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Ubisemiquinone in the NADH-ubiquinone reductase region of the mitochondrial respiratory chain

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Coupled bovine heart submitochondrial particles exhibit a rotenone-sensitive $g = 2.00$ low-temperature EPR signal attributable to ubisemiquinone which is observed during steady-state electron transfer from NADH to oxygen or from succinate to NAD^+ in $\Delta\tilde{\mu}H^+$-dependent reverse electron transfer. Quantitation of the signal under optimal conditions gives a value for maximal semiquinone of approx. 0.5 spins per spin of the fully reduced NADH dehydrogenase iron-sulfur center N-2. The intensity of the signal is drastically reduced when electron transfer from NADH to oxygen is blocked by cyanide or in the case of the reverse electron transfer from succinate to NAD^+ being prevented by anaerobiosis. Only those particles which contain 'turnover-preconditioned' NADH-ubiquinone reductase demonstrate the ubisemiquinone signal together with N-1, N-2, N-3 and N-4 iron-sulfur centers. The spin relaxation characteristics of the rotenone-sensitive ubisemiquinone signal point to its interaction with one of the rapidly relaxing (4Fe-4S) centers, most probably N-2.

NADH-ubiquinone reductase; Ubisemiquinone; Electron transfer; Mitochondrion

1. INTRODUCTION

The participation of ubiquinone (Q) as an obligatory redox component in the mitochondrial respiratory chain is well established [1–3]. There is strong evidence that, in addition to Q and QH_2, a substantial fraction of ubiquinone occurs as the semiquinone when functioning in the respiratory chain. Since the initial demonstration of ubiquinone free radicals in respiring mitochondrial preparations [4,5], details of the kinetic and thermodynamic properties of ubisemiquinone associated with succinate dehydrogenase [6–8] and the b-c_1 complex [8,9] have been reported.

Rotenone-sensitive proton-translocating NADH-ubiquinone reductase is coupled to ATP synthesis [10,11] suggesting that some component of complex I in addition to FMN [12] functions as a proton carrier during steady-state enzyme operation. Indeed, several lines of evidence, including the presence of 2–4 bound Q per FMN in purified complex I [13], the isolation of the protein containing low redox potential (< -125 mV) bound Q from the enzyme [14,15] and a direct observation of stable rotenone-sensitive ubisemiquinone radicals in isolated complex I [16], make the $QH^{\cdot}/Q$ couple an attractive candidate for the active form of Q in the NADH-ubiquinone reductase region of the respiratory chain. In fact, an ATP-dependent free radical with a midpoint potential of -25 mV in piericidin inhibited submitochondrial particles which might be attributed to ubisemiquinone has been claimed to appear [17].

Here, we present evidence for the existence of NADH-dehydrogenase-associated, stabilized ubisemiquinone in coupled submitochondrial particles during steady-state NADH oxidation or $\Delta\tilde{\mu}H^+$-dependent succinate-supported NAD^+ reduction.

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Abbreviations: Q, ubiquinone-10; BSA, bovine serum albumin; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone

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2. MATERIALS AND METHODS

2.1. *Submitochondrial particles*

All experiments were carried out using coupled AS-submitochondrial particles [18] (treated with oligomycin [19]). It was recently shown that such particles are able to catalyze aerobic $\Delta\tilde{\mu}H^+$-dependent NAD^+ (or ferricyanide) reduction by succinate provided that succinate dehydrogenase was activated by malonate and that NADH-ubiquinone reductase was 'preconditioned' via several turnover cycles with NADH or NADPH [20]. Therefore, 'active' and 'inactive' particles were prepared as follows: 1 ml AS particles (20–30 mg/ml) suspended in 0.25 M sucrose and stored in liquid N_2 were thawed, followed by incubation at 30°C for 90 min at 10–15 mg/ml in a mixture containing 0.25 M sucrose, 20 mM Hepes, 4 mM malonate, 0.1 mM EDTA (pH 8.0, potassium salts), BSA (1 mg/ml) and oligomycin (0.4 μg/ml). The suspension was cooled to room temperature, 1 mM NADPH (active particles) or 1 mM $NADP^+$ (inactive particles) was added and after incubation for 10 min, samples were slowly (~1 h) filtered through a 1.5 × 17 cm Sephadex G-50 (medium) column equilibrated with the above mixture containing 0.5 mM malonate but no NADPH or $NADP^+$. Particles were collected by centrifugation (at 200 000 × g for 20 min, 15°C), suspended (5–8 mg/ml) in a mixture containing 0.25 M sucrose, 0.1 mM EDTA, 50 mM Hepes (potassium salts, pH 8.0) and BSA (1 mg/ml) and used for assays. Enzymatic activities of active particles determined at 30°C and pH 8.0 (2 μg-electron equivalents/min per mg protein) were: NADH oxidase, 0.2; succinate oxidase, 0.4; aerobic rotenone-sensitive succinate-NAD^+ reductase, 0.5; aerobic rotenone-sensitive succinate-ferricyanide reductase, 0.7. Activities measured in the presence of 20 μM CCCP were 1.8, 0.9, 0.0, and 0.0, respectively. No $\Delta\tilde{\mu}H^+$-dependent, succinate-supported NAD^+ or ferricyanide reduction was observed when inactive particles were assayed, whereas NADH and succinate oxidase activities were identical for both active and inactive preparations.

2.2. *Preparations for EPR measurements*

0.2 ml particle suspension (5–8 mg/ml, see above) was transferred to an EPR tube, incubated with constant stirring at 30°C (incubation times and further additions indicated in the table and figure legends). Samples were rapidly (< 1 s) frozen in cold pentane (144 K) and stored in liquid N_2.

2.3. *EPR measurements*

EPR spectra were recorded with a Radiopan SE/X 2544 X-band spectrometer (Poland) equipped with a helium flow transfer line. Standard quartz tubes (3 mm inner diameter) were used.

Protein content was assayed by using a biuret reagent [21]. Enzymatic activities were assayed as described [20]. NADH and $NADP^+$ were obtained from Boehringer (FRG), NAD^+ and NADPH from "Reanal" (Hungary), potassium pyruvate and oligomycin from Serva (FRG), BSA from Sigma (USA), rotenone from Ferak (West Berlin), and lactate dehydrogenase from Merck (FRG). Other chemicals were of the purest commercially available grade.

3. RESULTS AND DISCUSSION

No EPR-detectable reduced Fe-S center or semiquinone components were observed in either active or inactive submitochondrial particles incubated in the presence of cyanide, thus indicating that the respiratory chain was oxidized in both preparations. When 2 mM NADH was added to particles and the samples frozen 15 s after respiration was initiated, a reduction of N-1, N-2, N-3 and N-4 Fe-S centers was evident from the EPR spectra (fig.1B,C). A prominent signal at $g = 2.02$ (S-3 center of succinate dehydrogenase) and a broad trough at $g \sim 2.0$ (cytochrome oxidase copper signal) indicate that aerobic conditions were maintained during incubation and hence a true 'coupled' steady state of NADH-ubiquinone reductase

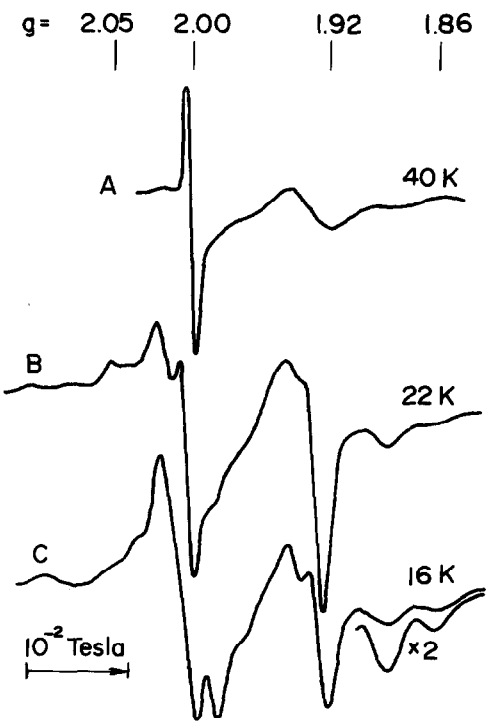

Fig.1. Low-temperature EPR spectra of coupled submitochondrial particles during steady-state NADH oxidation. Submitochondrial particles (7 mg/ml) were treated as described in section 2. NADH (2 mM) was added, the suspension then being frozen 15 s after the reaction had been initiated. Spectra recorded 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, 17.5×10^{-3} T/min; the wings on (C) were recorded at the time constant 3 s, scanning rate 8.75×10^{-3} T/min.

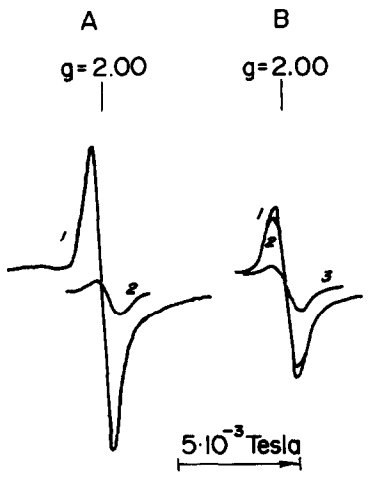

Fig.2. Low-temperature EPR spectra of ubisemiquinone in coupled submitochondrial particles. 2 mM NADH (A) or 20 mM succinate (B) was added to an aerobic suspension of coupled submitochondrial particles (7 mg/ml), and the samples were frozen 15 s after respiration had been initiated; curves 2 (A) and 3 (B), 10 μM rotenone present; curve 1 (B), 2 mM NAD^+, 5 mM pyruvate and lactate dehydrogenase (0.5 mg/ml) present. EPR spectra were recorded at 40 K. EPR conditions as in fig.1; modulation amplitude, 5×10^{-4} T; scanning rate, 7.5×10^{-3} T/min; time constant, 1 s.

was fixed. An intense free radical signal ($g = 2.00$) was observed when the spectrum of the same sample was recorded at 40 K (fig.1A). The signal width (< 1.0 mT) was similar to that of semiquinone associated with complex II [6,7] and III [8,9] and less than that of flavosemiquinones (1.2–1.6 mT [22]). The lines visible at 16 K (fig.1C) at $g = 1.99$ and 2.04 (the satellites of center S-3) indicate that the strongly dipole-dipole interacting ubisemiquinones associated with succinate dehydrogenase were also present. It might therefore be suggested that the rapidly relaxing (half-saturation power, $P_{1/2} \sim 70$ mW at 40 K) free radical signal shown in fig.1A was due to ubisemiquinone interacting with one of the iron-sulfur centers of NADH-dehydrogenase.

The relative intensity and shape of the signal seen in particles incubated under various conditions are depicted in fig.2. The signal was highly sensitive to rotenone; more importantly, the rotenone-sensitive radical was observed in the presence of succinate under conditions where steady-state $\Delta\tilde{\mu}H^+$-dependent NAD^+ reduction took place [20].

Quantitation of the ubisemiquinone free radical signal relative to those of the Fe-S centers for different conditions is presented in table 1. The ratio

Table 1

Relative content of ubisemiquinone free radical and reduced iron-sulfur centers in coupled submitochondrial particles

Samples	Radical/N-2[a]	N-1[b]	N-2[c]	N-3[d]	N-4[e]
1. NADH (2 mM), CN^- (1 mM)	0.08	100	100	100	100
2. NADH (2 mM), 15 s	0.50	85	80	75	75
3. Succinate (20 mM), 15 s	0.25	15	50	30	65
4. Succinate (20 mM), NAD^+ (2 mM), pyruvate (5 mM), lactate dehydrogenase, 15 s	0.28	0	50	10	45
5. Succinate (20 mM), 3 min, anaerobiosis	0.05	–	30	–	–
6. Same as 3, inactive particles	0.05	0	0	0	0

[a] Ratio of the free radical content (integral intensity of the $g = 2.00$ signal at 48 K) to that of iron-sulfur center N-2. N-2 spin content was determined as the integral intensity of the low-field component at $g_1 = 2.054$ [30] measured in the presence of NADH and cyanide

[b–e] Levels of reduction of iron-sulfur centers N-1–N-4 (% of fully reduced state in the presence of NADH and cyanide)

[b] Determined as the amplitude of the $g = 1.94$ signal at 16 K

[c] Determined as the amplitude of the $g = 1.92$ signal at 22 K

[d] Determined as the difference of the $g = 1.88$ signal amplitudes measured at 16 and 30 K

[e] Determined as the amplitude of the $g = 1.86$ signal at 16 K

of radical spins to spins of fully reduced center N-2 reaches a value of 0.5 under conditions of steady-state NADH oxidation (sample 2); it decreases when cytochrome oxidase is blocked (sample 1) or during anaerobiosis. An important feature of the signal is that it is observed during steady-state reverse electron transfer (sample 4). It is worth noting that signals of almost equal intensity were visible when NADH dehydrogenase was operating in the reverse direction in the presence or absence of NAD^+ (samples 3 and 4, respectively). The latter finding suggests that the low rate of succinate-supported aerobic superoxide generation at some site of NADH dehydrogenase [23] provides similar steady-state levels of the enzyme redox components (except for centers N-1 and N-3). The most conclusive evidence for the association of ubisemiquinone with NADH-ubiquinone reductase of the respiratory chain is provided by comparison of samples 6 and 3. The only difference between the samples is that the former contained inactive complex I (no free radical observed), whereas the latter (high level of radical observed) contained complex I which has been preconditioned by several turnovers [20]. This result and the complete oxidation of all iron-sulfur centers in inactive particles being in harmony with the enzymatic properties of the active and inactive particles (see section 2) indicate that the block of the electron transfer in inactive particles lies between the enzyme iron-sulfur centers and ubiquinone.

It has been shown that when dipole-dipole interaction between the free radical and an iron-sulfur center occurs, the rate of spin-lattice relaxation ($1/T_1$) of the radical is proportional to that of the Fe-S center [24]. Consequently, the value of $1/T_1$ for the radical is proportional to $\exp(-\Delta/kT)$ where k is Boltzmann's constant, and Δ denotes the difference between the ground state and first excited spin energy level of the iron-sulfur center [24,25]. Thus, the dependence of T_1 on temperature plotted in coordinates ln T_1 vs $1/T$ gives a straight line with a slope equal to Δ/k (for details see [24,25]). When saturation of the rotenone-sensitive ubisemiquinone signal was studied at 30–55 K, a Δ value of 90–100 cm^{-1} was determined.

This value is significantly different from those for the Rieske iron-sulfur center of the b-c_1 complex (Δ ~200 cm^{-1} [26]), or for N-1 and S-1 (Δ ~270 cm^{-1} [26]) or S-3 (Δ ~43 cm^{-1} [27]). On the other hand, it corresponds closely to the Δ value of some rapidly relaxing 4Fe-4S centers. Since S-2 does not undergo reduction under the conditions employed, then N-2, N-3 and N-4 (Δ ~80–100 cm^{-1} [24,27,28]), showing a significant degree of reduction for steady-state electron transfer through complex I, appear as likely candidates for the free radical–iron-sulfur center interaction. It is generally accepted that center N-2 serves as the terminal redox component in the NADH-ubiquinone region of the respiratory chain [29]. Thus, the stabilization of ubisemiquinone at the specific Q-binding site located close to the N-2 center appears quite reasonable.

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