate electron donor pulse" in the presence of substrate acceptor excess instead of traditionally used "substrate acceptor pulse" (oxygen, cytochrome *c*) in the presence of electron donor excess (succinate, NAD^+-dependent dehydrogenases substrates). The other prerequisite is a compensation of the electric potential difference ($\Delta\psi$) resulting from the vectorial proton movement by some antiport process such as charged K^+–valinomycin complex movement.

Figure 4 shows hydrogen ion change in the medium after the addition of a small amount of NADH to an aerobic suspension of fully activated SMP. The addition of valinomycin resulted in a significant increase of the pH change amplitude, as expected. It should be noted that when NADH is used as the substrate, the high respiratory control ratio under steady-state conditions (100-200 μM NADH) were seen only in the presence of BSA (1 mg/ml). The coupling effect of BSA is apparently due to some unknown non-trivial reasons, such as free fatty acid and other endogenous uncoupler binding. The details of coupling and other effects of BSA on the activity of Complex I will be reported elsewhere.

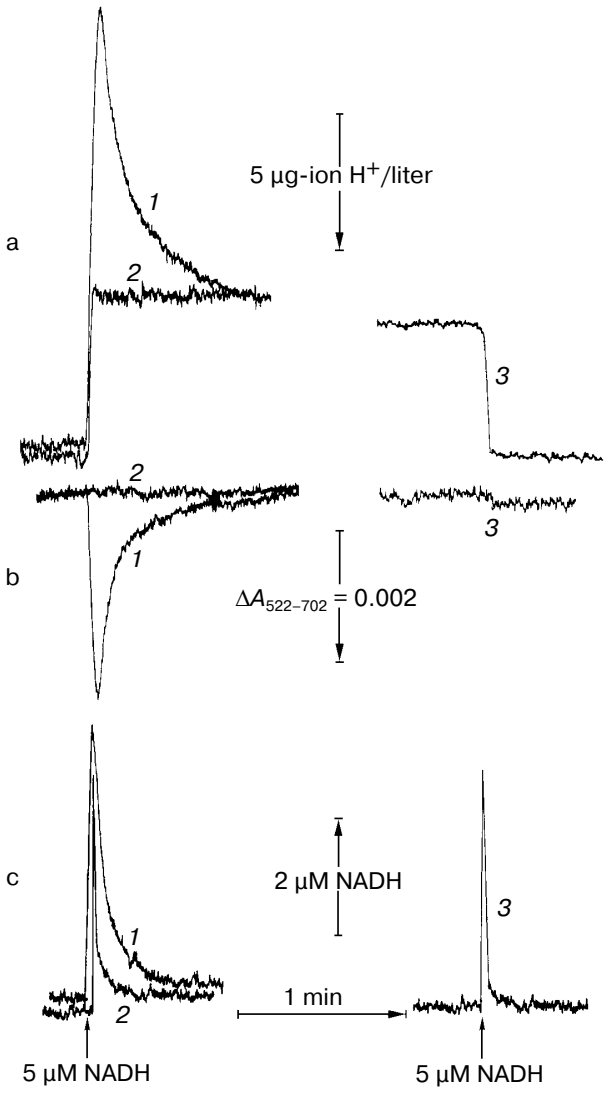

Fig. 5. Oxidation of NADH and vectorial proton translocation catalyzed by activated Complex I in submitochondrial particles. Activated SMP (5 mg/ml) were preincubated with myxothiazol (1 nmol/mg of protein) at 0°C for 1 h. a) pH changes. SMP (0.375 mg/ml) were placed in reaction mixture containing 0.25 M sucrose, 3 mM Tris-HCl (pH 8.0), 0.2 mM EDTA, BSA (1 mg/ml), 30 μM Phenol Red, and 100 μM Q_1 (curves *1* and *2*) or 100 μM ferricyanide (curve *3*). The reaction was initiated by addition of 5 μM NADH. FCCP (17 μM) was added to the mixture (curve *2*). b) Internal pH changes as followed by the response of 90 μM Neutral Red. The Neutral Red was added to the standard reaction mixture (see (a)) except that 50 mM Tris-HCl was present to increase the buffer capacity of the external medium to eliminate the pH-dependent response of the indicator outside the SMP. c) NADH oxidation. Other conditions, as described in (a).

Figure 5 shows a representative example of the proton-translocating activity of Complex I in NADH:Q_1 reductase reaction. The addition of 5 μM NADH resulted in rapid alkalinization of the medium (Fig. 5a, curve *1*), which was due to H^+ translocation into the inner vesicular space, followed by relatively slow decay of the pH to a constant level different from the initial level due to scalar proton consumption (Eq. (1)). Rapid proton uptake is accompanied by synchronous acidification of the inner space as evident from the Neutral Red response (Fig. 5b, curve *1*). Linear calibration of the indicator response in the medium (Phenol Red, Fig. 3) allowed quantification of the proton concentration outside, whereas the inside pH change is difficult to quantify (low and unknown inner space buffer capacity, no direct calibration of the indicator response). The pH changes observed were uncoupler-sensitive (Fig. 5, a and b, curves *2*). No internal pH change was observed in the presence of FCCP or gramicidin, and the final outside pH change (scalar H^+, Eq. (1)) was the same in the presence of the uncouplers. The number of protons consumed in the presence of the uncouplers was equal to that observed after complete equilibration of the external and internal pH (Fig. 5, a and b, curves *1*). The external alkalinization was always strictly proportional to the amount of NADH added. No external Neutral Red response was seen in the presence of the uncouplers because of the high buffer capacity of the reaction mixture that was employed when the Neutral Red response was followed. In contrast to NADH oxidase (Fig. 4), the proton-translocating activity of the NADH:Q_1 reductase reaction was not affected by valinomycin. At present, we have no plausible explanation for this unexpected finding. Formally, this observation suggests that under the conditions employed the coupling membrane has high conductivity for K^+ or other ions. We suspect that this may be due to the presence of Q_1 and albumin in the assay system; however, detailed studies of this phenomenon is beyond the scope of this paper.

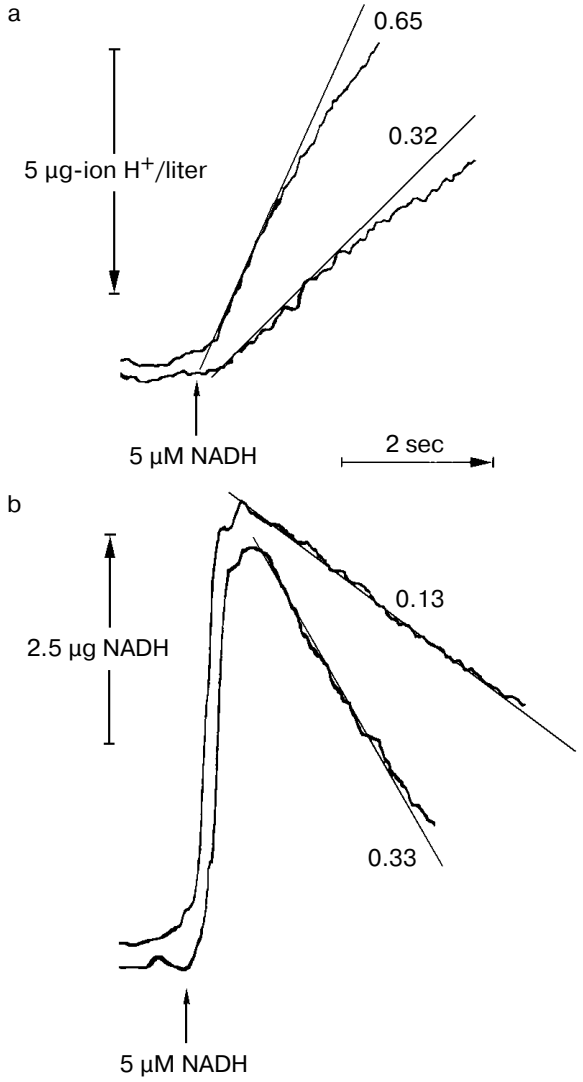

Fig. 6. Time course of oxidation of NADH and proton translocation at higher time resolution. a) pH change in the external medium; b) NADH oxidation. The experimental conditions were as described in Figs. 5a and 5c, respectively. Figures on the curves indicate the initial rates (μmoles or μg-ions/min per mg protein).

Figure 5c shows the synchronous registration of NADH oxidation. It worth mentioning that when the pH change reached its maximum, only about two-thirds of the NADH was oxidized. The rate of NADH oxidation was increased 2.5-3.5 times in presence of the uncoupler (Fig. 5c, curve *2*). The addition of 10 μM palmitoyl-CoA to inhibit possible transhydrogenase activity did not change the reaction pattern (data not shown).

It is known that no $\Delta\bar{\mu}_{H^+}$ generation occurs during the NADH:ferricyanide or NADH:hexaammine ruthenium (III) reductase reactions [25, 26]. The pH changes in the course of NADH oxidation by ferricyanide is shown in Fig. 5 (right panel). Acidification of the external medium (uncoupler insensitive) was evidently due to release of one scalar proton according to Eq. (2):

$$NADH + 2Fe(CN)_6^{3-} \leftrightarrow NAD^+ + 2Fe(CN)_6^{4-} + H^+. \quad (2)$$

The initial rates of NADH oxidation and external medium alkalinization were measured for calculation of the stoichiometric coefficient ***n*** for the vectorial proton translocation according to the following expression:

$$\boldsymbol{n}\ (\vec{H}^+/2\bar{e}) = (\nu_{H^+}/\nu_{NADH}) - 1, \quad (3)$$

where ν_{H^+} stands for the initial rate of the overall proton consumption, ν_{NADH} stands for the initial rate of NADH oxidation, and 1 in right part of Eq. (3) corresponds to disappearance of the scalar proton.

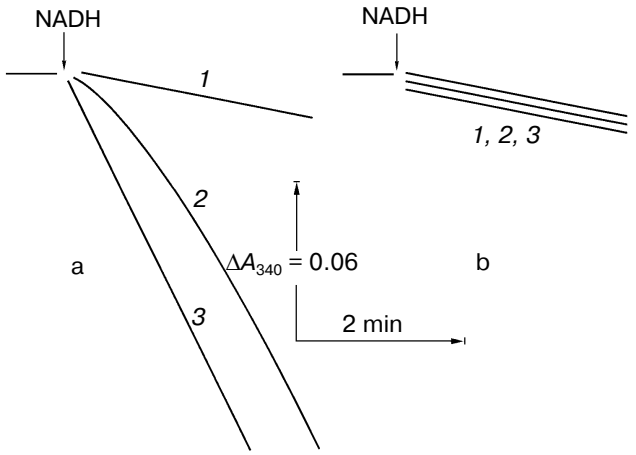

Fig. 7. Time course of NADH oxidation by the active and deactivated Complex I in myxothiazol-treated submitochondrial particles. SMP (5 mg/ml) were treated as described in "Materials and Methods" and assayed in standard reaction mixture containing 100 μM Q_1 and 0.2 μM FCCP. The reaction was initiated by addition of 100 μM NADH (indicated by arrows). a: *2*) deactivated SMP; *1*) de-activated SMP treated with 1 mM NEM (5 min, 20°C); *3*) as curve *2* but 5 μM NADH was oxidized for enzyme activation before assay; b) same as in Fig. 7a but 25 μM rotenone was present.

Figure 6 demonstrates the synchronous registration of NADH oxidation (lower part) and H^+ consumption (upper part) at better time resolution. The $\vec{H}^+/2\bar{e}$ ratio calculated according Eq. (3) was 4. This ratio remains the same when the initial rate of NADH oxidation was decreased by the competitive inhibitor ADP-ribose [35] (data not shown).

Characteristics of active and deactivated Complex I in the NADH:Q_1 reductase reaction. Figure 7a demonstrates the time course of the NADH:Q_1 reductase reaction catalyzed by differently treated Complex I preparations. After 30 min exposure of the particles at 30°C, complex I catalyzed the rotenone-sensitive reaction with a considerable lag phase (curve *2*). Preincubation of the deactivated preparation with 5 μM NADH and 100 μM Q_1 results in disappearance of the lag phase: the preparation thus treated catalyzed NADH oxidation at constant rate (Fig. 7a, curve *3*). When thermally deactivated preparation was further treated by NEM its NADH oxidase activity was lost, but small, rotenone-insensitive NADH:Q_1 reductase activity was still present (Fig. 7, a and b, curves *1*). Remarkably, the rotenone-insensitive fractions of the total activity were the same for both active and deactivated enzyme preparations (Fig. 7b). It was of interest to determine whether deactivated enzyme is capable of proton translocation.

The residual rotenone-insensitive NADH:Q_1 reductase activity of SMP is accounted for about 10% of the original uncoupled oxidation rate (or about 30% of that for the coupled reaction of NADH oxidation by Q_1). To prevent possible reactivation of the thermally deactivated enzyme during the assays, the preparations were treated with NEM. The time course of NADH oxidation and alkalinization of the medium for the deactivated, NEM-treated SMP is shown in Fig. 8. Both the NADH oxidation and proton consumption rates were significantly decreased; however the $\vec{H}^+/2\bar{e}$ coefficient determined as the ratio between the corresponding initial rates remained the same (4). Rotenone did not affect the pattern of the reactions shown in Fig. 8.

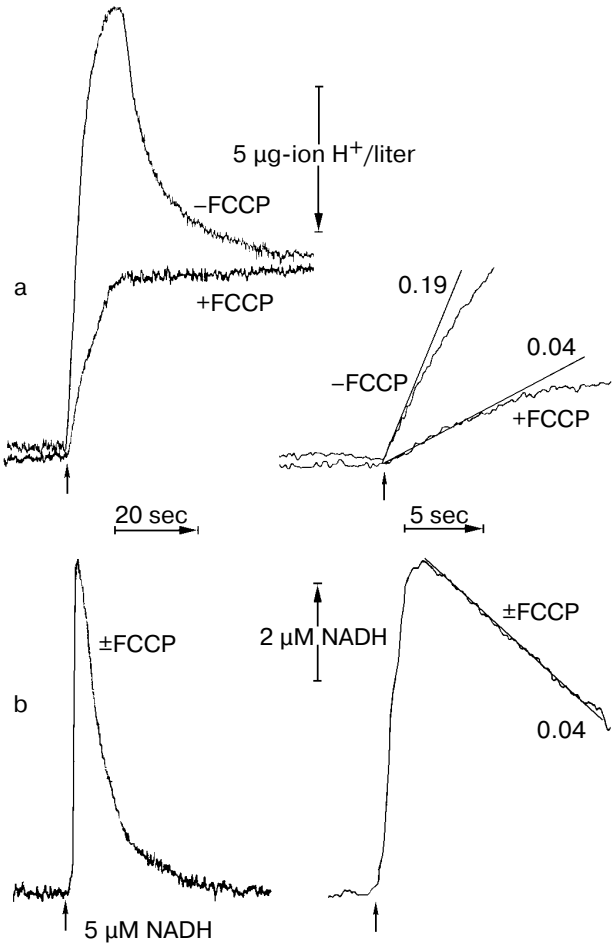

Fig. 8. Proton translocation by deactivated Complex I. SMP (5 mg/ml) were deactivated and treated with myxothiazol (1 nmole/mg protein) as described in "Materials and Methods". NEM (1 mM) was added and the suspension was further incubated for 5 min at 20°C. a, b) Proton translocation and NADH oxidation, respectively (for details, see the legends to Figs. 5 and 6). The oxidation of NADH was insensitive to 17 μM FCCP.

$\vec{H}^+/2\bar{e}$ stoichiometric coefficient (***n***) for the NADH:ubiquinone reductase reaction*

Preparation	Method used	Electron donor or acceptor**	Calculation method***	***n***	Reference
Escherichia coli cells	pH-sensitive electrode	DMSO	*A*	3	[37]
Bovine heart mitochondria	same	Q_1	*B*	2	[12]
Bovine heart submitochondrial particles	same	NADH	*B*	2	[13]
Complex I containing proteoliposomes	same	NADH	*B*	1.4	[13]
Rat liver mitochondria	spectrophotometry (pH indicator)	Q_1	*C*	4	[38]

* No data are included for those studies where the Site-1 stoichiometry was determined as the difference between coupling Sites 1 + 2 + 3 and 2 + 3 [10, 11, 41] or between Sites 1 + 2 and 2 [14, 39] or by other indirect methods [40].

** The substrate used to initiate the reaction.

*** *A*, linear extrapolation to zero time; *B*, H^+ release amplitude; *C*, ratio of initial rates of electron and proton transfer.

DISCUSSION

The methodology for the stoichiometry measurements originally introduced by Mitchell [9, 10] has been used and further developed in a number of other studies [11-14]. Several preparations have been employed to determine of the $\vec{H}^+/2\bar{e}$ stoichiometry of Complex I: prokaryotic cells [36, 37], intact mitochondria [12, 14], submitochondrial particles [11], and Complex I-containing proteoliposomes [13]. NADH (SMP [11], proteoliposomes [13]) or artificial acceptors oxidizing endogenous quinones (intact mitochondria [12, 38], cells [36, 37]) were used to initiate vectorial proton translocation. The stoichiometries determined in these studies have been extrapolated to variable values ranging from 2 to 4 (see table), and the generally accepted value of 4 [4] (although never directly measured) has been widely circulated in a number of recent reviews.

Intact mitochondria and cells are advantageous over other preparations because of relatively large highly buffered internal volumes and intactness of the coupling membrane. Their major disadvantage is that the inner mitochondrial or plasma membranes are not permeable for NADH, which makes it impossible to use the natural substrate of the reaction. Substrates of NAD^+-dependent dehydrogenases (endogenous and exogenous) were used as the electron donors. Both NAD and NADP were reduced before the addition of an electron acceptor under such experimental conditions. The presence of NADPH and NAD^+ is expected to activate the proton-translocating transhydrogenase reaction. Also, the presence of $\Delta\bar{\mu}_{H^+}$-dependent substrate translocases in the inner mitochondrial or plasma membranes may lead to some errors in the experimentally found stoichiometry. When mitochondria are incubated under deenergized conditions (before oxidant pulse), several endogenous ions are expected to move outside, and after energization the accumulation of previously released species (either via symport or antiport mechanisms) may significantly contribute to the observed proton movement [41, 42]. The ideal system for the stoichiometry measurement would be proteoliposomes containing fully active Complex I oriented in the coupling membrane as it is in intact mitochondria. No such preparations are available yet, and Complex I-containing proteoliposomes [13, 25] can be and have been used for qualitative demonstration of proton-translocating activity rather than for quantitative measurements.

The SMP used in the present study catalyze rapid (no lag phase) oxidation of NADH. To our knowledge, there is only one report in the literature where SMP were used to determine the Complex I proton translocation stoichiometry [11]. This is apparently because most preparations of inside-out SMP are loosely coupled and respond slowly to the addition of NADH [1].

In contrast to our approach, the ratio of H^+ ions released or consumed to the amount of the substrate added was used in most studies on quantification of the stoichiometry. The peak amplitude in the time course of pH changes induced by the substrate addition was taken as such to measure translocated H^+ [12, 13], or this value was quantified by extrapolation of the decay curve, presumed to be exponential, to zero time [10, 11, 42]. The direct comparison of NADH oxidation and H^+ consumption (Fig. 5) showed that a considerable amount of NADH was present at the moment when the pH change curve reached its maximum. This suggests that neither directly seen maximal amount of H^+ consumed nor the

extrapolated value can be used for reliable calculation of the actual stoichiometry.

The data presented here and in our previous short report [30] seem to provide, for the first time, the experimentally determined $\vec{H}^+/2\bar{e}$ stoichiometry for Complex I. It should be noted that a similar approach was used by Azzone's group using intact rat liver mitochondria [38]. We believe, however, that the consensus value of 4 for the Complex I catalyzed proton translocation stoichiometry was due to superposition of some overestimations (transhydrogenase contribution) and underestimations (secondary ion movement).

Interestingly, deactivated Complex I was demonstrated to be fully capable of the proton-translocating activity, although the reoxidation of the enzyme by endogenous ubiquinone was completely blocked (Fig. 8). We speculated previously that deactivated Complex I under certain conditions may provide a leakage of the coupling mitochondrial membrane [43]. The results presented here do not support this hypothesis. With the water-soluble ubiquinone homolog Q_1 as electron acceptor, completely deactivated Complex I is fully coupled.

All the properties of the rotenone-inhibited and thermally deactivated Complex I studied so far are identical. Thus, we believe that in both cases one of the redox components of the enzyme becomes accessible for the externally added Q_1. Most likely this component is tightly bound ubisemiquinone previously postulated to serve as the electron and proton carrier [17].

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