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Published in:
European Journal of Biochemistry

Citation for published version (APA):

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Ubisemiquinones as obligatory intermediates in the electron transfer from NADH to ubiquinone

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(Received February 2/April 5, 1994) – EJ9 94 0139/6

Until now ubisemiquinones associated with NADH:ubiquinone oxidoreductase (complex I) have been reported to occur in isolated enzyme and in tightly coupled submitochondrial particles. In this report it is shown that ubisemiquinones are always detectable during steady-state electron transfer from NADH to ubiquinone, independent of the type of inner-membrane preparation used. The EPR signal of the rotenone-sensitive ubisemiquinones could be detected not only in coupled MgATP submitochondrial particles, but also in routine preparations of uncoupled submitochondrial particles and in mitochondria. The ubisemiquinone formation in coupled preparations was completely insensitive to uncouplers. The maximal radical concentration during steady-state electron transfer from NADH to quinone was equal to that of iron-sulphur cluster 2. Experiments with antimycin, myxothiazol and 2-thenoyltrifluoroacetone demonstrated that about half of this radical was associated with complex I, giving a ubisemiquinone concentration of about 0.5 mol semiquinone/mol cluster 2. Uncoupled submitochondrial particles, prepared by extensive sonification, never showed radical signals within 100 ms after mixing with NADH. This was due to the reversible inactivation of the enzyme, caused by elevated temperatures during sonification. In preparations with deliberately heat-inactivated complex I, no radical signals were detected within 200 ms after mixing with NADH; at 1 s, however, radical formation was maximal. Yet, depending on the procedure of reactivation of the complex, in preparations previously treated to inactivate them ubisemiquinone concentrations were always less than in untreated particles. When complex I was in the active state the ubisemiquinone signal was maximal within 40 ms. The results described in this report lead to the conclusion that ubisemiquinones form obligatory intermediates in the reaction of NADH dehydrogenase with ubiquinone.

NADH:ubiquinone oxidoreductase (NADH:Q oxidoreductase; EC 1.6.5.3) is the largest and most complex enzyme of the mitochondrial respiratory chain. This enzyme, usually called complex I, catalyzes the oxidation of NADH and the transfer of electrons to ubiquinone (Qo), linked to the outward translocation of protons with a $\Delta$G° of $-5$ kcal/mol and a $\Delta$H° of $-4.5$ kcal/mol. The enzyme purified from bovine heart mitochondria contains about 41 different polypeptides, adding up to a minimal molecular mass of 880 kDa [3, 4]. The prosthetic groups are FMN and at least four EPR-detectable iron-sulphur clusters. One of these clusters, called cluster 1b or N-1b, is a binuclear Fe-S centre which had already been observed in 1960 [5]. The other Fe-S clusters are tetraneuclear clusters, called cluster 2, 3 and 4 (or N-2, N-4 and N-3 respectively) [6–14].

The electron transfer in complex I has been the topic of research for quite some time. It was observed a long time ago that all four Fe-S clusters are reduced within 5 ms after mixing with NADH, even at 4°C [9]. NADH:Q oxidoreductase is also capable of oxidizing NADPH, with a rather sharp pH optimum around pH 6. Pre-steady-state kinetics of the reaction of NADH dehydrogenase with NADPH have led to a model concerning the electron transfer pathway through NADH dehydrogenase [15–17]. It has been put forward that NADH:Q oxidoreductase exists as a structural and functional dimer (Fig. 1). NADH can react with both monomers, but NADPH can only react with the cluster-1b-deficient monomer. For steady-state oxidation of either NADH or NADPH the clusters 2a, 2b, 4a and 4b are all required. The model also explains why NADH oxidation in particles can be inhibited completely by one mole of piericidin A/two moles of cluster 2 [18]. Furthermore, proper quantifications of the EPR signals of the clusters 1b and 2 result in relative concentrations of one cluster 1b ($g_{1b}$ = 1.92, 1.94, 2.02) per two clusters 2 ($g_{2}$ = 1.92, 1.92, 2.05) in isolated complex I as well as in submitochondrial particles [10, 14, 19].

Iron-sulphur cluster 2 is generally considered to be the last electron acceptor of the series of Fe-S clusters within the complex. Reports on the midpoint potential of this cluster are confusing. It was found to be $-20$ mV at pH 7.0 in mitochondria from both pigeon heart and bovine heart [20, 21]. This has been the basis for the idea that energy conservation at site I involved the redox potential gap in between the Fe-S clusters with lower potentials and cluster 2 [20, 22]. There...
are also reports, though, on a much lower midpoint potential for cluster 2 [21, 23]. In this laboratory the midpoint potential of this cluster in submitochondrial particles was determined to be −270 mV at pH 8.0 using the redox couple NAD+/NADH and the couple of oxidized and reduced 3-acetylpyridine adenine dinucleotide (Van Belzen, R. and Albracht, S. P. J., unpublished experiments). The other clusters were somewhat lower in potential (between −320 and −330 mV). Another possibility therefore is that energy conservation in coupling site I is located between cluster 2 and ubisemiquinone. Weiss and co-workers proposed the existence of an internal quinone, functioning as an electronic connector between the clusters with lower potentials (1b, 3 and 4) and cluster 2. This idea was based on the fact that, although Fe-S cluster 2 is missing in the small form of Neurospora crassa complex I, electrons can still be transferred to quinone. As this reaction was not inhibited by rotenone, two quinone binding sites were suggested [24, 25]. This proposal is, however, not in agreement with rapid-mixing rapid-freezing experiments with Q$_{10}$-free submitochondrial particles [17].

Bovine heart mitochondrial inner membranes contain 4–6 nmol ubiquinone (Q$_{10}$)/mg protein. Isolated complex I also contains about 4 nmol Q$_{10}$/mg protein [26–29]. Ubiquinone is generally accepted as an obligatory component for electron transport from complex I to the QH$_2$:cytochrome c oxidoreductase. Up till now little has been known about the mechanism of reduction of ubiquinone by complex I. However, there is a considerable amount of evidence that semiquinones might be an essential intermediate in electron transfer from NADH to Q$_{10}$. Two reports were published as early as 1970 on free radical EPR signals in submitochondrial particles that were suggested to be due to semiquinones in NADH dehydrogenase [30, 31]. There have been several reports on a complex-I-associated ubiquinone-binding protein in isolated enzyme that could stabilize ubisemiquinones, giving rise to radical EPR signals [29, 32, 33]. Tightly coupled submitochondrial particles also showed a prominent rotenone-sensitive ubisemiquinone EPR signal upon steady-state electron transfer from NADH to O$_2$. This semiquinone signal was only seen in the presence of oligomycin, added to increase the respiratory control with NADH to values of 7–9. The semiquinone signal was not observed in the presence of uncouplers [34, 35].

In our dimeric model of complex I the electron-exit pathway of the complex consists of two Fe-S clusters 2 [18]. Experiments with the inhibitor ptericidin A in uncoupled submitochondrial particles strongly indicated that both clusters 2 are involved in the reaction with Q$_{10}$. Presumably they each donate one of the two electrons required for reduction of ubiquinone to ubisemiquinol [18]. In this laboratory significant radical signals have hitherto never been observed in pre-steady-state experiments with submitochondrial particles mixed with NADH [15–18].

In this report it is demonstrated that NADH-dehydrogenase-associated ubisemiquinones are formed in all preparations of mitochondrial inner membranes and an explanation is provided why this has not been detected in this laboratory before. It is shown that the ubisemiquinone formation, associated with complex I, is not related to the degree of coupling in the preparation.

**MATERIALS AND METHODS**

NADH, NADPH and NAD$^+$ were purchased in the purest form available from Boehringer (Mannheim). Antimycin A, ATP, digitonin, 2,4-dinitrophenol, gramicidin D, myxothiazol, oligomycin, 2-thenoyltrifluoroacetone and valinomycin were obtained from Sigma (USA) and carbonyl cyanide p-trifluoromethoxyphenyl hydrazone (FCCP) from Fluka AG. 5-Chloro-3-tert-butyl-2'-chloro-4'-nitro-salicylanilide (S13) was a kind gift from Dr S. J. Brewer of Monsanto Company (St Louis, USA). All other chemicals were of analytical grade.

MgATP submitochondrial particles were prepared from bovine heart mitochondria essentially by the method of Löw and Vallin [36]. Mitochondria were sonified in a medium containing 0.25 M sucrose, 10 mM Tris/acetate pH 7.5, 1.5 mM ATP, 1 mM succinate, 15 mM MgCl$_2$ and 1 mg/ml bovine serum albumin cooled in an ice/water mixture for 30 s-periods with intervals of 1 min. The sonification was performed with a Branson sonifier B-12 operating at 40–50% of its maximal output. Another type of submitochondrial particles, which will be called shortly-sonified submitochondrial particles, was prepared in exactly the same way as the MgATP submitochondrial particles but the medium did not contain succinate, ATP, Mg$^{2+}$ or albumin. A third type was prepared by sonifying mitochondria in 0.25 M sucrose and 10 mM Tris/acetate pH 8 cooled in an ice/water mixture with the sonifier operating at its maximal output for four 2-min periods, with intervals of 2 min. This preparation will be referred to as prolonged-sonified submitochondrial particles in this report.

Mitochondria free from internal substrates were prepared by treatment with either sodium deoxycholate or digitonin. For deoxycholate-treated mitochondria the optimal deoxycholate concentration was determined by titrating bovine heart mitochondria with deoxycholate until the NADH oxidation activity in the presence of 40 μM cytochrome c was maximal. The concentration was usually in the range from 0.15–0.25 mg sodium deoxycholate/mg mitochondrial protein. To a suspension of 3–5 mg mitochondrial protein/ml in 0.25 M sucrose, 10 mM Tris/acetate pH 7.5, the desired amount of deoxycholate was added. The suspension was centrifuged for 15 min at 10000×g. The pellet was resuspended in the same buffer, whereafter the procedure was repeated twice. Digitonin-treated mitochondria were prepared essentially according to [37]. Hovius et al. showed that for rat liver mitochondria a digitonin concentration of 0.25–0.3 mg digitonin/mg mitochondrial protein gives mitochondria with leaky inner membranes; these digitonin-treated mitochondria can still be spun down at 12000×g. Only with the highest concentration, 0.3 mg digitonin/mg protein, was a fluffy layer present on top of the pellet. To
obtain a similar preparation with bovine heart mitochondria digitonin concentrations in the range from 0.1–0.4 mg/mg mitochondrial protein were tested. In all cases internal substrates could be removed. A concentration of 0.1 mg digitonin/mg protein was used, however, since at higher concentrations a fluffy layer was present after centrifugation at 12,000Xg. After the digitonin treatment, the mitochondria were collected and washed as in the treatment with deoxycholate. Mitochondrial preparations treated with deoxycholate or digitonin remained oxidized even at the high concentrations (up to 60 mg/ml) required for EPR spectroscopy. No decrease of the typical EPR signal of Cu,(II) of cytochrome c oxidase, nor any other sign of reduction, could be observed even after 30 min at room temperature (22°C).

Inactivation of complex I was accomplished by incubating submitochondrial particles at 37°C for 10 min, which is reported to block NADH oxidation completely [38]. Submitochondrial particles were (re-)activated by incubation under oxygen at room temperature for 10 min at a protein concentration of 1 mg/ml in 0.25 M sucrose, 10 mM Hepes, 1 mM NADPH and 10 μM NADH at pH 7. Immediately after this activation procedure the particle suspension was chilled in ice and centrifuged. The particles were resuspended in the same buffer without NADPH and NADH and centrifuged again. Protein concentrations were determined with the biuret reaction [39]. Assays for the oxidation activity of NADH were performed polarographically at 30°C, using a Clark electrode (model 5331 of Yellow Springs Instrument Co.) in 0.25 M mannitol or sucrose, 10 mM Mops/KOH, 1 mM KCN, 10 mM MgCl2, 2 mM EDTA, 5 mM Na2HPO4, and 0.5 mM NADH (pH 7.5). Reverse electron transfer activity was determined spectrophotometrically at 340 nm in 0.25 M sucrose, 10 mM Mops/KOH, 1 mM KCN, 10 mM MgCl2, 1 mM EDTA, 50 mM succinate, 0.5 mM NAD+, and 0.75 mg/ml bovine serum albumin (pH 7.5). The reaction was started by the addition of 2.4 mM ATP. Rapid-mixing/rapid-freezing experiments were performed at room temperature (22°C) as described by De Vries et al. [40]. EPR samples with reaction times higher than 200 ms were prepared with the rapid-mixing apparatus, but the reaction mixture was collected directly in an EPR tube instead of being sprayed in cold isopentane (133 K). After the appropriate reaction time the EPR tube was rapidly immersed in cold isopentane. X-band (9GHz) EPR spectra were recorded with a Bruker ECS106 EPR spectrometer equipped with an Oxford Instruments ESR900 helium flow cryostat with an ITC4 temperature controller or with a cold finger with liquid nitrogen. The field-modulation frequency was 100 kHz. The magnetic field was calibrated with an AEG magnetic field meter. The frequency was measured with a HP 5246L electronic counter, supplemented with a HP 5255A frequency converter. Spectra were simulated as described earlier by Beinert and Albracht [11].

RESULTS

Time dependence of radical formation

In Fig. 2 EPR spectra at 50 K are shown of MgATP submitochondrial particles that had reacted with NADH for 10–200 ms. The spectrum of a control sample with rotenone and a reaction time with NADH of 40 ms shows part of the Cu2,(II) signal of the cytochrome c oxidase complex (g1 part, around g = 2) as well as the signal of cluster 1b of complex I (g11.23 = 1.92, 1.94, 2.02). In the other spectra these signals were also present, but an additional radical signal at g = 2 could be observed, which increased in time. The radical signal remained present for at least 15 s, but disappeared after 60 s (not shown).

As can be seen from the figure the radical formation was completely rotenone-sensitive. It was partly sensitive to a mixture of antimycin A, myxothiazol and 2-thenoyltrifluoroacetone, as is shown later on in this section. When recorded under optimal conditions (0.01 mW, 0.32 mT and 77 K), the radical signal was positioned at g = 2.0043 and had a line-width of 0.84 mT. The spectra in Fig. 2 were recorded with a microwave power of 2.6 mW in order to obtain an acceptable signal/noise ratio. However, with this power, the radical signal was partly saturated. This is demonstrated in Fig. 3, in which a power dependence plot of this radical signal is shown. Even at a temperature of 77 K, the signal already showed saturation at microwave powers higher than 0.026 mW.

Quantification of the radical signal

The ubisemiquinone concentration was reported to be maximally half that of cluster 2 in submitochondrial particles.
with a respiratory control of 7–9 with NADH [34, 35]. The MgATP submitochondrial particles used in the present experiments showed respiratory controls of 1–2 with NADH. The radical concentration for these MgATP submitochondrial particles was usually found to be 1 radical/cluster 2 in samples with reaction times between 40–200 ms. This value was determined by direct double integration of a spectrum of the radical signal at 77 K, recorded with non-saturating power (0.01 mW). The concentration of cluster 2 in the same tube was determined by comparing an experimental spectrum, recorded at 17 K with 2.6 mW and a modulation amplitude of 1.27 mT, with a calculated lineshape [parameters: \( g_{x,y,z} = 1.925, 1.925, 2.053 \) and widths \( \Delta w_{x,y,z} = 1.88, 1.88, 1.18 \) mT].

The effect of uncouplers on radical formation

The effect of uncouplers on radical formation during NADH oxidation in MgATP submitochondrial particles was studied by comparing particles incubated for 10 min at room temperature with 64 \( \mu \)M valinomycin and 2 mM K\(^+\) with particles without uncoupler. EPR spectra of samples frozen 100 ms after mixing with NADH showed comparable radical signals (not shown). This experiment has been repeated with a variety of uncouplers and inhibitors like oligomycin, 2,4-dinitrophenol, FCCP, S13, gramicidin D, stearic acid and even 0.1% sodium deoxycholate. From these experiments it was concluded that radical formation observed at 100 ms was completely insensitive to the state of coupling. This result contrasts with the findings of Kotlyar et al. [35], where the ubisemiquinone formation was reported to be completely sensitive to uncouplers. Since these results [35] were obtained with samples frozen 10 s or 20 s after the addition of NADH, we have extended our study on the effect of uncouplers to the time range from 100 ms to 15 s. The uncouplers used in these experiments were S13, gramicidin D and FCCP. Although the 100-ms samples all showed the same radical signal, there was a clear difference in radical concentration after reaction times of 2 s in the preparations with and without uncouplers, as is shown in Fig. 4 for S13 and FCCP.

Effect of sonification on the radical formation

In previous pre-steady-state experiments with submitochondrial particles mixed with NADH, no significant radicals have been observed in this laboratory [15–18]. The particles used in these experiments were prepared by extensive sonication. The experiments described thus far in the present report suggested already, that when a mild sonication step was applied, as with the preparation of MgATP submitochondrial particles, semiquinones could be detected.

For the preparation of MgATP submitochondrial particles, Mg\(^{2+}\), ATP, succinate and bovine serum albumin were added to the medium. Therefore the possible influence of these additions has been tested. To this purpose, one batch of mitochondria was divided in two and either treated to be MgATP submitochondrial particles or shortly-sonified submitochondrial particles. Upon mixing with NADH, the MgATP submitochondrial particles contained 1.0 radical/cluster 2 after a reaction time of 100 ms, while the shortly-sonified submitochondrial particles and NADH contained 0.7 radical/cluster 2. Apparently the medium was of little influence. Semiquinones were formed as long as the sonification was mild.

Fig. 3. Microwave-power dependence of the ubisemiquinone EPR signal in MgATP submitochondrial particles. MgATP submitochondrial particles (66 mg/ml) were mixed with 20 mM NADH. The reaction was quenched in cold isopentane after 100 ms. EPR conditions: microwave frequency, 9370 MHz; modulation amplitude, 1.27 mT; temperature, 77 K (0 dB = 260 mW). On the y-axis \( \log A_e \) (in arbitrary units) is plotted, which is the logarithm of the radical amplitude normalized for the differences in microwave power and receiver gain.

Fig. 4. Effect of uncouplers on the appearance and disappearance of ubisemiquinone signals. MgATP submitochondrial particles (72 mg/ml) were mixed with NADH and the reaction was quenched by freezing in cold isopentane after reaction times between 100 ms and 15 s. Radical/cluster 2 ratios were determined as described in Results on quantification of the ubisemiquinone signal. (C) No uncoupler present. (V) In the presence of 0.5 \( \mu \)M S13. (●) In the presence of 10 \( \mu \)M FCCP.

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To further investigate the possible effect of sonification, we have performed experiments with inner membranes, prepared without the use of ultrasound. As explained in Materials and Methods, both deoxycholate-treated and digitonin-treated mitochondria were found to be suitable for this purpose. In both types of preparation large radical signals were detected during steady-state electron transfer from NADH to O₂ (not shown). As with MgATP submitochondrial particles with activated complex I, the radical concentration was maximal within 40 ms. The ratio of radical/cluster 2, found in digitonin-treated mitochondria 10 s after mixing with NADH, was 1.0. This was the same as obtained with the MgATP submitochondrial particles.

Next the reaction of prolonged-sonified submitochondrial particles with NADH was followed from 10 ms to a few seconds. Between 10–200 ms there were hardly any radicals detectable. Only after a reaction time of 1 s was a prominent radical signal present. These results are in line with our previous experience that complex-I-associated semiquinones were never observed within 100 ms after mixing of extensively sonified particles with NADH.

There can be a number of possible explanations for this phenomenon. It is possible that the sonification step causes some damage to the native structure of complex I. Another plausible explanation could be that during extensive treatment with ultrasound, at which time the temperature of the suspension can rise to 35 °C, complex I becomes reversibly inactivated. According to Kotlyar et al., inactive complex I is not capable of transferring electrons from NADH to ubiquinone [38]. The enzyme can be activated after a number of turnovers. The process of reversible inactivation is accelerated at higher temperatures; the half-time of inactivation at 33°C is reported to be 2.5 min at pH 9 and 7 min at pH 7 [38]. Since in routine NADH-oxidation assays, the activities of the prolonged-sonified submitochondrial particles are equal to or even higher than those of MgATP submitochondrial particles, we conclude that temperature-induced inactivation of NADH dehydrogenase [38] (presumably taking place during the 2-min periods of sonification) can explain these observations.

We have also looked at the effect of sonification and subsequent reactivation of complex I on radical formation in MgATP submitochondrial particles and prolonged-sonified submitochondrial particles. Inactive particles quenched 200 ms after mixing with NADH showed EPR signals of reduced complex I. Cu₆(II) and a very small radical signal. After 1 s, however, a prominent radical was detectable. Its concentration was 0.4 radical/cluster 2 for both types of particles. It is concluded that, under these circumstances, both types of particles showed partial reactivation of complex I between 200 ms and 1 s after mixing with NADH. This did not, however, lead to radical concentrations higher than 0.4/cluster 2. The same preparations of heat-inactivated particles were subsequently activated by the method described in Materials and Methods. Samples of both preparations, mixed with 20 mM NADH, already showed a radical signal within 10 ms; after 40 ms, the concentrations of the radicals were 0.7/cluster 2 for MgATP submitochondrial particles and 0.5/cluster 2 for prolonged-sonified submitochondrial particles.

The effect of inhibitors of other complexes of the respiratory chain on radical formation

The possibility that quinone radicals attached to QH₂-cytochrome c oxidoreductase or succinate-Q oxidoreductase, or flavin radicals, would contribute to the observed radical signal, has been investigated. To that purpose the separate effects of antimycin, 2-thienyltrifluoroacetone, 1.3% ethanol (the highest ethanol concentration used in this experiment), myxothiazol and KCN were tested. Also the effect of a mixture of antimycin, myxothiazol and 2-thienyltrifluoroacetone was studied. The results are given in Fig. 5.

The presence of KCN had no effect on the radical formation, nor did the presence of 1.3% ethanol. The addition of 2-thienyltrifluoroacetone had only a slight effect. The effect of myxothiazol was somewhat greater. Antimycin appeared to be the inhibitor that caused the largest decrease in radical concentration (about 40%). The experiment with the mixture of antimycin, myxothiazol and 2-thienyltrifluoroacetone was performed twice, resulting in identical curves except for the last point. However, it is most likely that the point with the lowest radical concentration represents the correct value, since there was never much increase in radical concentration between 40–100 ms in comparable experiments with MgATP submitochondrial particles inhibited with a mixture of 2-thienyltrifluoroacetone and antimycin (not shown).

DISCUSSION

In submitochondrial particles EPR signals from ubisemiquinones have thus far only been detected in preparations that were tightly coupled [34, 35]. Kotlyar et al. [35] reported that ubisemiquinone formation induced by NADH in such submitochondrial particles was completely sensitive to uncouplers like carbonyl cyanide m-chlorophenyl hydrazone (CCCP). In uncoupled submitochondrial particles, as rou-
cally dependent on an intact coupling machinery at site I. In this report we have investigated the relationship between ubisemiquinones, electron transfer and coupling. Our experiments show that ubisemiquinone formation during the oxidation of NADH, both in the pre-steady-state and the steady-state, is insensitive to uncoupling agents. The experiments with mitochondria treated with deoxycholate or digitonin demonstrate that the formation of ubisemiquinones is not dependent on the intactness of the membrane. In such preparations ubisemiquinones were formed in the same concentration as in MgATP submitochondrial particles. We therefore conclude that the radical formation is not related to the degree of coupling of a mitochondrial innermembrane preparation at all. Uncouplers enhance the rate of electron transfer of coupled submitochondrial particles. The radical signal can only be observed during steady-state electron transfer from NADH to oxygen, but becomes much smaller when electron transfer comes to a halt [34, 35]. This is also demonstrated in Fig 4; the disappearance of the radical signals is enhanced by uncouplers. This could possibly (in part) explain the findings of Kotlyar et al. [35]. These investigators worked with submitochondrial particles which were much better coupled than the ones used here. Hence it might be expected that in the absence of uncoupler, the disappearance of the radical signals proceeded still more slowly than the corresponding trace in Fig 4, making the effect of uncouplers even more pronounced.

Although in the time range up till 200 ms the radical concentration is maximally 1 radical/cluster 2 for fully active preparations, higher concentrations are present at longer reaction times in the absence of uncoupler (Fig. 4). The samples at 2 s and 5 s especially show concentrations of more than 1 radical/cluster 2. This effect is not exactly understood. Since both the coupled and uncoupled samples reached aerobicities within a few seconds, the extra radicals are apparently not directly related to steady-state electron transfer. The rate of decay and possibly also the rate of formation appears to be uncoupler-sensitive. As yet, the origin of these extra radicals is not known.

We have also tried to understand why no ubisemiquinones have been observed previously in uncoupled submitochondrial particles in this laboratory [15–18]. The major difference in the preparation of MgATP submitochondrial particles and uncoupled prolonged-sonified submitochondrial particles is the procedure of sonification. The experiments performed here with the prolonged-sonified submitochondrial particles demonstrate that, when the observation time is in the appropriate range (seconds), these particles were also capable of showing ubisemiquinones during the oxidation of NADH. No radical formation was observed within 200 ms after mixing with NADH. Previous studies [15–18] almost exclusively involved reaction times up to 100 ms. No particular attention was paid to what happened at longer reaction times and therefore the ubisemiquinone formation escaped detection. The reason for the slow appearance of radicals in prolonged-sonified submitochondrial particles is presumably that, during the relatively long sonification procedure, NADH dehydrogenase will become inactivated by increased temperatures reached during sonification. The slow formation of radicals in submitochondrial particles with inactive complex I is in agreement with the views put forward by Kotlyar and Vinogradov [38] about the slow formation of the specific binding site for a ubisemiquinone as being responsible for the activation.

With active NADH dehydrogenase in submitochondrial particles the ubisemiquinone signal is maximal (1 spin/cluster 2) within 40 ms after mixing with NADH (Fig. 5). In samples of particles with in complex I was deliberately inactivated prior to mixing with NADH, the radical concentration was considerably lower than 1/cluster 2 and radicals only appeared at longer reaction times. In experiments with prolonged-sonified submitochondrial particles as isolated and in experiments with inactivated prolonged-sonified submitochondrial particles and inactivated MgATP submitochondrial particles the maximal concentration of radical was 0.4, even though a number of turnovers with NADH was reported to be sufficient to bring the enzyme into an active state [38].

A more effective activation procedure involved the aerobic incubation of the particles with NADPH prior to the reaction with NADH. Then, radical concentrations (at reaction times of 40 ms) of 0.5/cluster 2 were observed in prolonged-sonified submitochondrial particles and 0.7 in MgATP submitochondrial particles.

By comparing the rate of radical formation as well as the maximal concentration of radical after 100 ms in different preparations of MgATP submitochondrial particles as isolated, with the rate in deliberately inactivated and subsequently reactivated preparations of particles, it appeared that on the average in MgATP submitochondrial particles only a very small amount of complex I becomes inactivated during the preparation. The 200-ms sample of MgATP submitochondrial particles with NADH in Fig 2 contains radicals in a concentration of 0.8/cluster 2, while 100-ms samples of two other preparations of MgATP submitochondrial particles both showed 1.0 radical/cluster 2. In submitochondrial particles with optimally activated complex I, the concentration of radical/cluster 2 was usually found to be 1.

With X-band EPR it is not possible to discriminate between different radical signals. A contribution of flavin radicals of FMN in complex I and FAD in succinate dehydrogenase is not very likely, since flavosemiquinones have linewidths in the range of 1.2–2.0 mT. Furthermore, the line shape of flavosemiquinones at temperatures below 0°C features well-resolved shoulders or asymmetry in the wings [41]. In order to investigate the possible contribution of radicals formed elsewhere in the mitochondrial respiratory chain, the effects of several inhibitors were tested. KCN, 2-thenoyltrifluoroacetone or 1.3% ethanol had no effect on the kinetics or extent of radical formation (Fig. 5). Both myxothiazol and antimycin did affect the radical formation and the effects appeared to be additive. From Fig. 5 it is concluded that at least 40% of the radical signal is due to NADH-dehydrogenase-associated ubisemiquinones. This would bring the concentration to at least 0.4 spins/cluster 2.

One possible reason for the diminished intensity in the presence of the inhibitors antimycin and myxothiazol could be that the electron transfer comes to a halt in a time where the complex-I-associated radical has not been fully developed yet. Another possibility is that the antimycin-sensitive semiquinone of the QH$_2$:cytochrome c oxidoreductase (Q$_{-}$) [42] contributes to the g = 2 signal, and to a lesser extent the antimycin-insensitive semiquinone of the same complex. It is not possible to distinguish between these possibilities from the present experiments. In view of these considerations, we believe that an amount of 0.5 radical/cluster 2 is a reasonable minimum for the complex-I-associated ubisemiquinones. Such a value can be easily accommodated in the
dimeric model proposed for the functioning of complex I (Fig. 1). The dimeric complex might contain one binding site where a semiquinone could be stabilized. From previous experiments we know both clusters 2 are involved in electron transfer to ubiquinone. One possibility is that the rates of electron transfer from the clusters 2 to ubiquinone would be different for both monomers; this would result in the detection of semiquinones. From the experiments described in this report we conclude that ubisemiquinones are obligatory intermediates in the reaction between NADH dehydrogenase and ubiquinone.

We thank Dr A. B. Kotlyar and Professor K. van Dam for their stimulating interest during this research and for critically reading the manuscript. Mr P. J. Molenaar is kindly acknowledged for his help with some of the rapid-mixing rapid-freezing experiments. S. P. J. A. is indebted to the Netherlands Organization of Pure Research (NWO) for grants, supplied via the Netherlands Foundation for Chemical Research (SON), which enabled the purchase of the Bruker ECS106 EPR spectrometer.

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