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Electron spin resonance measurement on ubiquinone-depleted and ubiquinone-replenished submitochondrial particles

Submitochondrial particles show a number of ESR-signals originating from different components of the electron transport system, the most distinct of which originate, in the oxidized state, from Cu^{2+}, and, in the reduced state, from free radicals and non-heme iron[1,2]. The radical signal situated at $g = 2.00$ is generally considered to be principally due to flavosemiquinone[1]. Another possible contributor to the signal is the semiquinone of Q, as suggested by studies with both the pure substance[3,4] and mitochondria[5]. In this communication we wish to report ESR measurements with Q-depleted and Q-replenished submitochondrial particles[6,7] which indicate that Q contributes to a substantial part of the $g = 2.00$ signal.

Submitochondrial particles were derived from heavy beef-heart mitochondria by sonication in the presence of EDTA[8] Lyophilized, Q-depleted and Q-replenished particles were prepared as previously described[7]. The particles were suspended in 0.25 M sucrose in a final concentration of approx. 40 mg protein/ml (determined with the biuret method). 0.35 ml of the suspension was mixed with 20 μl buffer (Tris–acetate, pH 7.5, final concn. 50 mM, unless otherwise indicated) and 3 μl KCN solution (final concn. 1 mM), and the sample was incubated for 3 min at 0°. 15 μl NADH (final concn. 10 mM) or succinate (final concn. 16 mM) were then added and the incubation continued for 2 and 3 min, respectively, during which time the mixture was transferred into an ESR sample tube (inner diameter 3 mm) whereupon the sample was quickly frozen in liquid nitrogen. ESR measurements were made with a Varian model V4500-10A X-band ESR Spectrometer at 0.3 and 7 mW microwave power, 6.5 gauss modulation amplitude, 25 gauss/min scanning rate and a temperature of 77°K.

Fig. 1 shows the $g = 2.00$ and $g = 1.94$ signals of submitochondrial particles with NADH as substrate. For quantitative evaluations, the $g = 2.00$ signal was also measured at 0.3 mW, which minimizes the degree of saturation and interference by non-heme iron and copper but still gives a good signal/noise ratio. Replacement of Tris–acetate by potassium phosphate lowered the signal height slightly at $g = 1.94$ and very markedly at $g = 2.00$. Preliminary experiments indicate that the difference in signal height at $g = 2.00$ is due both to a stimulating effect of the Tris ion and an inhibitory effect of the phosphate ion; the effect of phosphate was not abolished by oligomycin.

ESR spectra of lyophilized Q-depleted and Q-replenished particles in Tris–acetate buffer with NADH as substrate are shown in Fig. 2. The lyophilized particles revealed a spectrum similar to the normal particles (*cf.* Fig. 1), except that the signal-height ratio $g = 2.00/g = 1.94$ was somewhat higher. In the Q-depleted particles the $g = 2.00$ signal was diminished by more than 50%. This decrease was restored in the Q-replenished particles, which exhibited a $g = 2.00$ signal even higher than the particles before Q extraction. The $g = 1.94$ signal was somewhat diminished in both the Q-depleted and Q-replenished particles, possibly more so in the former than in the latter. The curved background for the spectrum of Q-depleted particles indicates

Abbreviations. ESR, electron spin resonance, Q, ubiquinone.

that part of the Cu was in the oxidized form, which is in accordance with the virtual lack of electron transport between NADH and cytochrome oxidase.

A similar decrease and restoration of the $g = 2.00$ signal in the Q-depleted and Q-replenished particles was found with succinate as substrate or with a combination of succinate and NADH (Fig. 3).

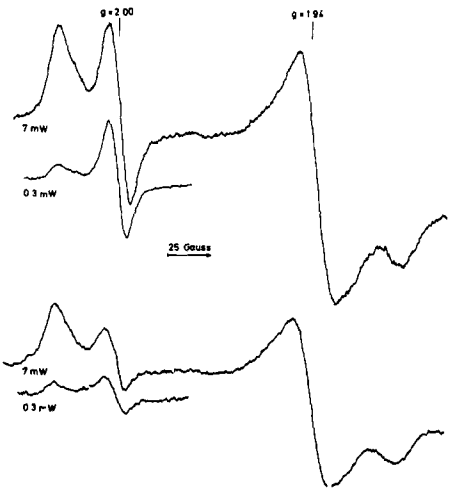

Fig. 1. ESR spectra of submitochondrial particles in 50 mM Tris–acetate (pH 7.5) (upper) or in 50 mM potassium phosphate buffer (pH 7.5) (lower) treated with NADH as substrate in the presence of KCN.

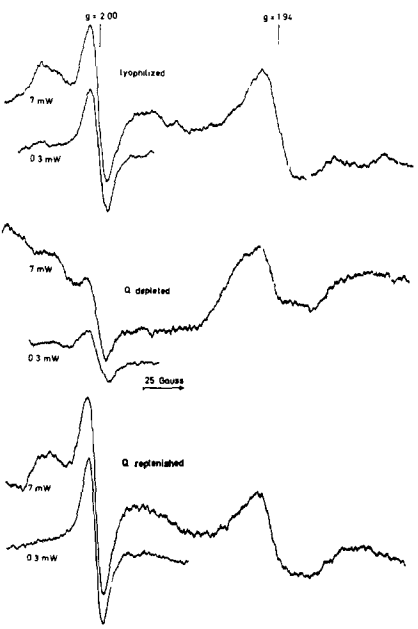

Fig. 2. ESR spectra of lyophilized, Q-depleted and Q-replenished submitochondrial particles in 50 mM Tris–acetate buffer, pH 6.5, treated with NADH as substrate in the presence of KCN.

The $g = 2.00$ signals of the Q-containing and Q-depleted particles also differed qualitatively. Whereas, before Q extraction, the signal height was much greater in Tris–acetate than in potassium phosphate buffer (*cf.* Fig. 1), the height of the residual signal after Q extraction was not influenced by the composition of the buffer. Similar effects of phosphate have been reported previously[5]. Furthermore, in the Q-containing particles, the $g = 2.00$ signal increased with increasing pH (6.5–8.5), whereas in the Q-depleted particles it was independent of the pH in the range indicated. Finally, the width of the $g = 2.00$ signal was 10 gauss in the Q-containing particles in Tris–acetate buffer, and somewhat larger (approx. 12 gauss) both in the Q-containing particles in potassium phosphate buffer and in the Q-depleted particles in either Tris–acetate or potassium phosphate.

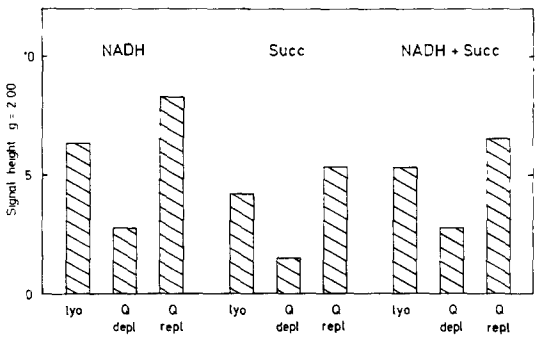

Fig. 3. Signal heights at $g = 2.00$ obtained with lyophilized, Q-depleted and Q-replenished submitochondrial particles in 50 mM Tris–acetate buffer (pH 6.5) treated with NADH, succinate, or NADH + succinate as substrate in the presence of KCN. The treatment with both substrates was made as follows: addition of 15 μl succinate (final concn. 16 mM), incubation for 1 min at 0°, addition of 15 μl NADH (final concn. 10 mM), incubation for 2 min, and finally quick freezing in liquid nitrogen.

It appeared from these results that the fraction of the $g = 2.00$ signal which disappears upon the extraction of Q (or the addition of phosphate) originates from the semiquinone of Q. Support for this conclusion was obtained in experiments with pure Q. Reduction of Q in an ethanol solution with $NaBH_4$ gave rise to a signal at $g = 2.00$ similar to that obtained with submitochondrial particles in regards to both line shape and width. With a Cu–EDTA standard as a reference, the radical concentration was estimated in a 1 mM Q solution. Double integration and g-value correction[9] gave the result that about 1–2 μM Q was in the radical state in the frozen mixture. On comparison of the height of the Q-radical signal with that of the $g = 2.00$ signal obtained with the Q-containing particles in Tris–acetate buffer, the concentration of the radical in the particles was estimated to be 0.5–1.5 μM in a particle suspension containing about 40 mg protein/ml. With a Q content of 3–6 nmoles/mg protein[7] this would correspond to a free radical yield of 0.2–1.5 % of the total Q.

In summary these results indicate that a substantial part (>50 %) of the $g = 2.00$ signal observed with submitochondrial particles suspended in Tris–acetate buffer in the presence of NADH or succinate as substrate and KCN as respiratory inhibitor, originates from the semiquinone of Q. That part of the $g = 2.00$ signal which is independent of Q—and which probably originates from flavin—differs from

the Q semiquinone signal in several qualitative respects such as signal width, and sensitivity to pH and ionic environment. The definite identification of the Q semiquinone and the clarification of its role in the respiratory chain will require further experimentation.

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Department of Biochemistry,
University of Stockholm,
Box 6409, S-113 82 Stockholm (Sweden)

DAN BÄCKSTRÖM
BIRGITTA NORLING
ANDERS EHRENBERG
LARS ERNSTER

and

Department of Biophysics,
University of Stockholm,
Karolinska Institutet,
S-104 01 Stockholm (Sweden)

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