$\rightarrow H^+/2\bar{e}$ stoichiometry in NADH-quinone reductase reactions catalyzed by bovine heart submitochondrial particles

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Abstract Tightly coupled bovine heart submitochondrial particles treated to activate complex I and to block ubiquinol oxidation were capable of rapid uncoupler-sensitive inside-directed proton translocation when a limited amount of NADH was oxidized by the exogenous ubiquinone homologue Q_1. External alkalization, internal acidification and NADH oxidation were followed by the rapidly responding ($t_{1/2} \leq 1$ s) spectrophotometric technique. Quantitation of the initial rates of NADH oxidation and external H^+ decrease resulted in a stoichiometric ratio of 4 H^+ vectorially translocated per 1 NADH oxidized at pH 8.0. ADP-ribose, a competitive inhibitor of the NADH binding site decreased the rates of proton translocation and NADH oxidation without affecting $\rightarrow H^+/2\bar{e}$ stoichiometry. Rotenone, piericidin and thermal deactivation of complex I completely prevented NADH-induced proton translocation in the NADH-endogenous ubiquinone reductase reaction. NADH-exogenous Q_1 reductase activity was only partially prevented by rotenone. The residual rotenone- (or piericidin-) insensitive NADH-exogenous Q_1 reductase activity was found to be coupled with vectorial uncoupler-sensitive proton translocation showing the same $\rightarrow H^+/2\bar{e}$ stoichiometry of 4. It is concluded that the transfer of two electrons from NADH to the Q_1-reactive intermediate located before the rotenone-sensitive step is coupled with translocation of 4 H^+.

Key words: NADH-ubiquinone oxidoreductase; Complex I; Energy transduction; Respiratory chain; Bovine heart mitochondrion

1. Introduction

The mitochondrial NADH-ubiquinone oxidoreductase (EC 1.6.5.3, NADH dehydrogenase, complex I, coupling site 1) catalyzes the energy-transducing reaction:

$$NADH + Q + H^+ + \boldsymbol{n}\, H^+_m \leftrightarrow NAD^+ + QH_2 + \boldsymbol{n}\, H^+_c \qquad (1)$$

where Q is either endogenous ubiquinone-10 or added quinone homologue (analogue) and $\boldsymbol{n}\, H^+_m$ and $\boldsymbol{n}\, H^+_c$ are $\boldsymbol{n}$ protons vectorially translocated from matrix (m) to cytoplasm (c) to build up $\Delta\bar{\mu}_{H^+}$ across the inner mitochondrial membrane. The mammalian enzyme is an extremely complex multisubunit component of the respiratory chain and neither the sequence of electron transfer nor the mechanism of proton translocation are known (see [1] for most recent reviews). Numerous working hypotheses on the redox-dependent vectorial proton translocation mechanism, which included FMN [2], iron-sulfur center N-2 [3], ubiquinone [4], a combination of FMN and iron-sulfur centers [5], and a combination of FMN, iron-sulfur center and ubiquinone [6] as the proton carriers, have been proposed; all of them remain purely speculative. Knowledge of the stoichiometric coefficient $\boldsymbol{n}$ in Eq. 1 is of crucial importance for any mechanistic model of chemiosmotic energy transduction [2] and tremendous efforts have been undertaken to determine the number of H^+ or charges translocated per $2\bar{e}$ transferred through the coupling sites in the mammalian respiratory chain. The experimental determination of the $\rightarrow H^+/2\bar{e}$ ratio for any coupling site and especially for complex I is far from being trivial. The direct measurement of proton translocation by the 'pulse' technique as originally introduced by Mitchell and Moyle [7] consists of additions of limited oxygen to anaerobic mitochondria resulting in a burst of protons in the surrounding medium which can be monitored by a pH-sensitive electrode. Depending on the substrate reductant (succinate in the presence of rotenone, or NAD-linked substrates) the total number of protons translocated per pair of electrons transferred at sites 1+2+3 or 2+3 can be quantitated as the $\rightarrow H^+/O$ ratio. The values obtained by this approach can be easily obscured by the secondary rapid movements of charged species such as inorganic phosphate and/or Ca^{2+} [8]. Further development of the oxygen or reductant 'pulse' technique as applied for intact mitochondria [9,10], submitochondrial particles [11], reconstituted cytochrome oxidase [12] and complex III [13] containing proteoliposomes have led to the establishment of charge/$2\bar{e}$ ratios of 2 and 4 for the coupling sites 2 and 3, respectively.

The oxidant 'pulse' method with duroquinone, Q_1 and Q_2 as electron acceptors has been applied for intact rat liver and bovine heart mitochondria [10]. The experimentally observed $\rightarrow H^+/2\bar{e}$ values of 0.75 and 1.75 for liver and heart mitochondria, respectively, were interpreted as suggesting the actual site 1 $\boldsymbol{n}$ value of 2 [10]. The same value was found in submitochondrial particles and purified reconstituted complex I vesicles [14] using a 'pulse' of NADH in the presence of Q_1 as electron acceptor. The value of 2 was also suggested by the elegant studies of Brand et al. who introduced a new steady-state method for quantitation of unequal charge separation by different coupling sites [15]. They found that numbers of H^+ translocated with isoascorbate, succinate and β-hydroxybutyrate as the substrates corresponded to 4:6:8 which is consistent with models where complex I functions as a proton pump (or loop) with a $\rightarrow 2H^+/2\bar{e}$ stoichiometry of 2 [15]. A compar-

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Abbreviations: PhR and NR, pH indicators phenol red and neutral red, respectively; FCCP, carbonyl cyanide *p*-(trifluoromethoxy) phenyl-hydrazone; BSA, bovine serum albumin; DB, 2,3-dimethoxy-5-methyl-6-decylbenzoquinone; Q_n, homologues of ubiquinone having *n* isoprenoid units in position 6 of the quinone ring; NEM, *N*-ethylmaleimide

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ison of the stoichiometries at coupling sites 1 and 2 in rat liver mitochondria led Pozzan et al. to conclude that the $\rightarrow H^+/2\bar{e}$ ratio for site 1 is 4 [16]. The latter value was confirmed by Wikström who measured the extents of intramitochondrial alkalization and electrical membrane potential ($\Delta\psi$) with succinate and β-hydroxybutyrate as a function of amounts of ferricyanide added as oxidant 'pulse' [17].

Our inspection of the literature showed that the $\rightarrow H^+/2\bar{e}$ stoichiometry for the NADH-ubiquinone reductase region of the respiratory chain remains controversial and it would be important for further development in the field to measure n value in Eq. 1.

In this report we will present data showing that tightly coupled submitochondrial particles treated with activate NADH oxidation catalyze the NADH-quinone reductase reaction with a stoichiometry $\rightarrow H^+/2\bar{e}$ of 4. Both NADH oxidation by endogenous ubiquinone and proton translocation coupled with this reaction are completely prevented by rotenone. NADH oxidation by exogenous Q_1 is only partially rotenone-sensitive. With exogenous Q_1 as electron acceptor the residual rotenone-insensitive NADH-Q_1 reductase reaction is coupled with proton translocation and shows the same $\rightarrow H^+/2\bar{e}$ stoichiometry of 4.

2. Materials and methods

Submitochondrial particles artificially coupled by oligomycin (respiratory control ratio in the NADH oxidase assay 7–10) were prepared as described [18] and stored as a suspension (40–50 mg/ml) in liquid nitrogen. To avoid a sluggish response to NADH (lag phase in the NADH-quinone reductase activities) [19] the particles were activated by preincubation with NADPH. The samples were thawed and diluted to a protein content of 5 mg/ml with 0.25 M sucrose, 0.2 mM potassium EDTA, and BSA (1 mg/ml), pH 8.0. 0.4 mM NADPH was added and the suspension was incubated for 25 min at 20°C. Myxothiazol (1 nmol/mg protein) was then added and the mixture was further incubated before any measurements for 1 h in ice.

All NADH-dependent proton translocating activities were measured in a simple double-wavelength photometer specially designed for sensitive pH registration in light-scattering samples. The wavelength pairs were selected by the interference filters (full width-half maximum of 10 nm). The light beams were modulated (350 Hz) by a rotating shutter. The cuvette (2 ml, 5 mm optical path), equipped with a continuously operating mixing device (vibrating plastic rod), was placed close ($\approx$2 mm) to the window of the photomultiplier. Alternating photocurrent was amplified, directed by a phase-sensitive detector and the signal was recorded on a Kipp and Zonen BD111 paper recorder. A time constant for the instrument response (including mixing) was $\leq$1 s. A gentle stream of argon gas was continuously applied on the open surface of the reaction cuvette to avoid pH drift caused by CO_2. All the experiments were done at room temperature (18–20°C). The reactions were started by rapid injections of the reagents (prepared as concentrated solutions in complete reaction mixture) from Hamilton syringes. The linear instrument responses were calibrated by the addition of analytically titrated HCl (indicator responses) or NADPH (NADH response).

Phenol red (PhR) (30 μM) was used as external pH indicator [20] in the standard reaction mixture containing: 0.25 M sucrose, 3 mM Tris-Cl^-, 0.2 mM potassium EDTA, and BSA (1 mg/ml) at pH 8.0. Neutral red (NR) (90 μM) was used as internal pH indicator in the same reaction mixture except for a higher Tris-Cl^- concentration (50 mM) to increase the buffer capacity and to avoid the indicator response to the external protons [21]. The following wavelength pairs were used: PhR response (external pH): 555–620 nm; NR response (internal pH): 522–702 nm; NADH oxidation: 366–405 nm.

NADH, NADPH, rotenone, myxothiazol, FCCP, BSA, and ADP-ribose were from Sigma. Q_1 was kindly provided by Esai Co. (Japan). DB was a kind gift from Dr. H. Weiss (Dusseldorf, Germany). The purities of quinones were checked by thin-layer chromatography. Other chemicals were of the highest purity commercially available.

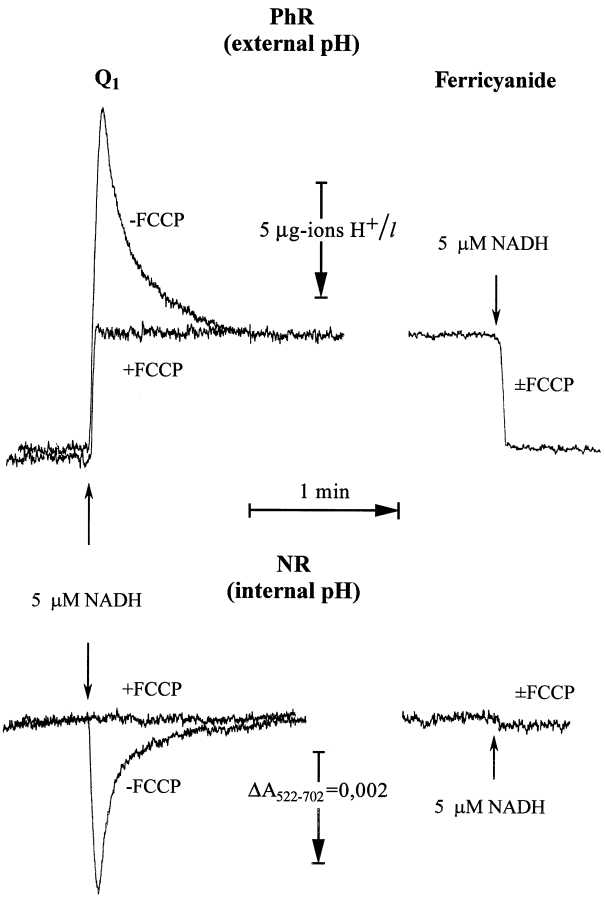

Fig. 1. NADH-induced proton translocation in submitochondrial particles. Activated particles (0.4 mg/ml) treated as described in Section 2 were suspended in the standard reaction mixture; 100 μM Q_1 or 100 μM potassium ferricyanide was added as the electron acceptor for complex I. The concentration of FCCP was 17 μM. The additions of 6 μM valinomycin or 6 μM valinomycin plus 20 mM KCl did not significantly change the reaction patterns.

3. Results

Fig. 1 shows the results of typical experiments where H^+ changes in medium (PhR response, upper panel) and internal acidification (NR response, lower panel) after the addition of NADH were recorded. Rapid proton consumption from the medium accompanied by synchronous acidification of the intravesicle space followed by relatively slow equilibration were observed when a limited amount of NADH was oxidized by externally added Q_1. In the presence of FCCP the internal acidification as followed by the NR response was completely abolished as well as the external alkalization burst (PhR response). Although the NR response perfectly corresponded qualitatively to the outer pH changes, the quantitation of internal acidification was difficult and was not used for further measurements. The amount of H^+ consumed during the reaction in the presence of uncoupler was exactly the same as that seen after complete NADH oxidation and slow equilibration of external and internal pH. This amount was strictly proportional to the NADH concentration and always corresponded to a stoichiometric coefficient of 1 for scalar H^+ according to Eq. 1 when $n = 0$. When ferricyanide was used as electron acceptor no vectorial proton translocation was

detected and uncoupler-insensitive acidification of the medium was observed according to the reaction:

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

The results shown in Fig. 1 seemed to be self-consistent and were further used to determine the value of ***n*** in Eq. 1. According to the oxidant (reductant) pulse method [7,11] the ***n*** value for vectorial proton translocation can be determined from the peak amplitude (see PhR response in Fig. 1) if the amount of NADH oxidized is known and appropriate corrections for scalar proton uptake (if needed, as in the case of Q_1 as electron acceptor) are made [14]. However, the amount of H^+ consumed during the initial rapid phase is a matter of several possible pitfalls. First, it can only be calculated by extrapolation of the slow decay curve which is presumed to be exponential. Because of its rather high steepness the extrapolated value is evidently subject to significant errors. Secondly, the amount of protons vectorially translocated after *complete* NADH oxidation (or *complete* oxidant reduction when an oxidant pulse is used) ideally must be determined for the true $\rightarrow H^+/2\bar{e}$ stoichiometry calculation. This amount

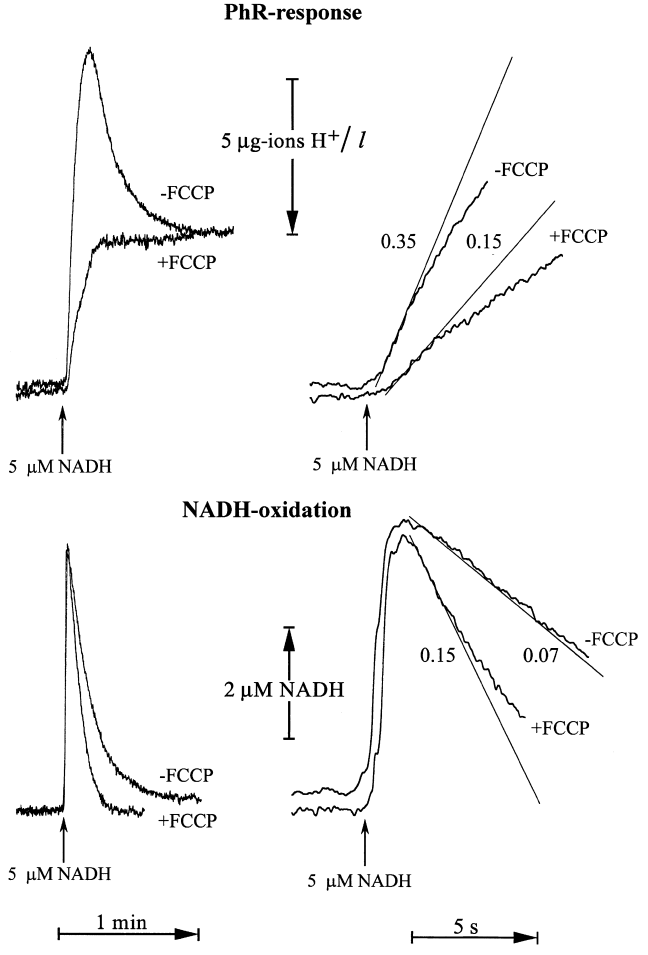

Fig. 2. Quantitation of proton translocation and NADH oxidation catalyzed by complex I. Particles (0.4 mg/ml) were placed in the standard reaction mixture containing 100 μM Q_1 and 175 μM ADP-ribose; external alkalization (upper panels) and NADH oxidation (lower panels) were followed at different time resolutions. Numbers on the curves are the initial rates of proton consumption and NADH oxidation (μg ions or μmol/min/mg protein).

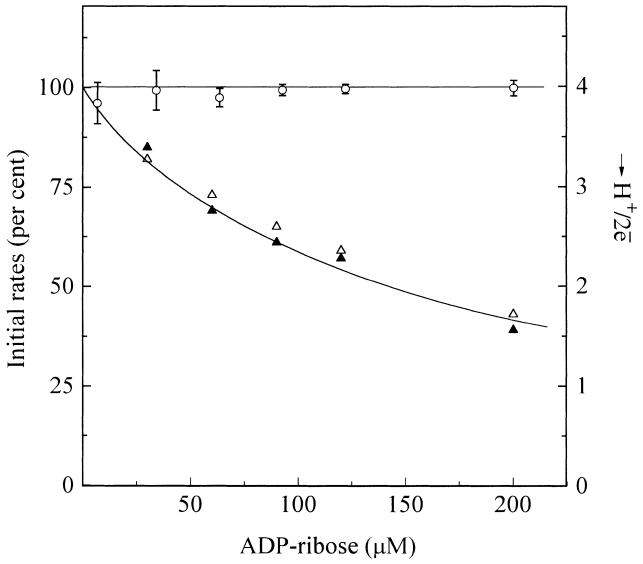

Fig. 3. Effect of ADP-ribose on NADH oxidation and proton translocation reactions catalyzed by submitochondrial particles. 100 μM Q_1 was present as electron acceptor. ▲, initial rates of NADH oxidation; △, initial rates of external proton uptake; ○, ***n*** quotient calculated as described in the text for Fig. 2.

is difficult to determine when only one reactant (H_m^+ or H_c^+ according to Eq. 1) is followed as was usually done in most previous studies on $\rightarrow H^+/2\bar{e}$ stoichiometries [7,11,14] (see, however [15,16] for a more reliable methodology). An alternative way to determine ***n*** in Eq. 1 is to measure simultaneously the initial rates of NADH oxidation and H^+ change. It follows from Eq. 1 that:

$$n\ (\rightarrow H^+/2\bar{e}) = (v\ H^+/v\ NADH) - 1 \quad (3)$$

where $v\ H^+$ is the initial rate of total H^+ consumption and v (NADH) is the initial rate of coupled NADH oxidation; the value of 1 on the right side of Eq. 3 corresponds to 'scalar' stoichiometry as predicted and experimentally determined (Fig. 1). In order to be able to measure the initial rate of NADH oxidation the latter was decreased by the addition of ADP-ribose, a competitive inhibitor of NADH binding sites [22]. Fig. 2 shows representative tracings of H^+ uptake (upper panels) and NADH oxidation (lower panels) at different time resolutions. When the $\rightarrow H^+/2\bar{e}$ stoichiometry was calculated (Eq. 3) using the data obtained as depicted in Fig. 2 (right panel), a value of 4 ± 0.05 was obtained. To assure that the quotient does not rely on the use of ADP-ribose and to cancel any systematic errors the experiments conducted as shown in Fig. 2 (on the right) were performed in the presence of different concentrations of the inhibitor. Fig. 3 demonstrates that the higher the ADP-ribose concentration added, the slower were the rates of NADH oxidation and proton translocation observed whereas the $\rightarrow H^+/2\bar{e}$ ratio of 4 was constant.

We were specifically interested to relate the proton translocating activity of complex I to the other NADH acceptor reductase reactions [19]. When hexaammineruthenium [23] was used no proton translocation was observed (the actual tracings were the same as those shown in Fig. 1 for ferricyanide). Fig. 4 demonstrates NADH oxidation and proton translocating activities measured in the presence of other qui-

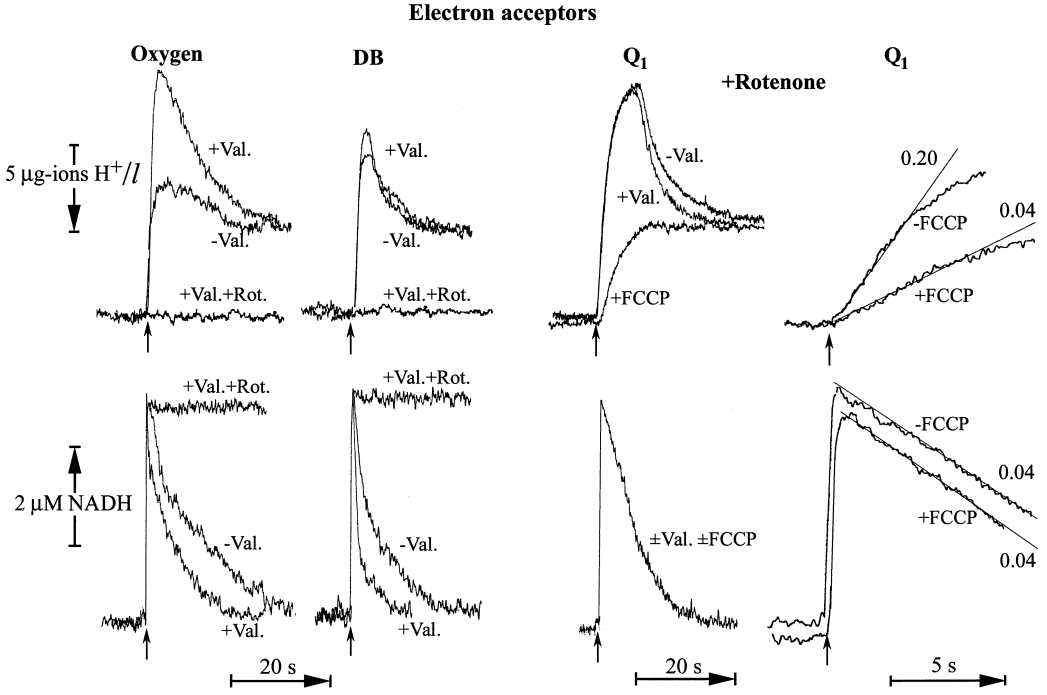

Fig. 4. NADH-induced proton translocation reactions in the presence of different electron acceptors. When the NADH oxidase reaction was followed the treatment of particles with myxothiazol as described in Section 2 was omitted. Particles (0.5 mg/ml) were placed in the standard reaction mixture containing 20 mM KCl. 100 μM DB or Q_1 was present where indicated. The reaction was initiated by the addition of 5 μM NADH (indicated by the arrows). The concentrations of rotenone, valinomycin, and FCCP (where indicated) were 25 μM, 6 μM, and 17 μM, respectively. Note that rotenone (25 μM) was present in the samples where Q_1 was used as electron acceptor. Numbers on the curves are the initial rates of proton consumption and NADH oxidation (μg ions or μmol/min/mg protein).

none acceptors for complex I. Complete NADH oxidase (sites 1+2+3, endogenous ubiquinone as electron acceptor for complex I and oxygen as terminal acceptor) was accompanied by proton translocation which was significantly increased by valinomycin. The latter effect is well known and it is evidently due to a collapse of the $\Delta\psi$ component of $\Delta\bar{\mu}_{H^+}$. As expected, rotenone completely prevented both NADH oxidation and coupled proton translocation. Qualitatively the same pattern was observed when DB was used as electron acceptor in the presence of myxothiazol. In contrast to NADH oxidase and NADH-DB reductase, NADH-Q_1 reductase activities of either isolated complex I or SMP are known to be only partially rotenone-sensitive [24,25]. When the residual rotenone-insensitive NADH-Q_1 reductase activity was analyzed it was unexpectedly found that it did catalyze vectorial proton translocation (Fig. 4). Although the rate of NADH oxidation by exogenous Q_1 in the presence of saturating rotenone was about 20% of that measured in the presence of uncoupler without the inhibitor, the small residual reaction showed the same $\rightarrow H^+/2\bar{e}$ ratio when the initial rates of H^+ uptake and NADH oxidation were quantitated (Fig. 4, right panels). It is worth stressing that the residual proton translocating activity of NADH-Q_1 reductase was due to neither lack of rotenone nor restoration of the rotenone-sensitive reaction by an excess of electron acceptor (Q_1); rotenone was added in great excess and an increase of its concentration did not change the reaction patterns shown in Fig. 4. Moreover, the NADH-Q_1 reductase reaction was fully capable of proton translocation with the same $\rightarrow H^+/2\bar{e}$ stoichiometry after treatment with piericidin A (0.7 nmol/mg protein, the residual NADH-Q_1 reductase was also about 20% of the control (no inhibitor) value). When thermally deactivated particles treated with NEM to prevent enzyme activation [26] were used the residual (about 20% of the original) NADH-Q_1 reductase was also capable of uncoupler-sensitive proton translocation (the results, which appeared qualitatively the same as those shown in Fig. 4 for Q_1, are not shown). Another difference between the proton translocation reactions when exogenous Q_1 (in the absence or presence of rotenone) and endogenous ubiquinone operating together with complex III and cytochrome *c* oxidase was different effects of valinomycin which slightly decreased the peak amplitude in the former reaction and significantly increased it, as expected, in complete NADH oxidase reaction.

4. Discussion

Any working hypothesis on the mechanism of redox-coupled proton translocation must rely upon well established stoichiometric relations. We feel that until now the data on such relations for complex I have been lacking. It is not surprising that in most speculative proposals some plausible mechanisms which account for any $\rightarrow H^+/2\bar{e}$ ratio from 2 to 5 were suggested [4,27]. We believe that the $\rightarrow H^+/2\bar{e}$ ratio of 4 is now experimentally determined. This value is in agreement with the site 1 stoichiometry previously suggested by Pozzan et al. [15] and Wikström [17] from their indirect measurements and contradicts the value of 2 suggested from more direct measurements in intact mitochondria [10] and submitochondrial particles [11,14]. The inconsistency of our data with those obtained in the pioneering studies of Lawford and Garland [10] on intact mitochondria is most likely due to secondary ion movements as was discussed [8]. The low $\rightarrow H^+/2\bar{e}$ quotient (1.8) found by Ragan and Hinkle in submitochondrial particles in the presence of cyanide and Q_1 as electron

acceptor [14] is not easy to explain. It should be mentioned, though, that they used the pulse extrapolation methodology, which might give erroneous values, and high potassium (100 mM KCl) at pH 6.4 and valinomycin were present in their studies. Much more experimental work, which would include tracing of K^+ movements at different pH with and without Q_1, is evidently needed. Whatever the explanation for the possible effect of valinomycin will be, it seems safe to conclude that at pH 8.0 in low potassium-containing medium the ***n*** value in Eq. 1 is 4. However strong the circumstantial evidence for the $\rightarrow H^+/2\bar{e}$ stoichiometry of 4 presented by Pozzan et al. [16] and Wikström [17], the final conclusion seems based more on the data summarized in the present report than elsewhere. It would be of a great interest to measure site 1 stoichiometry for prokaryotic type I NADH dehydrogenase using the technique applied in these studies. The values of 2 (*Escherichia coli* [28], *Paracoccus denitrificans* [29]) and more recently 3 (*E. coli* [30], were suggested from the oxidant pulse experiments.

Perhaps the most intriguing finding reported here is that rotenone- (also piericidin- and thermally induced inactivation-) insensitive NADH-Q_1 reductase activity is coupled to $\rightarrow H^+/2\bar{e} = 4$ proton translocation. It has been shown many years ago that depending on the chemical nature of the quinone acceptor NADH oxidation by the mammalian respiratory chain is either completely or partially sensitive to rotenone and piericidin [24]. It is generally believed that only the rotenone-sensitive quinone reductase reaction is capable of energy transduction, although two sites of interaction of ubiquinone analogues with complex I, having different proton pumping capacities, have been suggested recently [31]. Remarkably, only the rotenone-sensitive NADH-quinone reductase was reported to support oxidative phosphorylation [32]. The inability of the residual, low specific activity ($\leq 20\%$), rotenone-insensitive NADH-Q_1 reductase to drive oxidative phosphorylation is most likely due to its low power capacity. When a significant load (saturating ADP and P_i) is applied ATP synthase would likely be switched off. This proposal is corroborated by the well known effects of energization on chloroplast ATP synthase [33,34] and by our recent findings on $\Delta\bar{\mu}_{H^+}$-dependent transformation of the mitochondrial $F_0{\cdot}F_1$-ATPase [35].

In light of the findings reported here an obvious question arises: what is the nature of the rotenone-insensitive Q_1 reactive site and where it is located? In terms of our previously published speculative scheme [36] tightly bound ubiquinol located at the site designated Q_{b-i} would be the most likely candidate. It should be emphasized that our scheme needs to be modified to account for the $\rightarrow H^+/2\bar{e}$ stoichiometry of 4.

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