\[ \frac{\text{H}^+}{2e^-} \text{ Stoichiometry of the NADH:Ubiquinone Reductase Reaction Catalyzed by Submitochondrial Particles} \]

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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 \( \frac{\text{H}^+}{2e^-} \) (\( n \)) 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 \( n = 4 \). 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 transmembrane proton translocation (\( n = 4 \)). 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 \( \sim 10^6 \text{ daltons} \)) 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:

\[
\text{NADH} + Q + H^+ + nH_m^+ \leftrightarrow \text{NAD}^+ + QH_2 + nH_c^+, \quad (1)
\]

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 \( n (\frac{\text{H}^+}{2e^-}) \) 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 (\( \Delta \text{pH} \) 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
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 H₂O formation). The H⁺/2e⁻ 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 H⁺/2e⁻ 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 implicitly 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 watersoluble 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 absence 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 hexaaammine 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. Only the active form can catalyze the rotenone-sensitive and NEM-insensitive forward (NADH oxidase or NADH:Q₁ reductase) and reverse (ΔH⁺/2e⁻-dependent NAD⁺ reduction by ubiquinol) electron transfer reaction at a constant rate (the enzyme turnover number is about 1·10⁴ 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:Q₁ 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 of the reaction was 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:Q₁ 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 Q₁, and BSA (1 mg/ml). Phenol Red (30 μM)