Coupling site I and the rotenone-sensitive ubisemiquinone in tightly coupled submitochondrial particles

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The rotenone-sensitive $g=2.00$ low temperature EPR signal attributed to ubisemiquinone is observed in submitochondrial particles during coupled electron transfer from NADH to oxygen and from succinate to NAD^+. The signal is seen only in the presence of oligomycin added to induce the respiratory control (7–9 with NADH and 3–4 with succinate) and it disappears in the presence of uncouplers (CCCP or gramicidin D). No reduction of the iron-sulfur center N-2 in the presence of 20 mM succinate and cyanide is observed, thus suggesting that N-2 is not in equilibrium with the ubiquinone pool. A hypothesis is proposed on $\Delta\tilde{\mu}_{H}+$ generation coupled with electron transfer between iron-sulfur center N-2 and the ubiquinone pool.

NADH-ubiquinone reductase; Ubisemiquinone; Electron transfer; Mitochrondria

1. INTRODUCTION

NADH-ubiquinone oxidoreductase (Complex I, coupling site I [1]) is an oligomeric lipoprotein complex of the mammalian respiratory chain which catalyzes electron transfer from NADH to the ubiquinone pool coupled with vectorial translocation of protons from the mitochondrial matrix to the cytoplasm (see [1–4] for reviews). FMN [5,6] and at least four Fe-S centers [7–9] of the enzyme are involved in electron transfer from NADH to the ubiquinone pool. The isolated Complex I contains also 2–4 bound ubiquinone per FMN [1,10]. The stoichiometry of 2-5 $H^+/2e^-$ in the reaction of NADH oxidation by the ubiquinone pool in mitochondria and submitochondrial particles has been reported [11–14], i.e., the values which can hardly be explained by the simple loop mechanism involving $FMN/FMNH_2$ as hydrogen carrier as originally suggested by Mitchell [15]. Several more complex hypothetical mechanisms involving vectorial translocation of 4 H^+ by FMN per pair of electrons transferred from NADH to the most positive iron-sulfur center N-2 ($E_{m,7} \sim -20$ mV [7]) have been proposed [16–19].

Recently we have reported that coupled submitochondrial particles exhibit a rotenone-sensitive $g = 2.00$ low temperature EPR signal attributed to site I associated ubisemiquinone magnetically interacting with one of the rapidly relaxing Fe-S centers [20]. Although the EPR characteristics of Complex I (isolated or in mitochondrial membrane) have been thoroughly investigated by several groups [7,10,21–23], no definite conclusions on participation of ubisemiquinone in the electron transfer in coupling site I have been reached. Obviously it seemed of interest to know whether site I associated ubisemiquinone is sensitive to $\Delta\tilde{\mu}_{H+}$ as it might have been expected for a component whose oxidation or reduction is coupled with an energy accumulation step. In this report we will show that the rapidly relaxing rotenone-sensitive free radical signal is indeed sensitive to the uncouplers. Moreover, in our preparations of submitochondrial particles the Fe-S center N-2 is not reduced by succinate unless $\Delta\tilde{\mu}_{H+}$ is applied, thus suggesting a much lower midpoint redox potential for N-2 (< -100 mV) than the generally agreed value (~ -20 mV). A preliminary scheme of energy accumulation mechanism in site I during the electron transfer between center N-2 and the ubiquinone pool is proposed.

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Abbreviations: Q, ubiquinone-10; BSA, bovine serum albumin; Hepes, *N*-(2-hydroxyethyl) piperazine-*N*-(2-ethanesulfonic acid); CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone

2. MATERIALS AND METHODS

Turnover 'preconditioned' AS-particles were prepared as described [20]. The preparations catalysed NADH (spectrophotometric assay) and succinate oxidase (oxygen electrode assay) reactions with respiratory control ratios of 7–9 and 3–4, respectively. EPR spectra were recorded with a Radiopan SE/X 2544 X-band spectrometer (Poland) equipped with a helium flow transfer line. The suspension of submitochondrial particles (3–8 mg of protein per ml (biuret assay [24])) in medium containing 0.25 M sucrose, 50 mM Hepes, 0.2 mM EDTA (pH 8.0, potassium salts), and 1 mg/ml BSA was placed in

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standard quartz EPR tubes (further additions and incubation times are indicated in table and figure legends). The samples were rapidly (~1 s) frozen in cold pentane (144 K) and stored in liquid nitrogen. NADH was obtained from Boehringer (FRG); NAD^+, NADPH, ATP and pyruvate kinase were from 'Reanal' (Hungary); phosphocreatine, potassium pyruvate and oligomycin were from Serva (FRG); BSA was from Sigma (USA); rotenone was from Ferak (West Berlin); lactate dehydrogenase was from Merck (FRG); gramicidin D was from Fluka (Switzerland).

3. RESULTS AND DISCUSSION

The intensity and shape of the rotenone-sensitive free radical signal seen in the particles under various conditions of the steady-state NADH oxidation [20] are shown in Fig. 1. It is worthwhile noting that the signal depicted in Fig. 1 was observed in the particles artificially 'coupled' by oligomycin [25] thus having the respiratory control ratio of 8 when NADH was used as a substrate. A much smaller signal was observed in the particles which were not treated with oligomycin (respiratory control of 1.3) and the signal was eliminated when an uncoupler (20 μM CCCP) was added to the oligomycin treated particles.

The relative content of the signal shown in Fig. 1 under various conditions is given in Table I. In accord with the data of Krishnamoorthy and Hinkle [19] and our previous results [20] center N-2 was almost completely reduced upon steady-state NADH oxidation independently of $\Delta\tilde{\mu}_{H+}$. In contrast, the relative intensity of the $g = 2.00$ signal strongly depends on $\Delta\tilde{\mu}_{H+}$; it increases with an increase of oligomycin and disappears in the presence of uncoupling agents (samples 1–4). The qualitatively same results were obtained with succinate as the source for both electrons and energy to feed site I redox components although the amplitude of free radical and the steady-state level of N-2 reduction were considerably lower (samples 5–7). The uncoupler sensitive free radical and center N-2 reduction were also observed in ATP-dependent succinate supported NAD^+ reduction (sample 8).

The data shown in Fig. 1 and Table I suggest that site I associated ubisemiquinone behaves as an energy transducing component. Since $\Delta\tilde{\mu}_{H+}$ inhibits the steady-state electron flow through site I an increased level of ubisemiquinone in the coupled state indicates that the decreased rate of $QH^\cdot$ or $Q^{\cdot -}$ reduction to ubiquinol rather than the increased rate of one electron Q reduction is responsible for the crossover type behaviour. Formally it means that the oxidation of ubisemiquinone(s) stabilized at some site(s) close to the rapidly relaxing iron-sulfur center by the ubiquinone pool is coupled with energy accumulation. Since the most positive iron-sulfur N-2 center has been reported to have the midpoint potential ~ −20 mV [7,23], no energy seems to be available for the proton translocation function at site I between N-2 and ubiquinone pool ($E_{m,7}$ ~ +65 mV [26]). However, it should be emphasized that the midpoint potential of center N-2 is

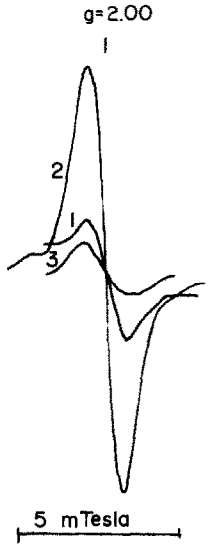

Fig. 1. Low-temperature EPR spectra of the rotenone-sensitive free radical signal in submitochondrial particles. 2 mM NADH was added to aerobic suspension of the particles (3 mg/ml) in the standard reaction mixture; the samples were incubated for 20 s in EPR tubes and rapidly frozen. EPR was recorded at 40 K at: microwave frequency, 9.310 MHz; microwave power, 50 mW; modulation frequency, 100 kHz; modulation amplitude, 5×10^{-4} T; time constant, 3 s; scanning rate, 3.75×10^{-3} T/min. Curve 1, particles as prepared; curve 2, particles were preincubated for 30 min with oligomycin (0.5 μg/mg of protein); curve 3, as curve 2, 20 μM CCCP was added.

'preparation dependent' and in some preparations a value as low as −140 mV has been reported [23]. It is also relevant to point out that coupling site I in *Paracoccus denitrificans* contains iron-sulfur clusters similar to mitochondrial N-1, N-2, N-3 and N-4 [27,28] and a pH-dependent midpoint potential of N-2 as low as −130 mV has been measured [29].

Taking into account the uncertainty of the N-2 midpoint potential we decided to find out whether cluster N-2 in our preparations can be reduced by succinate through the ubiquinone pool in deenergized particles. Fig. 2 demonstrates the low-temperature EPR spectra of our particles reduced by succinate or NADH in the presence of cyanide. No N-2 reduction was observed in the presence of succinate whereas a well-resolved N-2 signal was seen in the presence of NADH. The data shown in Fig. 2 suggest that the low midpoint potential center N-2 ($E_{m,8} < -100$ mV) is present in our preparations. The reason for 'preparation dependency' of the N-2 midpoint potential (see [23]) is not clear. One clue for the possible explanation is the 'history' of different mitochondrial particulate preparation. It should be emphasized that our 'activated' particles are used after two passages through a Sephadex column at high temperature [20] and they are devoid of NAD^+, fumarase and malate dehydrogenase (our unpublished observation), i.e., the enzymes which together with succinate dehydrogenase may be responsible for some N-2 reduction from the substrate side of coupling site I.

Table I

Relative content of the rotenone-sensitive ubisemiquinone free radical and iron-sulfur center N-2 in submitochondrial particles upon the steady-state electron flux through coupling site I

Substrate	Oligomycin added to the particles (μg/mg protein)	Radical[a]/N-2		Reduction of N-2[b] (%)	
		−CCCP	+CCCP[c]	−CCCP	+CCCP[c]
1. NADH (2 mM)	none	0.1	–	80	–
2. NADH (2 mM)	0.15	0.25	–	90	–
3. NADH (2 mM)	0.50	0.4	0.05	90	80
4. NADH (2 mM) + gramicidin D (0.5 μM)	0.50	0.05	–	80	–
5. Succinate (20 mM)	none	0.1	–	5	–
6. Succinate (20 mM)	0.15	0.15		10	–
7. Succinate (20 mM)	0.50	0.35	0.03	55	0
8. Succinate (20 mM), NAD^+ (2 mM), Mg^{2+}-ATP (2 mM)[d]	0.15	0.1	0.04	50	–

[a] Integral intensity of $g = 2.00$ signal measured as described in Fig. 1

[b] 100% N-2 spin content was determined as the integral intensity of the low-field component at $g = 2.054$ [31] in the presence of 2 mM NADH and 2 mM potassium cyanide

[c] 20 μM CCCP was added

[d] The standard reaction mixture (see section 2) contained: 2 mM cyanide, 20 mM pyruvate (potassium salts), 20 mM creatine phosphate, creatine kinase (5 mg/ml) and lactate dehydrogenase (0.5 mg/ml). Samples were incubated for 10 s (1–7) and 30 s (8) before freezing

If the midpoint potential of center N-2 in mitochondrial site I is taken as −130 mV, then the electron flow between N-2 and Q-pool may energetically be coupled with proton translocation. Our current working hypothesis on the mechanism of energy accumulation in site I which involves FMN as a hydrogen carrier [15] and a transformer from two electron donor NADH to one electron Fe-S carriers [16,17,19], two ubiquinone binding sites [10], the existence of $\Delta\tilde{\mu}_{H^+}$-dependent stabilized ubisemiquinone [20] and energetically favourable ubisemiquinones dismutation [20] is shown in Fig. 3. An operation of 'subsite' I (from N-2 to the ubiquinone pool) according to this scheme will provide

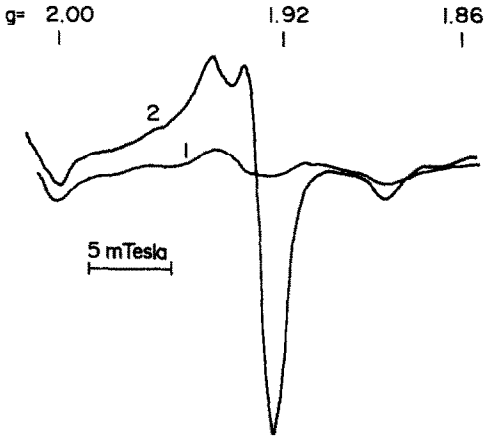

Fig. 2. Low-temperature EPR spectra of submitochondrial particles reduced by 20 mM succinate (curve 1) or 2 mM NADH (curve 2). Submitochondrial particles (7 mg/ml) treated with oligomycin (0.5 μg/mg of protein) were incubated for 3 min in the standard reaction mixture containing 2 mM potassium cyanide and 40 μM CCCP. EPR spectra were recorded at 20 K at: microwave frequency, 9.310 MHz; microwave power, 50 mW; modulation frequency, 100 kHz; modulation amplitude, 1×10^{-3} T; time constant, 1 s; scanning rate, 1.75×10^{-2} T/min.

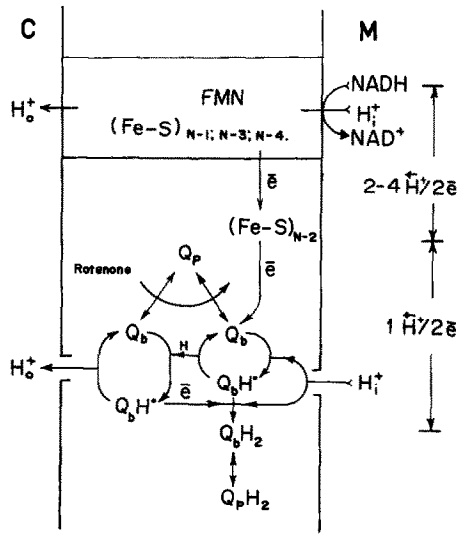

Fig. 3. Scheme for proton translocation at coupling site I. M and C, matrix and cytoplasmic sides of the coupling membrane, respectively. Two protons are absorbed at M-side to form bound protonated ubisemiquinones ($Q_bH^•$) at the specific binding sites as the result of sequential reduction of bound Q_b by N-2 Fe-S center. Additional proton is absorbed at M-side when the enzyme controlled dismutation takes place. Three protons are taken up from the M-side: two are accumulated in the membrane (Q_pH_2) and one is released at the C-side with the overall stoichiometry of 1 translocated H^+ per 2 electrons transferred from N-2 to the ubiquinone pool (Q_p). The electron transfer sequence between NADH and N-2 center is not specified (see [15–19] for possible schemes).

for the translocation of 1 H^+ per pair of electrons transferred from NADH to ubiquinone. This value added to 2-4 H^+ translocated per $2e^-$ transferred from NADH to N-2 center (flavin simple loop [15] or flavin cycle schemes [16–19]) seems to be in reasonable accord with the published data on the stoichiometry of net vectorial H^+ translocation in site I [11–14].

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