Energy-dependent Complex I-associated ubisemiquinones in submitochondrial particles

A.D. Vinogradov[a], V.D. Sled[b], D.S. Burbaev[c], V.G. Grivennikova[a], I.A. Moroz[c], T. Ohnishi[b,*]

[a]*Department of Biochemistry, School of Biology, Moscow State University, 119899 Moscow, Russian Federation*
[b]*Department of Biochemistry and Biophysics, School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA*
[c]*Institute of Chemical Physics, Russian Academy of Science, 117977 Moscow, Russian Federation*

Received 21 June 1995

Abstract Two distinct species of Complex I-associated ubisemiquinones (SQ_{Nf} and SQ_{Ns}) were detected by cryogenic EPR analysis of tightly coupled submitochondrial particles oxidizing NADH or succinate under steady-state conditions. The $g = 2.00$ signals from both fast-relaxing SQ_{Nf} ($P_{1/2} = 170$ mW at 40 K) and slow-relaxing SQ_{Ns} ($P_{1/2} = 0.7$ mW) are sensitive to uncouplers, rotenone and thermally induced deactivation of Complex I. At higher temperatures the SQ_{Nf} signal is broadened and only the SQ_{Ns} signal is seen ($P_{1/2} = 7$ mW at 105 K). The spin–spin interaction between SQ_{Nf} and the iron–sulfur cluster N2 was detected as split peaks of the $g_{//}$ 2.05 signal with a coupling constant of 1.65 mT, revealing their mutual distance of 8–11 Å. The data obtained are consistent with a model in which N2 and two interacting bound ubisemiquinone species are spatially arranged within the hydrophobic domain of Complex I, participating in the vectorial proton translocation.

Key words: NADH-ubiquinone oxidoreductase; Energy-coupling; Ubisemiquinone; Iron–sulfur cluster; EPR-spectroscopy; Bovine heart submitochondrial particle

1. Introduction

The NADH-ubiquinone oxidoreductase (EC 1.6.99.3), usually termed Complex I, catalyzes electron transfer from NADH to the ubiquinone pool coupled with vectorial translocation of protons across the mitochondrial inner membrane [1–3]. There has been a great deal of progress during recent years in the structural studies of the mammalian [4], *Neurospora crassa* enzymes [5], and their simpler bacterial counterparts [6,7]. Little is known, however, about the sequence of electron transfer within the Complex I, which is composed of more than 40 subunits (bovine heart) [2,4], containing one flavin mononucleotide (FMN) [8], at least six EPR-detectable iron–sulfur clusters [9,10] and at least two bound ubiquinone (Q) species. Among the intrinsic redox components, FMN and Q may function as classical redox-linked proton translocators providing their appropriate spatial locations within the enzyme [11–14]. The net rotenone-sensitive 2 ē reduction of the bulk Q must be accompanied by $2H^+$ uptake into the hydrophobic phospholipid bilayer. As a result, this makes somewhat difficult to reconcile proton translocation with the quinone reduction step(s). To overcome this problem, several schemes have been put forward where bound Q was placed between low- and high-redox potential iron–sulfur clusters, thus making the ubisemiquinone $Q^{\bullet-}(H^+)$ an obligatory participant of the electron/proton transfer reaction, preceding the terminal reduction of bulk ubiquinone by the most positive iron–sulfur cluster (N2) [14–16]. Indeed, the formation of ubisemiquinone upon reduction of isolated Complex I by NADH [17] or during the steady-state [18,19] and presteady-state [20] NADH oxidation by the submitochondrial particles has been documented.

However, such schemes seem to be unlikely due to an apparent lack of redox components on the oxygen side of the rotenone-sensitive Q reduction step, the thermodynamic properties of the iron–sulfur clusters [10], and the kinetic analysis of the cluster N2 reduction in Q-depleted submitochondrial particles [21]. Recently, an energy-coupled vectorial dismutation between specifically bound paired ubisemiquinones has been proposed as the terminal proton translocation step in Complex I [19,22]. The essential features which follow from this model [22] are: (i) no terminal electron acceptors other than the bulk ubiquinone are required; (ii) two species of bound ubisemiquinone with different spatial arrangement relative to the iron–sulfur cluster N2 exist during the aerobic steady-state NADH oxidation; and (iii) the relative concentration of ubisemiquinones is $\Delta\bar{\mu}_{H^+}$-dependent. Point (iii) has been documented using tightly coupled submitochondrial particles [19]. The existence of two EPR-detectable species of Complex I-associated ubisemiquinones (point (ii)) has also been briefly reported [23]. Recently, our conclusions were questioned by Albracht and his collaborators [24].

In this paper we will present data describing two types of the Complex I-associated, $\Delta\bar{\mu}_{H^+}$-sensitive ubisemiquinone species (SQ_{Nf}, fast relaxing; and SQ_{Ns}, slowly relaxing). The distance of 8–11 Å between SQ_{Nf} and the iron–sulfur cluster N2 was estimated based on their spin–spin interaction.

2. Materials and methods

2.1. Submitochondrial particles

Tightly coupled submitochondrial particles were prepared [25] and treated for activation of Complex I and succinate dehydrogenase [18] as previously described. Deactivation of Complex I was achieved by incubation of the particles for 30 min at 37°C [25].

2.2. Preparation of samples for EPR measurements

The suspension of submitochondrial particles (15 mg/ml, determined by the biuret procedure) in 0.25 M sucrose, 50 mM Hepes (pH 8.0) and 1 mg/ml of bovine serum albumin was placed in an EPR tube (further additions are indicated in the legends to the figures) at room tempera-

*Corresponding author. Fax: (1) (215) 573-3748;
E-mail: ohnishi@a1.mscf.upenn.edu

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N*-enanesulfonic acid; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; SMP, submitochondrial particles.

SSDI 0014-5793(95)00803-9

ture. The steady-state electron transfer was initiated by the addition of 1.5 mM NADH and/or 20 mM succinate (potassium salts). The mixture was incubated for 5 s. Samples were rapidly (~1 s) frozen in cold pentane/cyclohexane mixture (5:1, v/v) and stored in liquid nitrogen until EPR measurements were carried out.

2.3. EPR-measurements

EPR measurements were performed with Bruker 300 spectrometer operating at X-band (9.2 GHz). The sample temperature was regulated by Oxford instrument ESR-9 helium flow cryostat.

The power saturation data was analyzed by computer fitting to the equation:

$$A = \sum_{i=1}^{n} A_i = \sum_{i=1}^{n} C_i \sqrt{P}/(1 + P/P_{1/2(i)})^{0.5b_i}$$

where A_i is an amplitude of the i-th type free radical; C_i is a coefficient for the actual content of the i-th type free radical in the sample; $P_{1/2(i)}$ is the half-saturation power and b_i is the 'inhomogenity parameter' [26]. Simulation of the power saturation curves was performed using the software Origin (MicroCal Software, Inc.), utilizing the Marquardt-Levenberg algorithm and simplex method for non-linear least-square fitting.

3. Results

Fig. 1 presents low temperature EPR spectra of uncoupled (A) and tightly coupled (B) bovine heart submitochondrial particles, oxidizing NADH. The following two prominent features are clearly discernible in the spectrum of coupled particles, but not in that of uncoupled particles: (i) the presence of an intense semiquinone free radical signal (g = 2.00), and (ii) the line shape change of the $g_{//}$ signal of the cluster N2: the decrease of the $g_{//}$ 2.05 peak is concomitant with the appearance of two new split- peaks at g = 2.044 and 2.064 (for further details see below).

It has been previously reported [18] that 40 K is the optimal sample temperature for recording of the rapidly relaxing ubisemiquinone free radical signal (peak to peak width of ~0.96 mT). At this temperature the signal amplitude was only partially saturated even at the highest microwave power setting level (200 mW) (Fig. 2A, curve 1). In the presence of an uncoupler, the intensity of this signal is about 20 times decreased at the maximum power level (Fig. 2A, curve 2). The residual small uncoupler insensitive signal (SQ_x) has an extremely slow spin relaxation. The power saturation curve 1 fits to the sum of three semiquinone species with $P_{1/2}$ values of $P_{1/2(1)}$ = 0.06 mW, $P_{1/2(2)}$ = 0.7 mW and $P_{1/2(3)}$ = 170 mW with a predominant contribution from the very rapidly relaxing species (SQ_{Nf}) in the high microwave power range. An increase in temperature (T > 55 K) results in a greatly decreased intensity of the uncoupler-sensitive signal at high microwave power. This decrease significantly deviates from the Curie law (an intensity is proportional to 1/T) and the deviation becomes quite significant at 105 K. This abnormal temperature dependence of the free radical signal was not seen at low microwave power, where contribution of SQ_{Nf} is not predominant. Thus, it can be concluded that SQ_{Nf} is not observed at high temperature (105 K), because of its lifetime broadening due to the extremely rapid spin relaxation. The power saturation curve for the uncoupler-sensitive free radical signal (SQ_{Ns}) observed at a high temperature (105 K) is presented in Fig. 3A (curve 1). This signal has a narrower line width (0.78 mT) than that of the SQ_{Nf}, and a $P_{1/2}$ value of 7.4 mW, which evidently corresponds to the $P_{1/2}$ = 0.7 mW component at 40 K. Curve 1 also contains an uncoupler-

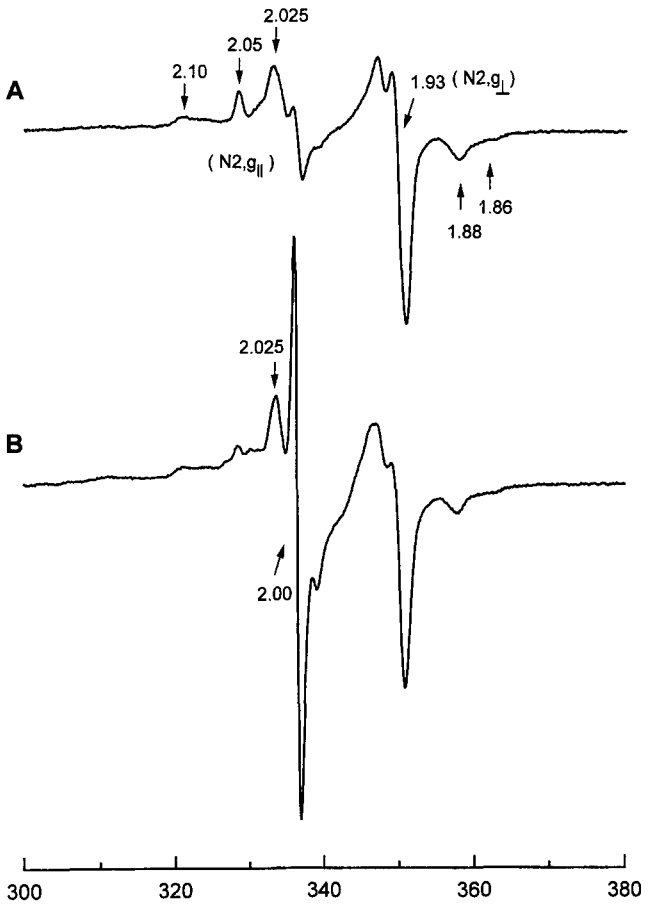

Fig. 1. EPR spectra of uncoupled (30 μM CCCP) (A) and tightly coupled (B) submitochondrial particles during steady-state NADH oxidation. SMP were mixed with 1.5 mM NADH and frozen 5 s after as described in 'section 2'. EPR condition: microwave frequency, 9.452 GHz; microwave power, 2.01 mW; modulation frequency, 100 kHz; modulation amplitude, 0.63 mT; scanning rate, 17.9 mT/min; sample temperature, 16 K. Characteristic g-values of iron–sulfur clusters of Complex I are indicated with arrows.

insensitive SQ_x species ($P_{1/2}$ = 0.1 mW). Both SQ_{Nf} and SQ_{Ns} signals were absent in the particles oxidizing succinate, in which Complex I has been deactivated [25] (see section 2). In addition, both SQ_{Nf} and SQ_{Ns} signals are quenched by rotenone with one order of magnitude difference in their sensitivity (Sled' et al., unpublished observations). Thus we conclude that both SQ_{Nf} and SQ_{Ns} are associated with Complex I.

In order to quantitatively evaluate the uncoupler-sensitivity of the Complex I-associated ubisemiquinones, the peak to peak amplitude of SQ_{Nf} and SQ_{Ns} signals were plotted as a function of $\sqrt{P}$ under non-power saturated conditions, where their amplitude is a linear function of $\sqrt{P}$ (Figs. 2B and 3B). Both SQ_{Nf} and SQ_{Ns} contents (which are proportional to the slopes of the lines) are significantly diminished in the presence of an uncoupler. The same difference in the relative contents of SQ_{Nf} and SQ_{Ns} in coupled and uncoupled preparations was obtained by the double-integration of the signals recorded using 8 μW power at 40 K and 105 K, respectively.

A striking difference exists in the spectral features of the iron–sulfur cluster N2 during steady-state NADH oxidation in

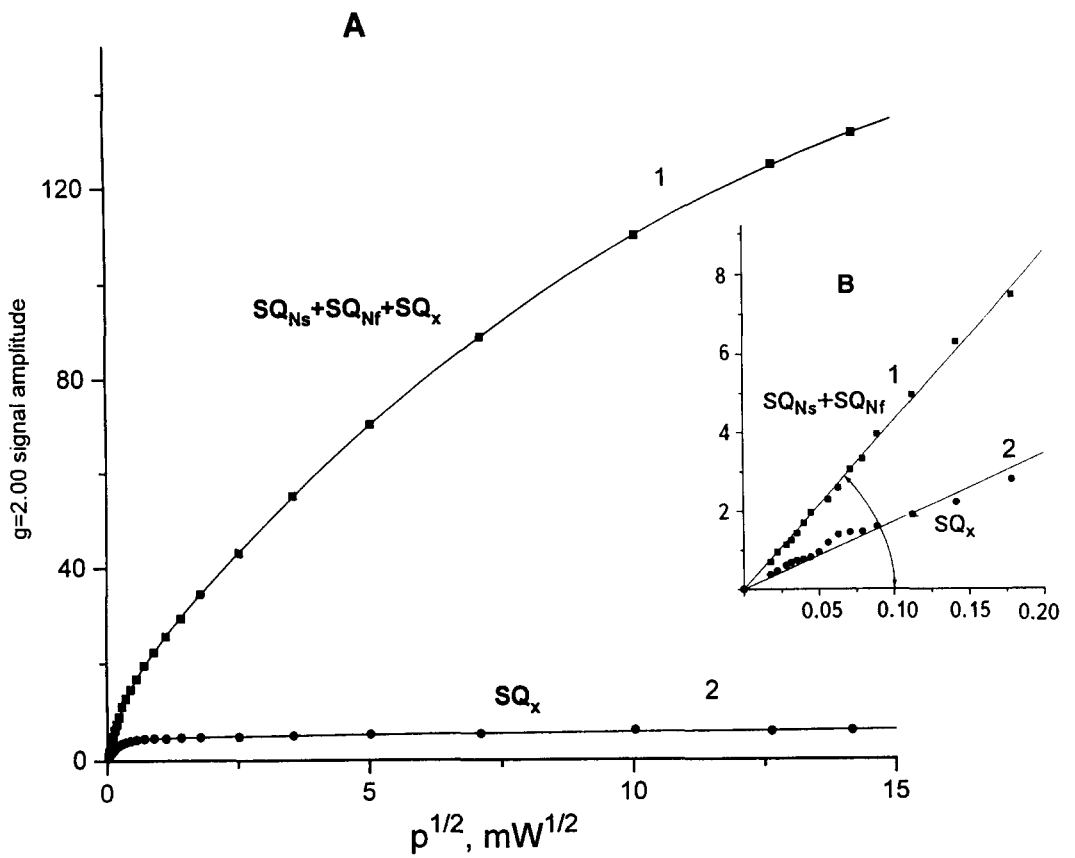

Fig. 2. Progressive power saturation of the ubisemiquinone $g = 2.00$ radical EPR signal recorded at 40 K during steady-state NADH oxidation in the absence (1) and presence of 30 μM CCCP (2). Samples were prepared as described in 'section 2'. The panel (B) shows the zoomed linear region of the power saturation curves at low microwave power levels. EPR conditions: microwave frequency, 9.452 GHz; modulation frequency, 100 kHz; modulation amplitude, 0.63 mT; scanning rate, 7.15 mT/min. Best fit theoretical curves are drawn through the experimental points, corresponding to the sum of individual power saturation curves (see 'section 2') with the following parameters: for curve (1): $C_1 = 18.7 \pm 0.7$; $P_{1/2(1)} = 0.057 \pm 0.008$ mW; $b_1 = 0.98 \pm 0.02$ (SQ_x); $C_2 = 13.2 \pm 0.7$; $P_{1/2(2)} = 0.65 \pm 0.12$ mW; $b_2 = 1.38 \pm 0.05$ (SQ_{Ns}); $C_3 = 12.7 \pm 0.1$; $P_{1/2(3)} = 167.4 \pm 6.8$ mW; $b_3 = 1.00$ (SQ_{Nf}); for curve (2): $C_1 = 18.7 \pm 0.7$; $P_{1/2(1)} = 0.057 \pm 0.008$ mW; $b_1 = 0.98 \pm 0.02$ (SQ_x).

tightly coupled and uncoupled particles as has been shown by Albracht and his collaborators [23] and is confirmed in the present report (Fig. 1). For further investigation of this difference, the spectra of the $g_{//}$ 2.05 region were examined in more details (Fig. 4). When cluster N2 is reduced by NADH in tightly coupled particles (spectrum B), the amplitude of the $g = 2.054$ signal is significantly decreased as compared with that of the uncoupled particles (spectrum A). Concomitantly, two new peaks at $g = 2.044$ and $g = 2.064$ appeared. The spectrum (C) of the split signal can be obtained by the weighted subtraction of spectrum (A) from (B). A close magnetic interaction between spins of the reduced cluster N2 and ubisemiquinone (SQ_{Nf}) has been suggested previously, based on the very rapid spin relaxation of the specifically bound ubisemiquinone [18]. The dipolar coupling between two spins leads to a broadening or splitting of the line. The latter is proportional to r^{-3}, where r is the distance between interacting dipoles [27–29]. Considering point dipolar approximation and the fact, that the difference in Zeeman terms for N2 and SQ_{Nf} is much larger than the spin coupling term, a distance between N2 and SQ_{Nf} can be calculated using the equation: $\Delta B = \beta \cdot g \cdot r^{-3} \cdot (1-3\cos^2\theta)$, where ΔB is the split peak separation (in magnetic field units), r is the distance between the interacting dissimilar species, g is the g-factor of the free radical and θ is the angle between interdipolar vector and $g_{//}$ direction of N2 [27]. ΔB is 3.3 mT for the $g_{//}$ splitting of N2. If spherically averaged value for the angular term is considered, an expectation value for the distance r is ~8 Å; the upper value (when $\theta = 0$) is 10.4 Å. Since the $g_{//}$ of the cluster N2 is colinear with the membrane normal [30], this number corresponds to the N2–SQ_{Nf} distance when the interdipolar vector is perpendicular to the membrane plane. The abscence of significant broadening of the observable isotropic SQ_{Nf} signal is apparently originated from very rapid relaxation of N2 [29]. Some asymmetry of the split signals is mostly due to different intensities of the background signals in samples A and B from the spin-coupled $SQ^{-\bullet}$ pair in the succinate-Q oxidoreductase segment of the respiratory chain.

4. Discussion

A consensus on the ubisemiquinones as the obligatory intermediates during the electron transfer from NADH to the ubiquinone pool in the mitochondrial membranes has been reached [18–21]. The previous failure to observe Complex I-associated ubisemiquinone in presteady-state kinetic experiments [31] was apparently due to the use of inappropriate submitochondrial

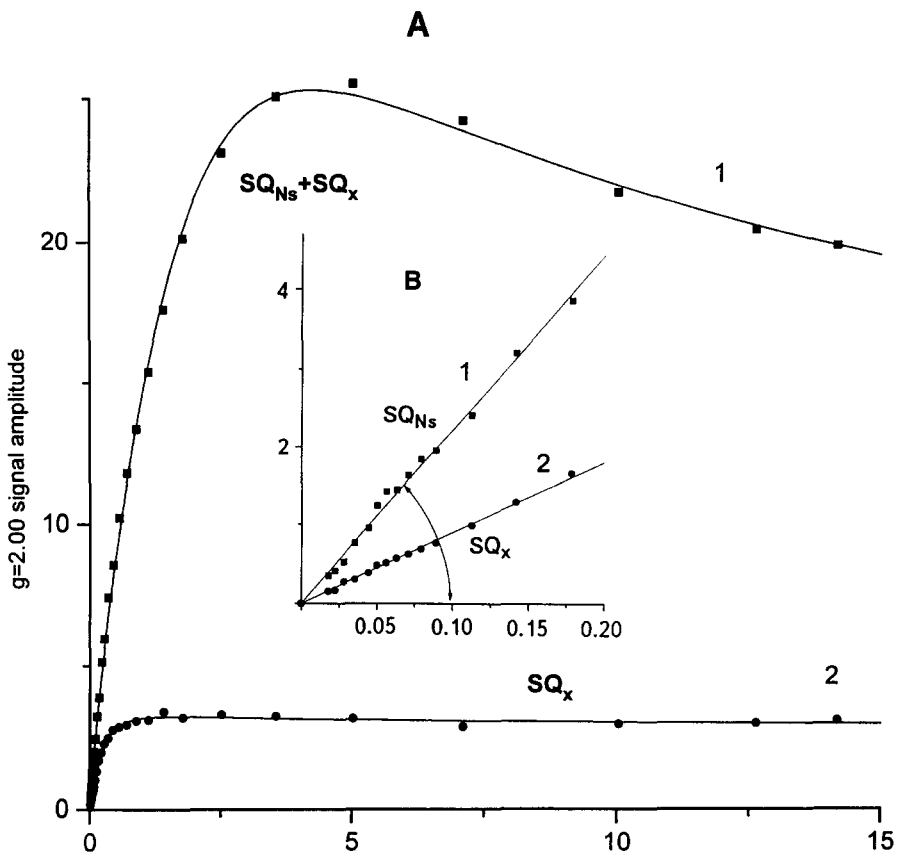

Fig. 3. Progressive power saturation of the $g = 2.00$ radical EPR signal recorded at 105 K during steady-state NADH oxidation in the absence (1) and presence of 30 mM CCCP (2). Samples were prepared as described in 'section 2'. The panel (B) shows the zoomed linear region of the power saturation curves at low microwave power levels. EPR conditions were the same as in Fig. 2 except for the sample temperature (105 K). Best fit theoretical curves are drawn through the experimental points, corresponding to the sum of individual power saturation curves (see 'section 2') with the following parameters: for curve (1): $C_1 = 10.1 \pm 0.4$; $P_{1/2(1)} = 0.12 \pm 0.01$ mW; $b_1 = 1.04 \pm 0.01$ (SQ_x); $C_2 = 12.4 \pm 0.2$; $P_{1/2(2)} = 7.42 \pm 0.53$ mW; $b_1 = 1.40 \pm 0.03$ (SQ_{Ns}); for curve (2): $C_1 = 10.1 \pm 0.4$; $P_{1/2(1)} = 0.12 \pm 0.01$ mW; $b_1 = 1.04 \pm 0.01$ (SQ_x).

particle preparations which may have contained almost completely deactivated Complex I [25]. In this paper we have presented experiments aimed at further investigation of the Complex I-associated ubisemiquinones and to provide an explanation for the apparent discrepancies reported for the differences between tightly coupled and uncoupled preparations. Original studies on the rotenone-sensitive ubisemiquinones were performed at rather high microwave power and low temperature [18,19] and it was concluded that the steady-state semiquinone concentration is $\Delta\bar{\mu}_H$+-dependent. In this report we have presented evidence that two distinct species of the specifically bound ubisemiquinone, with different spin relaxation properties, are present during steady-state NADH oxidation. Two independent approaches, computer de-convolution of the power saturation curves (Figs. 2A and 3A) and direct spin quantitation of individual SQ species under non-power saturated conditions (Figs. 2B and 3B), showed strong effect of an uncoupler on both spin-relaxation behavior and the concentration of SQ free radicals. The results obtained are in agreement with the previously postulated participation of two ubisemiquinone molecules (SQ_{Nf} and SQ_{Ns}) bound at spatially separated and environmentally different sites, participating in the terminal proton translocation step in Complex I [22].

The striking change in the spectral feature of the $g_{//}$ peak of the cluster N2 in coupled particles originally detected by Albracht's group was interpreted as a shift to higher fields due to a structural difference of the cluster coordination in the energized and non-energized particles [24]. We have shown here that the alteration of the $g_{//}$ peak arises from the spin–spin interaction of the N2↔SQ_{Nf} pair. In the case of least background interference the splitting seems to be symmetric, indicating mostly dipolar spin coupling. The distance between 8 and 11 Å calculated from the coupling constant of 1.65 mT is in accordance with only slight broadening of the SQ_{Nf} signal under non-power saturated conditions. However, if exchange component of the coupling is significant, the distance could be as long as 12 Å. A consistent distance estimate was obtained based on the enhanced spin relaxation of the SQ_{Nf} by the paramagnetic cluster N2 (not shown). The lack of significant change in the $g_{\perp}$ transition of the N2 signal (Fig. 1) can be explained by a strong anisotropy of the system.

Determination of the distance between N2 and SQ_{Nf} is an important first step in the studies of the spatial location of the redox components in the terminal region of Complex I. It would be of great interest to evaluate the spatial arrangement of all relevant EPR-detectable species (SQ_{Ns}, SQ_{Nf} and N2)

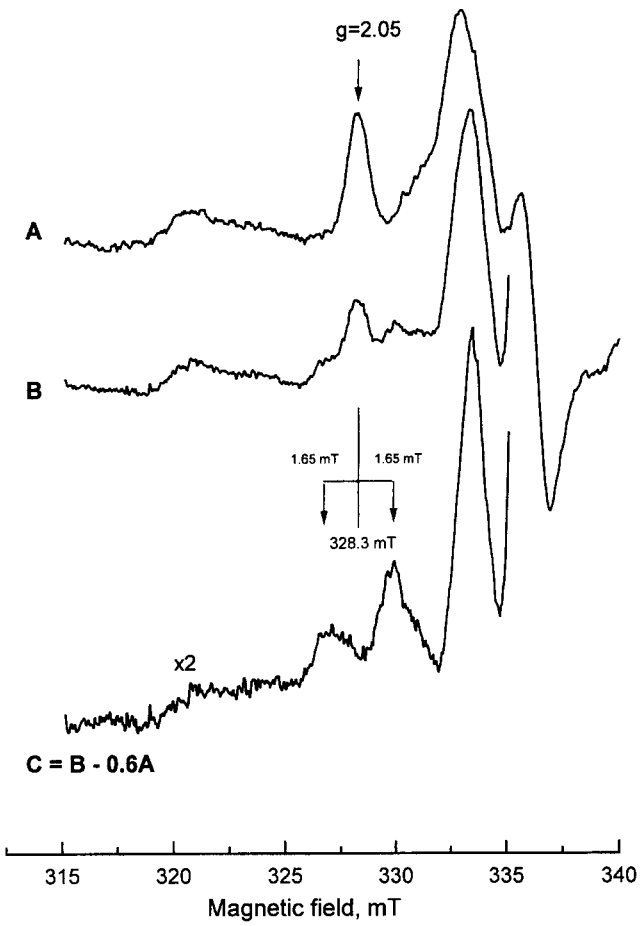

Fig. 4. EPR spectra of the $g_{//}$ region of the N2 iron–sulfur cluster in SMP during steady-state oxidation of NADH. (A) SMP were treated with 30 μM CCCP prior to mixing with NADH; (B) coupled SMP as a control; (C) difference between spectra (B) and (A) weighted in order to eliminate the central $g = 2.05$ signal and to show the split signal, indicated with arrows. Numbers correspond to the field position of the central $g_{//}$ peak and the coupling constants. EPR conditions were as in Fig. 1.

relative to each other and to the C- and M-side surfaces of the energy transducing membrane. Work aimed to reach this goal is currently under way in our laboratories.

Acknowledgements: This work was partly supported by National Institute of Health Fogarty International Research Collaborative Grant RO3 TW 00140-01A2 to T.O. and A.D.V., by Russian Foundation for Fundamental Research Grant 93-04-20214 and by the Russian Federal program 'Russian Federation Universities' to A.D.V., and by the NIH Grant GM 30376 to T.O. Thanks are due to Drs. J.C. Salerno and P. Angelolillo for their helpful and stimulating discussions.

References

[1] Hatefi, Y. (1985) Annu. Rev. Biochem. 54, 1015–1069.
[2] Walker, J.E. (1992) Quart. Rev. Biophys. 25, 253–324.
[3] Mini-review series 'NADH-Quinone Oxidoreductase: the most complex Complex' (T. Ohnishi, ed.) (1993) J. Bioenerg. Biomembr. 25, 323–391.
[4] Fearnley, J.M. and Walker, J.E. (1992) Biochim. Biophys. Acta 1140, 105–134.
[5] Weiss, H., Friedrich, T., Hofhaus, G. and Preis, D. (1991) Eur. J. Biochem. 197, 563–576.
[6] Yagi, T., (1993) Biochim. Biophys. Acta, 1141, 1–17.
[7] Friedrich, T., Weidner, U., Nehls, U., Fecke, W., Schneider, R. and Weiss, H. (1993) J. Bioenerg. Biomembr. 25, 331–337.
[8] Rao, N.A., Felton, S.P., Huennekens, F.M. and Mackler, B. (1963) J. Biol. Chem. 238, 449–455.
[9] Ohnishi, T. and Salerno, J.C. (1982) in Iron-sulfur proteins, Vol. IV (T. Spiro, ed.) Wiley Publishing Co. Inc., New York, pp. 285–327.
[10] Sled, V.D., Friedrich, T., Leif, H., Weiss, H., Meinhardt, S.W., Fukumori, Y., Calhoun, M., Gennis, R.B. and Ohnishi, T. (1993) J. Bioenerg. Biomembr. 25, 347–356.
[11] Krishnamoorthy, G. and Hinkle, P. (1988) J. Biol. Chem. 263, 17566–17575.
[12] Sled, V.D., Rudnitzky, N.I., Hatefi, Y. and Ohnishi, T. (1994) Biochemistry 33, 10069–10075.
[13] Vinogradov, A.D. (1994) Biophys. J. 66, A1 22.
[14] Mitchell, P. (1979) David Keilin's Respiratory Chain Concept and Its Chemiosmotic Consequences, Nobel Foundation, Stockholm.
[15] Ragan, C.I. (1987) Curr. Top. Bioenerg. 15, 1–36.
[16] Weiss, H. and Friedrich, T. (1991) J. Bioenerg. Biomembr. 23, 743–754.
[17] Suzuki, H. and King, T.E. (1983) J. Biol. Chem. 258, 352–358.
[18] Burbaev, D.Sh., Moroz, I.A., Kotlyar, A.B., Sled, V.D. and Vinogradov, A.D. (1989) FEBS Lett. 254, 47–51.
[19] Kotlyar, A.B., Sled, V.D., Burbaev, D.Sh., Moroz, I.A. and Vinogradov, A.D. (1990) FEBS Lett. 264, 17–20.
[20] De Jong, A.M.P. and Albracht, S.P.J. (1994) Eur. J. Biochem. 222, 975–982.
[21] Van Belzen, R. and Albracht, S.P.J. (1989) Biochim. Biophys. Acta 974, 311–320.
[22] Vinogradov, A.D. (1993) J. Bioenerg. Biomembr. 25, 367–375.
[23] Vinogradov, A.D., Sled, V.D., Maklashina, E.O., Burbaev, D.Sh. and Moroz, I.A. (1991) Biol. Chem. Hoppe-Seyler 372, 553.
[24] De Jong, A.M.P., Kotlyar, A.B. and Albracht, S.P.J. (1994) Biochim. Biophys. Acta 1186, 163–171.
[25] Kotlyar, A.B. and Vinogradov, A.D. (1990) Biochim. Biophys. Acta 1019, 151–158.
[26] Rupp, H., Rao, K.K., Hall, D.O. and Cammack, R. (1978) Biochim. Biophys. Acta 537, 255–269.
[27] Abragam, A. and Bleaney, B. (1970) Electron paramagnetic resonance of transition ions, Calendar Press, Oxford.
[28] Eaton, G.R. and Eaton, S.S. (1976) in Spin labeling, theory and application (L.J. Berliner, ed.) Academic Press, New York, v.3, pp. 339–397.
[29] Leigh, J.S. (1970) J. Phys. Chem. 52, 2608–2612.
[30] Salerno, J.C., Blum, H. and Ohnishi, T. (1979) Biochim. Biophys. Acta 547, 270–281.
[31] Albracht, S.P.J., Van Belzen, R. and De Jong, A.M.P. (1991) Biol. Chem. Hoppe Seyler 372, 547.