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Energy-induced structural changes in NADH : Q oxidoreductase of the mitochondrial respiratory chain

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Abstract

The reaction of coupled submitochondrial particles (SMP) with NADH was studied in the absence and presence of the uncoupler gramicidin, both in pre-steady-state and steady-state experiments. It was shown that the formation of ubisemiquinones associated with NADH : Q oxidoreductase is insensitive to uncouplers. It was found, however, that in the absence of gramicidin the ubisemiquinone showed a noticeably faster relaxation than in the presence of this uncoupler. During steady-state oxidation of NADH by coupled submitochondrial particles, the EPR signal of iron-sulphur cluster 2 of complex I, the cluster that is generally believed to be the electron donor for ubiquinone, showed some remarkable changes. Its g_z line seemed to disappear from the spectrum, although the g_{xy} line remained clearly present. Detailed EPR analysis indicated that (a component of) the g_z line shifted to higher field. The temperature dependence of the EPR signal of cluster 2 was affected as well. In the presence of uncoupler the EPR properties of cluster 2 were indistinguishable from those in particles that showed no intrinsic coupling. These experiments strongly indicate that the coordination of cluster 2 is different in energized and non-energized SMP. The pre-steady-state reaction between these submitochondrial particles and NADH showed that the uncoupler-sensitive changes in both the ubisemiquinone and cluster 2 became effective between 9 ms and 30 ms. Similar changes were observed during succinate-driven reverse electron transfer. This report shows, for the first time, energy-induced structural changes in NADH : Q oxidoreductase.

Key words: NADH : Q oxidoreductase; Ubisemiquinone; Energy coupling; Pre-steady-state kinetics; Fe-S cluster; (Bovine heart mitochondria)

1. Introduction

NADH : Q oxidoreductase (EC 1.6.5.3; also named complex I) is the largest enzyme of the mitochondrial respiratory chain and is now thought to consist of at least 41 polypeptides [1–3], adding up to a molecular mass of at least 880 kDa, assuming that each subunit is present once [2]. The enzyme catalyzes the oxidation of NADH and the transfer of two electrons to ubiquinone. The electron transfer through complex I is coupled to vectorial proton translocation with a $H^+/2e^-$ stoichiometry of 4 to 5 [1,4]. The electron carriers of the redox enzyme are FMN and at least four EPR-detectable Fe-S clusters. One of these clusters, cluster 1b, is a binuclear centre; clusters 2, 3 and 4 are tetranuclear centres (for reviews, see [5–8]; note that Ohnishi et al. [5] name these clusters N-1b, N-2, N-4 and N-3 respectively).

The electron transfer through complex I has been the subject of many investigations. There is a considerable amount of evidence, presented by some of us previously, that NADH dehydrogenase is a structural and functional dimer (for model, see e.g. [9]). The detected concentration of one of the Fe-S clusters, cluster 1b ($g_{x,y,z} = 1.92, 1.94, 2.02$) is one half of the concentration of cluster 2 ($g_{x,y,z} = 1.92, 1.92, 2.05$), as was shown for both isolated enzyme and submitochondrial particles [6,10,11]. Pre-steady-state kinetic studies have shown that NADPH can only reduce the cluster 1b-lacking protomer [12–14]. Furthermore, it is

Abbreviations: BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; Q_{10}, ubiquinone; RC, respiratory control; SMP, submitochondrial particles.

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possible to completely inhibit NADH oxidation in particles with one molecule of piericidin A per two clusters 2 [9].

The mechanism of the reaction of complex I with quinone is still not known. It is becoming clear, however, that the formation of ubisemiquinones is an obligatory step in electron transfer from complex I to ubiquinone. The first reports on radical signals in SMP, proposed to be located in the complex I region of the respiratory chain, date back to 1970 [15,16]. In 1983 Suzuki and King reported on semiquinone radicals in isolated complex I [17]. They suggested that these radicals, that were almost abolished by addition of rotenone, were bound to the protein. Later Suzuki and Ozawa reported on a ubiquinone-binding protein in isolated complex I, containing one mole of quinone per mole of enzyme [18,19]. More recently one of us was involved in a study on complex-I-associated ubisemiquinones in coupled submitochondrial particles [20,21]. The EPR radical signal was detected only in the presence of oligomycin, which was added in order to increase the respiratory control to 7–9. The signal disappeared in the presence of uncouplers. The stoichiometry of ubisemiquinone per cluster 2 was found to be maximally 0.5.

Recently some of us have brought forward kinetic evidence that ubisemiquinones form an obligatory intermediate in the reaction of NADH dehydrogenase with ubiquinone in submitochondrial particles, independent of the type of preparation used or the degree of coupling of the preparation [22]. Both in submitochondrial particles (coupled or uncoupled) and in digitonin-treated mitochondria the concentration of ubisemiquinone, showing up within 40 ms after mixing with NADH, was at least half the concentration of cluster 2. It was also demonstrated that after temperature-induced reversible inactivation of complex I [23] no ubisemiquinones could be detected within 200 ms in the reaction with NADH.

In this paper experiments are described that provide an explanation for the apparent discrepancies reported for the effect of uncoupling upon ubisemiquinones in submitochondrial particles [20–22]. Further investigations of the ubisemiquinone formation in coupled submitochondrial particles are described, both during the NADH oxidation reaction and during reverse electron transfer. For the first time we have observed a pronounced effect of energization on the EPR characteristics of the ubisemiquinone signals and the Fe-S clusters of NADH : Q oxidoreductase.

2. Materials and methods

NADH, NADPH and NAD^+ were obtained in the purest form available from Boehringer (Mannheim). Gramicidin D was purchased from Sigma (USA). All other chemicals were of analytical grade. Coupled submitochondrial particles were prepared, treated with oligomycin (0.5 μg/mg protein) for optimal coupling and activated with 1 mM NADPH under oxygen at room temperature as described earlier [20]. Succinate dehydrogenase was activated with malonate which was present during the column filtration [20]. The active SMP were stored in liquid nitrogen until use. The particles thus prepared were capable of ATP-independent succinate-driven reverse electron transfer. For the experiments described in this report different preparations of SMP were used; one of these preparations showed a respiratory control of 11 with NADH, the other preparations showed a respiratory control of 3–4 with NADH. Reversible inactivation of the NADH dehydrogenase was established by incubating the active SMP at 37°C for 10 min. Enzymatic activities were assayed at room temperature (25°C). The ATP-independent reverse electron transfer activity was measured by following NADH formation at 340 nm in a Zeiss spectrophotometer (type M4 QIII) in 2 ml 0.25 M sucrose, 1 mg/ml BSA, 5 mM NAD^+, 20 mM succinate, 0.2 mM EDTA and 20 mM Hepes (potassium salts, pH 8.0). The oxidation of NADH was followed at 340 nm in a Zeiss spectrophotometer in 0.25 M sucrose, 1 mg/ml BSA, 0.2 mM NADH, 1 μg/ml gramicidin, 0.2 mM EDTA and 20 mM Hepes (potassium salts, pH 8.0). The catalytic activities of the preparations used here were nearly identical to those described in [23]. The respiratory control (RC) was calculated as the ratio between the rates of NADH oxidation after and before the addition of gramicidin. Protein concentrations were determined with the biuret reaction [24]. Rapid-mixing rapid-freezing experiments were performed at room temperature (25°C) as described by De Vries et al. [25]. EPR-samples with reaction times longer than 200 ms were usually prepared with the rapid-mixing apparatus, but for these samples the reaction mixture was directly collected in an EPR tube instead of being sprayed into cold isopentane (133 K). After a certain time the EPR tube was rapidly immersed in cold isopentane. Some of the samples, with reaction times longer than 3 s were prepared by mixing the substrate by hand with the SMP in the EPR tube and subsequent freezing in cold isopentane. X-Band EPR measurements (9 GHz) were obtained with a Bruker ECS106 EPR spectrometer, equipped with an Oxford Instruments ESR900 helium flow cryostat with an ITC4 temperature controller. The magnetic field was calibrated with an AEG Magnetic Field Meter. The frequency was measured with a HP 5350B Microwave Frequency Counter. Spectra were simulated as described by Beinert and Albracht [8]. Ubisemiquinone concentrations were determined by direct double integration of an experimental spectrum

(recorded with 0.01 mW at 77 K). The concentration of cluster 2 was determined from a comparison of an experimental spectrum (recorded at 17 K, a microwave power of 2.6 mW and a modulation amplitude of 1.27 mT) with a simulated lineshape. The parameters used for the simulation were $g_{x,y,z} = 1.925$, 1.925 and 2.053 and widths $(x,y,z) = 1.88$, 1.88 and 1.18 mT.

3. Results

3.1. Radicals during NADH oxidation, under coupled and uncoupled conditions

As mentioned in the introduction, there is an apparent discrepancy between the results obtained with coupled SMP prepared according to [20,21] and those obtained with MgATP SMP [22] with respect to the effect of uncoupling on the formation of ubisemiquinones associated with NADH dehydrogenase. To further investigate this, the reaction of active coupled SMP with NADH was studied in comparison with the reaction of these SMP uncoupled by the addition of 20 μM gramicidin. EPR samples of the preparations were made with a reaction time of 1 s. SMP in the presence or absence of uncoupler showed a prominent radical signal under these conditions. In either case the radical gave rise to an EPR signal with $g = 2.0041$, with a linewidth of 0.83 mT, that reached a concentration of one radical per cluster 2 within 30 ms (Table 1). The presence of 20 μM rotenone completely abolished this radical signal. Also after reaction times of 120 s, when the sample was fully anaerobic, no radicals were present anymore (not shown). It is concluded that both coupled SMP and SMP uncoupled with gramicidin showed radical signals during steady-state electron transfer from NADH to ubiquinone; these radicals were the same with respect to g-value, linewidth and intensity.

A notable difference between the radical formed in the absence and presence of gramicidin, however, was the saturation behaviour of the EPR signal. In Fig. 1 the power dependence at 40 K of the radical signal in samples of coupled SMP with NADH in the presence and absence of gramicidin (reaction time 1 s) is shown. The radical showed a faster relaxation in the absence of gramicidin. The same measurements were done at different temperatures between 17 and 80 K. The difference in saturation behaviour was most prominent at 40 K.

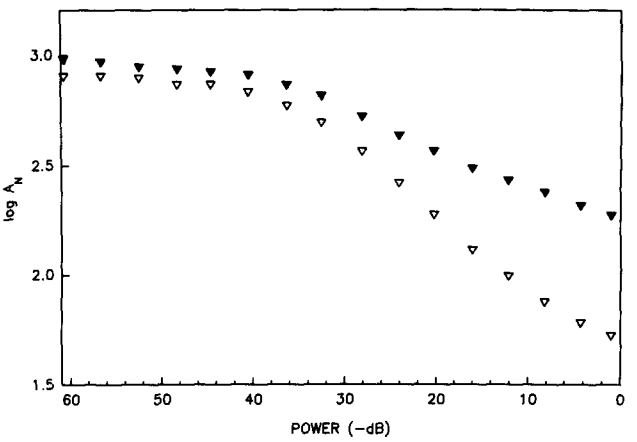

Fig. 1. Microwave-power dependence of the radical EPR signal during NADH oxidation in the absence and presence of uncoupler. Coupled SMP (RC 3–4) were mixed with 10 mM NADH in the rapid-mixing apparatus. The reaction was quenched in cold isopentane after 1 s. (▼) Without uncoupler. (▽) The syringe with SMP contained 20 μM gramicidin in DMSO (final DMSO concentration 0.2%). The control sample contained an equal amount of DMSO. On the y-axis the logarithm of the radical amplitude, normalized for the differences in microwave power and receiver gain (log A_N, in arbitrary units) is plotted. EPR conditions: microwave frequency, 9430 MHz; modulation amplitude, 0.45 mT; temperature, 40 K. The g_{xy} amplitude of the cluster 1b signal recorded at 50 K and 2.6 mW was used as an internal standard.

In order to distinguish in this report between the two types of radicals the fast relaxing radical will be called 'coupled', the other one 'uncoupled'.

3.2. Radicals in the succinate-driven reverse electron transfer reaction

The submitochondrial particles used for the experiments described in this report, with a respiratory control of at least 3–4, were capable of ATP-independent succinate-driven reverse electron transfer (250 nmol NADH min^{-1} mg^{-1}, 25°C). To study the reaction of coupled SMP with succinate, EPR samples were made with reaction times of 1 or 4 s. For the samples with a reaction time of 4 s the SMP were diluted to 6–12 mg/ml, in order to maintain aerobic conditions. EPR spectra of these samples showed a radical signal, which was exactly the same as observed during NADH oxidation with respect to g-value, linewidth and saturation behaviour of the coupled radical. Steady-state succinate oxidation in uncoupled SMP also led to the ap-

Table 1
Radical concentration in different samples of coupled submitochondrial particles during oxidation of NADH; the concentrations are given relative to that of Fe-S cluster 2

Sample		−Gramicidin	+Gramicidin
	Reaction time	radical per cluster 2	radical per cluster 2
SMP (RC 11)+NADH	10 s	2.0	–
	30 s	2.1	–
SMP (RC 3–4)+NADH	9 ms	0.9	0.6
	30 ms	1.1	1.0
	1 s	1.5	1.3
SMP (RC 3–4)+succinate	1 s	2.5	–

pearance of radical signals, the significance of which is discussed later.

3.3. The effect of coupling on the Fe-S clusters of complex I

During NADH oxidation in coupled SMP in the presence of gramicidin, the EPR signals of the Fe-S clusters of NADH dehydrogenase had lineshapes indistinguishable from those in isolated complex I, uncoupled SMP or MgATP SMP, whereas the radical signal was present in its uncoupled form. In the absence of gramicidin, however, the signal of cluster 2 showed a clear difference. When a coupled sample was frozen after anaerobiosis had set in, the signal of cluster 2 was normal again. The first difference concerned the g_z line of cluster 2 ($g = 2.053$). In coupled particles the g_z line seemed to disappear from the EPR spectrum, as can be seen in Fig. 2. In this figure EPR spectra are shown of active or inactive SMP with a respiratory control of 3–4 or 11, reduced with NADH in the absence and presence of uncoupler; the spectra were recorded such as to focus on the EPR signals of the Fe-S clusters.

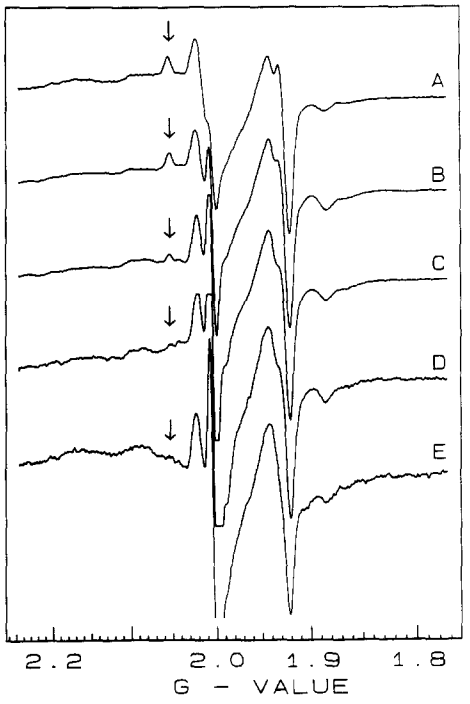

Fig. 2. EPR spectra of coupled submitochondrial particles during NADH or succinate oxidation. Coupled SMP were mixed with substrate to a final concentration of 5 mM NADH (A–D) or 20 mM succinate (E). Samples B, C and E were prepared by the rapid-mixing rapid-freezing technique; for samples A and D the SMP and substrate were mixed by hand. (A) Inactivated SMP (RC 3–4), frozen 5 s after mixing with NADH. (B) Active SMP (RC 3–4) in the presence of 20 μM gramicidin, frozen in 1 s after mixing with NADH. (C) As (B), but in the absence of gramicidin. (D) Active SMP (RC 11), frozen in 10 s after mixing with NADH. (E) As (C), but frozen in 1 s after mixing with succinate. EPR conditions: microwave frequency, 9430 MHz; microwave power incident to the cavity, 2.6 mW; modulation amplitude, 1.27 mT; temperature, 17 K. The spectra were plotted with the same amplitude for the trough at the $g = 1.92$ position. The arrows in the spectra indicate the position of the g_z line of unperturbed cluster 2.

Fig. 2 contains two spectra of samples with a normal cluster 2 lineshape, namely inactive SMP (A) and SMP with gramicidin (B), both reduced with NADH. The other spectra, of samples of both NADH and succinate reduced samples, showed the changes in the cluster 2 lineshape to a variable extent. Note that at 17 K and 2.6 mW the radical signal (especially the uncoupled form) is saturated to a considerable extent. When the spectra of SMP mixed with NADH in the absence and presence of gramicidin are compared (spectra B and C), it can be seen that the g_{xy} lines of cluster 2 at $g = 1.92$ are present with the same apparent linewidth in both samples. The g_z line in trace C is, however, considerably smaller than expected. In the sample with a respiratory control of 11 (trace D) no apparent g_z line could be observed at all, although the g_{xy} line was clearly present. To further investigate this point some detailed spectra of the $g = 2.05$ region have been recorded.

From Fig. 3 it can be seen that the g_z line of cluster 2 ($g = 2.053$) is present in the spectrum of coupled SMP which had reacted with NADH for 1 s (spectrum C). The amplitude of this line seemed to be much smaller than that observed in the presence of uncoupler (trace B) or that observed with inactive SMP (trace A). It seemed as if an extra line in the active coupled sample had appeared at position $g = 2.044$, which was missing in the inactive sample and also in the uncoupled sample. This could be a possible candidate for (a component of) the g_z of a modified cluster 2. Also the succinate sample of 4 s clearly showed the changed features in the $g = 2.04$–2.05 region (Fig. 3, spectrum D).

The temperature dependence of the EPR signal of cluster 2 was also different in coupled SMP during steady-state oxidation of NADH. The remaining $g = 2.053$ line in the coupled samples seemed to disappear when going down from 17 K to 4.2 K at a non-saturating microwave power (Fig. 4-II), unlike the control sample of inactive SMP (Fig. 4-I) or a sample taken after 2 min (not shown). The lineshape of the g_{xy} line of cluster 2 seemed to be hardly affected in the coupled form, but the temperature dependence of the g_{xy} was different from that in the uncoupled form. This could be seen from the line shapes of the g_{xy} line of this cluster between 4.2 and 25 K. Unlike in inactive SMP, where the signal started broadening only at temperatures from 20 K upwards, the g_{xy} line in the coupled form broadened considerably above 16 K already, whereas optimal sharpening was only detected below 8 K. Note that only a minor radical signal was

present in inactive SMP (Fig. 4-I) and that the g_z line of cluster 2 appeared normal.

Comparison of the spectra of coupled SMP at temperatures of 4.2 K to 25 K (Fig. 4-II) with those recorded at 17 K and 2.6 mW (Fig. 2, traces C and D), raised the question whether the EPR conditions used in Fig. 2 were suitable for optimal detection of the cluster 2 signal in coupled SMP. Fig. 4-II suggested that at least the g_{xy} line of cluster 2 in energized preparations should be recorded at lower temperatures. In Fig. 5 a spectrum is shown of the same sample of active SMP mixed with NADH (reaction time of 5 s; also used for trace D in Fig. 4-II). The spectrum was recorded with a modulation amplitude of 0.64 mT, while the scan time and time constant were increased 4-fold, and the scan width 1.7-fold. Recorded under these conditions, the structure of the g_z-region of cluster 2 is more clear.

There is an additional feature in the EPR spectrum that is different in coupled preparations during steady-state oxidation of NADH: the lineshape in the $g = 1.93$–1.96 region (Fig. 4 and traces A–D of Fig. 2). The changes observed here are not due to cluster 1b, the lineshape of which did not change, when measured at 50 K (data not shown). The other Fe-S clusters of NADH dehydrogenase do not seem to be affected by the presence of the coupled radical signal.

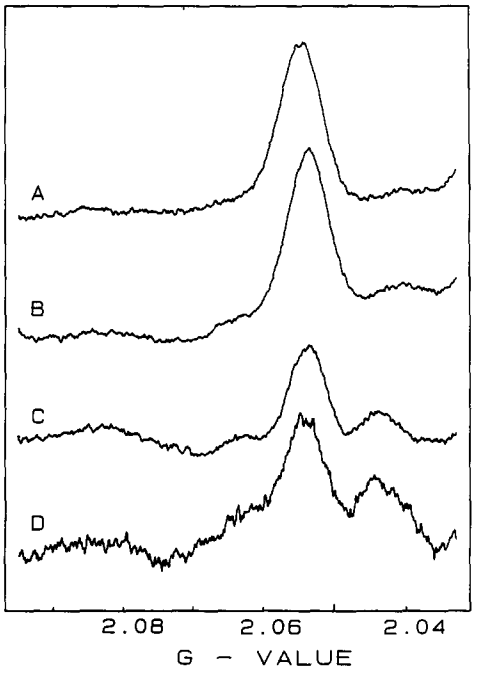

Fig. 3. Detailed EPR spectra of the g_z region of cluster 2 in coupled submitochondrial particles frozen during NADH or succinate oxidation. Coupled SMP (RC 3–4) were mixed with substrate to a final concentration of 5 mM NADH (A–C) or 20 mM succinate (D). (A–C) Same tubes as used for the traces A, B and C in Fig. 2. (D) Active SMP (RC 3–4), frozen in 4 s after mixing with succinate; for this sample SMP and succinate were mixed by hand. EPR conditions: microwave frequency, 9427 MHz; microwave power, 2.6 mW; modulation amplitude, 0.64 mT; temperature, 17 K. The spectra were plotted in such a way that they showed the same amplitude (not shown) for the trough at the $g = 1.92$ position.

3.4. Time dependence of the uncoupler-sensitive changes

The time dependence of the uncoupler-sensitive changes was studied via pre-steady-state kinetics of the reaction between coupled SMP and NADH, both in the absence and in the presence of an uncoupler. The reaction was quenched after reaction times of 9 and 30 ms and 1 s. In Table 1 the concentrations of the radicals relative to that of cluster 2 in these samples are given. At 9 ms the radical concentration was not yet maximal. At 30 ms the radical concentration was equal to that of cluster 2, both in the absence and in the presence of gramicidin. When the 9 and 30 ms samples of coupled SMP were compared, differences in the saturation behaviour of the radical, as well as differences in the lineshape of cluster 2 were observed. The difference in saturation behaviour of the radical is depicted in Fig. 6.

After 9 ms there was already a slight difference in saturation behaviour between the coupled and uncoupled radical signals. At 30 ms this difference noticeably increased. When the difference in saturation behaviour after 30 ms is compared to the difference observed at 1 s (Fig. 1), it can be seen that the effect hardly changed between 30 ms and 1 s. In section 3.3 it was described that the lineshape of cluster 2, more specifically the g_z line of this cluster, is different in energized complex I, when the coupled radical signal is present. In the 9 ms sample of coupled SMP oxidizing NADH, the lineshape of cluster 2 seems to be the same as in the presence of uncoupler (Fig. 7). At 30 ms the g_z of cluster 2 shows uncoupler-sensitive changes in the $g = 2.05$ region. The lineshape obtained after 1 s is virtually the same as observed at 30 ms.

From these experiments it is concluded that the uncoupler-sensitive changes appear much more slowly than the reduction of complex I and are maximal after about 30 ms.

3.5. Reverse electron transfer and the effect of rotenone

Reverse electron flow from succinate through complex I was also studied with freeze-quench experiments with coupled SMP, in which both complex I and succinate dehydrogenase were fully activated. After a reaction time of 30 ms there was, however, only a small radical signal and hardly any reduction of the [2Fe-2S] cluster of succinate dehydrogenase. No reduction of any of the clusters of complex I was observed at this time. After reaction times of 1 s, a prominent radical signal and partial reduction of succinate dehydro-

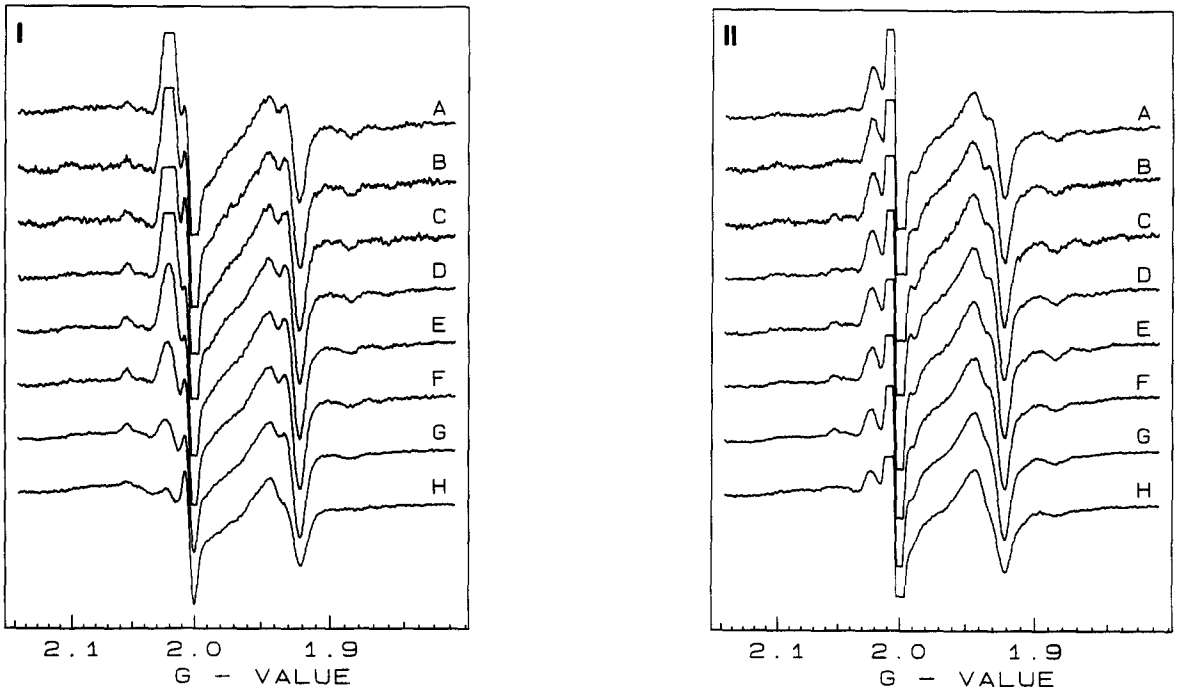

Fig. 4. Temperature dependence of the EPR signal of cluster 2 in active and inactive coupled SMP frozen during oxidation of NADH. Coupled SMP (RC 3–4) were mixed by hand with 5 mM NADH in the EPR tube and the samples were frozen after 5 s by immersing the tubes in cold isopentane. Left-hand panel (I): SMP were inactivated before mixing with NADH. Right-hand panel (II): Active SMP (same batch). EPR spectra were recorded at (A) 4.2 K, (B) 6 K, (C) 8 K, (D) 10 K, (E) 13 K, (F) 16 K, (G) 20 K and (H) 25 K. The spectra are plotted normalized for the differences in temperature, microwave power and receiver gain. EPR conditions: microwave frequency, 9427 MHz; microwave power (non-saturating), 2.6 μW (A–C), 26 μW (D), 41 μW (E), 65 μW (F), 260 μW (G,H); modulation amplitude, 1.27 mT.

genase were observed. Apparently the reaction between succinate dehydrogenase and succinate was slow (seconds), even though the enzyme was activated in the usual way [20]. The size of the radical signal obtained at 1 s was almost independent of the presence of rotenone; the power dependence changed, however. The presence of the inhibitor converted the radical signal from a coupled type to an uncoupled type. The inhibitor prevented reduction of the clusters of complex I.

3.6. *Inactive NADH dehydrogenase*

When complex I in coupled active SMP was reversibly inactivated at 37°C, the behaviour of the Fe-S clusters (including cluster 2) after short reaction times with NADH, was exactly the same as in SMP from the same batch inhibited with rotenone. A sample of inactivated SMP and NADH with a reaction time of 5 s, however, did show a small radical signal (0.3 spins per cluster 2). It was of the uncoupled type, as verified by its power dependence at 77 K, taking a sample of untreated SMP (same batch) under otherwise identical conditions as a reference.

The reaction between succinate and SMP with inactive complex I, but with active succinate dehydrogenase, was also studied. A sample frozen after 2–3 s showed a prominent radical signal, having a power dependence of an uncoupled type. Hardly any reduction of the Fe-S clusters of NADH dehydrogenase was observed. This observation bears a resemblance to that

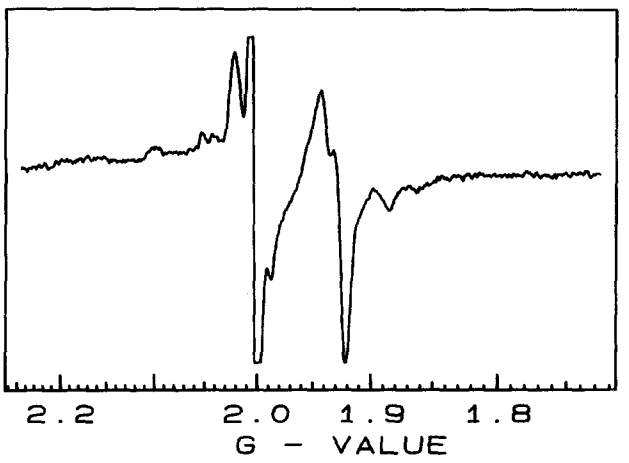

Fig. 5. The optimal lineshape of cluster 2 in energized preparations. Coupled SMP (RC 3–4) were mixed with 5 mM NADH in the EPR tube and the sample was frozen after 5 s by immersing it in cold isopentane. EPR conditions: microwave frequency, 9425 MHz; microwave power, 26 μW; modulation amplitude, 0.64 mT; temperature, 10 K.

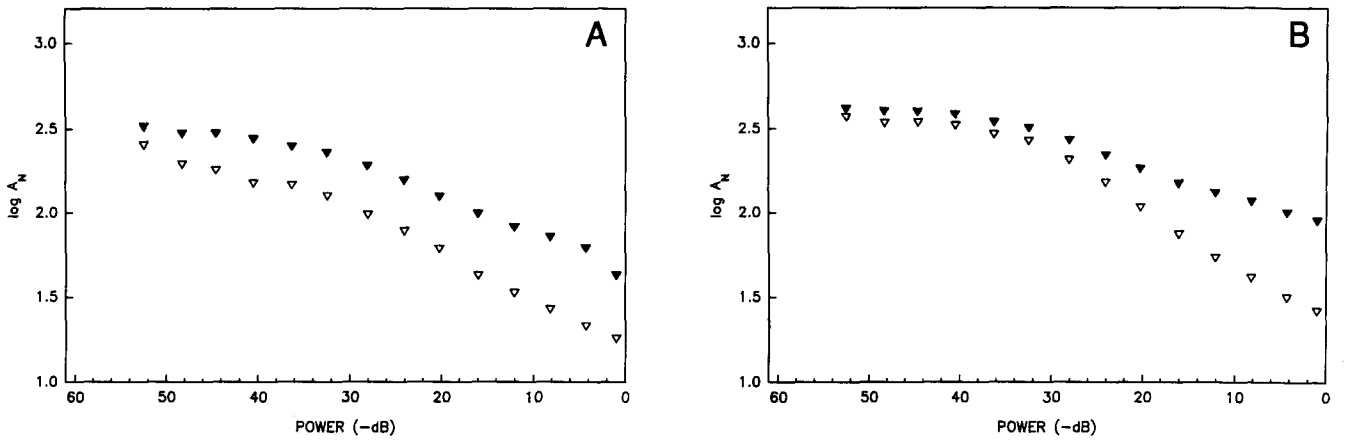

Fig. 6. Power dependence of the radical EPR signal derived during NADH oxidation in the absence and presence of uncoupler. Coupled SMP (RC 3–4) were mixed with 10 mM NADH with the rapid-mixing rapid-freezing technique. The reaction was quenched in cold isopentane after (A) 9 ms and (B) 30 ms. (▼) No gramicidin present. (▽) The syringe with SMP contained 20 μM gramicidin in DMSO (final DMSO concentration 0.2%). The control sample contained an equal amount of DMSO. On the *y*-axis the logarithm of the radical amplitude, normalized for the differences in microwave power and receiver gain (log A_N, in arbitrary units) is plotted. The EPR conditions are the same as in Fig. 1, except for the modulation amplitude, which was 1.27 mT. The g_{xy} amplitude of the cluster 1b signal recorded at 50 K and 2.6 mW was used as an internal standard.

observed with SMP and succinate in the presence of rotenone.

A coupled radical could be obtained in these SMP again as follows. The SMP preparation (20 mg/ml) with inactive complex I was treated with a small amount of NADH (0.67 mM). After 10 min at room temperature (25°C) a sample was taken for EPR measurements to check the redox state of the complexes. The sample proved to be fully oxidized; no decrease of the typical EPR signal of Cu_A(II) of cytochrome *c* oxidase or any other signs of reduction could be observed. With this preparation the reaction with succinate was studied again. After a reaction time of 2–3 s a radical signal appeared which was comparable in size to the signal in

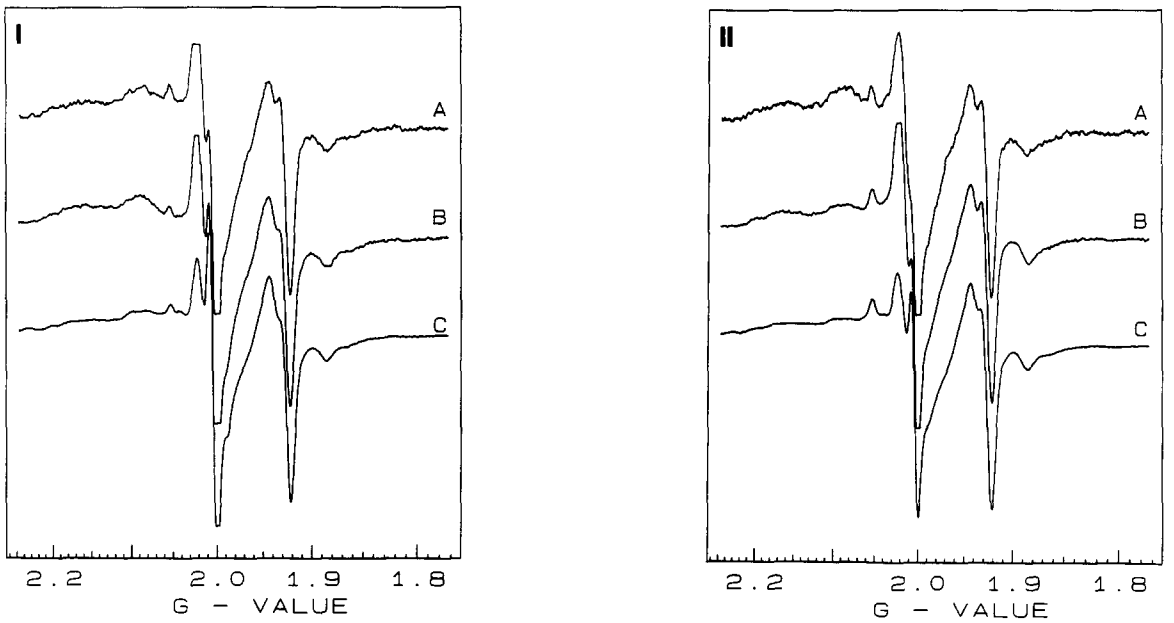

Fig. 7. EPR spectra of coupled SMP oxidizing NADH in the absence and presence of uncoupler. Coupled SMP (RC 3–4) were mixed with 10 mM NADH with the rapid-mixing rapid-freezing technique. The reaction was quenched in cold isopentane after (A) 9 ms, (B) 30 ms and (C) 1 s. Left-hand panel (I): without uncoupler. Right-hand panel (II): the syringe with SMP contained 20 μM gramicidin in DMSO (final DMSO concentration 0.2%). The control sample contained an equal amount of DMSO. The spectra are plotted with the same amplitude for the g_{xy} trough of cluster 2. EPR conditions: microwave frequency, 9430 MHz; microwave power, 2.6 mW; modulation amplitude, 1.27 mT; temperature, 17 K.

the same preparation before the treatment with NADH. Its saturation behaviour had changed, however, to that of a coupled radical. At the same time clear reduction of cluster 2 of complex I (about 85%) was observed. Apparently complex I needs to be in the active state before coupled radicals can be observed.

4. Discussion

In this report experiments have been described that resolved the apparently conflicting results on the uncoupler sensitivity of NADH-induced ubisemiquinones [20–22]. The formation of the ubisemiquinones is insensitive to uncouplers, but their spin-lattice relaxation is not (Fig. 1). Hence care must be taken to use non-saturating conditions to study these radicals. Previous studies on the coupled ubisemiquinone [20,21] were performed at a high microwave power and a rather low temperature (50 mW, 40 K). Using these conditions for the uncoupled radical will lead to a large degree of saturation (compare the intensities at 6 dB in Fig. 1). This has led to erroneous conclusions about the effect of uncoupler on the intensity of the radical signal [20,21].

A striking observation in the coupled SMP during steady-state electron transfer from NADH to quinone is the behaviour of Fe-S cluster 2 of complex I. The g_z line of this cluster apparently shifted to higher fields, where it was no longer discernible from the g_z lines of clusters 1b and 4. The g_{xy} line of cluster 2 was less affected, but showed a slight difference in temperature dependence. Both effects were uncoupler-sensitive. The change in g_z of cluster 2 reflects a structural difference of this cluster in energized and non-energized SMP. The spectra in Fig. 2 suggest that the changes in lineshape of cluster 2 are more pronounced in preparations with a high respiratory control (RC 11). These experiments demonstrate, for the first time, energy-induced changes in complex I which directly affect the structure around cluster 2. This cluster is generally believed to donate electrons directly to quinone.

Both the change in saturation behaviour of the ubisemiquinone and the change in lineshape of cluster 2 fully developed between 9 ms and 30 ms after mixing of SMP with NADH (Figs. 6 and 7). This suggests that these changes might be somehow related. One possibility is that they might be dependent on the build-up of $\Delta\mu_{H^+}$.

The studies on the reaction between coupled active SMP and succinate, in the absence and presence of rotenone, suggest that complex I apparently is able to stabilize ubisemiquinones from the Q-pool, even when its Fe-S clusters are oxidized. The experiments show that rotenone cannot prevent the formation of stable ubisemiquinones, suggesting that the rotenone-binding site is not the same site as the Q-binding site. This is in line with the present knowledge on inhibition by piericidin A; piericidin binds most effectively when both reduced cluster 2 and Q_{10} are present [9]. In the presence of rotenone, or in inactive SMP, only the uncoupled type of radical signal could be observed. In the absence of rotenone the coupled radical signal was detected. This could mean that complex I (especially cluster 2) needs to be reduced and 'energized' (changed lineshape) to generate the coupled version of the radical.

When preparations of coupled SMP, in which complex I was deliberately inactivated, were mixed with NADH and frozen after 5 s, only a small radical signal could be detected (0.3 spins per cluster 2; uncoupled type). This is comparable with the behaviour of inactive MgATP SMP, which only showed small radical signals after prolonged reaction with NADH, when partial activation of complex I could take place [22]. Apparently, in the inactivated coupled preparations, it is not possible to obtain coupled radical signals within 5 s.

A quantification of the complex I-associated ubisemiquinone concentration is rather difficult, as has been indicated before [22]. It was reported by some of us that in SMP prepared in several ways the radical concentration observed during steady-state oxidation of NADH was about equal to that of cluster 2 [22]. EPR spectra at X-band do not permit discrimination between ubisemiquinones associated with complex I and other radical signals at the $g = 2$ position, e.g. semiquinones in the ubiquinol : cytochrome c oxidoreductase and succinate dehydrogenase and flavin radicals. It was found that in MgATP SMP contributions of radical signals that were sensitive to antimycin and myxothiazol are present. It was concluded that the maximal concentration of complex I-associated radicals was about half that of cluster 2 [22]. The quantification of cluster 2 in the coupled samples likewise provides a problem, since the cluster 2 lineshape is different from the lineshape in uncoupled SMP. In that case the cluster 2 concentration was taken from the same preparation in the presence of an uncoupler. In view of these uncertainties we feel that radical concentrations obtained 30 ms after mixing of active coupled SMP with NADH, probably reflect the best estimate of the amount of ubisemiquinones: 1 radical per cluster 2 (Table 1). This is in agreement with earlier experiments [22]. In view of the effects of antimycin and myxothiazol [22] on the amount of radical produced within 40 ms after addition of NADH, we conclude that the best estimate for complex I-associated ubisemiquinones is one per two clusters 2.

In section 3.3 it has been described that the $g =$ 1.93–1.96 region of the EPR spectrum of coupled preparations during steady-state oxidation of NADH looked different from that in uncoupled preparations.

The changes observed here are not due to any of the known four Fe-S clusters of complex I. One possibility could be the involvement of an unknown cluster. From the sequences there is evidence for at least four cubane clusters, one in both the 51 kDa [26] and 75 kDa subunit [27], and two more in the TYKY subunit [28]. A binuclear cluster is located in the 24 kDa subunit [29], and there could be one more in the 75 kDa subunit [27]. Furthermore, the PGIV subunit, with eight conserved cysteines, could possibly contain an Fe-S cluster [30] and so could the PSST subunit, with three strictly conserved cysteines [31,32].

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