# THE INTERACTION OF UBISEMIQUINONES WITH THE IRON–SULPHUR CENTRE S-3 OF SUCCINATE DEHYDROGENASE IN PLANT MITOCHONDRIA

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## 1. Introduction

At low temperatures (< 20 K) complex EPR spectra are observed in various plant and mammalian mitochondria which have been attributed to an interaction of quinones in the partially reduced form and oxidized centre S-3 of succinate dehydrogenase [1–5]. From computer simulations [2] and potentiometric titrations [6] it was concluded that the semiquinone signals arise from a dipolar interaction between a pair of ubisemiquinones. In order to explain the low temperatures necessary to observe these signals an additional interaction of the ubisemiquinone pair with centre S-3 was assumed [2,6]. However, the EPR spectrum of the postulated ubisemiquinone pair alone without the presence of centre S-3 could not be observed. In an attempt to resolve such complex spectra we have investigated their temperature dependence and microwave power saturation in mitochondria from mung beans. Since these mitochondria possess a cyanide- and antimycin A-insensitive alternative route of substrate oxidation [7] it was also of interest to study the relationship of the complex signal to the alternative oxidation step. Particularly, since recent studies have indicated that the branchpoint of the alternative pathway from the main respiratory chain may be located at the ubiquinone level [8,9].

Small, but detectable differences were observed in the electron spin relaxation behaviour which have allowed us to further characterize the interaction between centre S-3 and the ubisemiquinone species. Power saturation studies revealed that centre S-3 has the fastest relaxation, whereas the unsplit and split semiquinones exhibit a similar but slower relaxation. The spectra were further complicated by the presence of centre bc-3, which has been distinguished from centre S-3 at higher temperatures by the differential effects of ethanol and salicyl-hydroxamic acid (SHAM). Difference EPR spectra indicated that the effect of SHAM is solely on the dipolar coupled ubisemiquinones whereas ethanol also perturbs the signals of centres S-3 and bc-3. The relevance of these findings is discussed with respect to the location of the alternative pathway in plant mitochondria.

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## 2. Materials and methods

Etiolated mung bean hypocotyls (*Phaseolus aureus*) were grown for 5–7 days in a dark room maintained at 28°C. Fresh beef hearts were obtained from commercial sources. Mitochondria were prepared as in [10]. Steady state experiments were carried out with an air-saturated resuspension medium (medium A) containing 0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$ and 10 mM potassium phosphate buffer, pH 7.2. The salicyl-hydroxamic acid (SHAM) stock solution was 5 mM in medium A. Enough time was allowed (10–15 s for 15 mg/ml protein) so that equilibrium had been reached. Chemicals were of the highest grade available commercially. Protein was measured by the Lowry method [11] using bovine serum albumin as standard. Samples for EPR measurements were frozen in isopentane/methylcyclohexane (5:1, v/v) freezing mixture (81 K) and stored in liquid

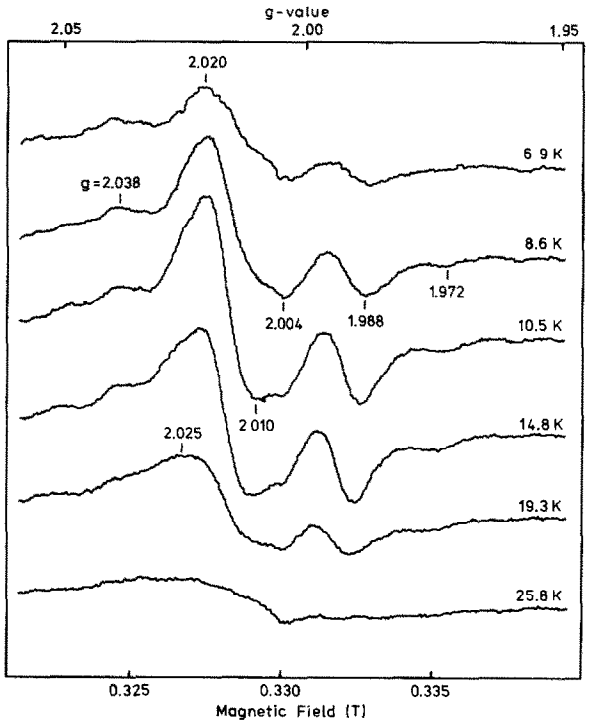


Fig.1. EPR spectra of aerobic mung bean mitochondria at different temperatures and at constant microwave power (10 mW). Mung bean mitochondria were suspended in medium A to a final conc. 15 mg/ml. EPR conditions: modulation amplitude 1 mT; microwave frequency 9.26 GHz.

nitrogen. EPR spectra were recorded on a Varian E4 spectrometer. Low temperature studies were performed using an ESR-9 continuous flow cryostat (Oxford Instruments, Oxford). The temperature was monitored with a gold/iron chromel thermocouple below the sample position and was checked periodically with a carbon resistor thermometer placed at the sample position. Difference EPR spectra were obtained with a Nicolet 1074 signal averager. Quartz sample tubes were calibrated with a copper–EDTA solution.

## 3. Results

### 3.1. *Temperature dependence and microwave power saturation*

Figure 1 shows EPR spectra of mung bean (*Phaseolus aureus*) mitochondria in the aerobic state. All spectra were measured with 10 mW microwave power. At 6.9 K the signal of centre S-3 of succinate dehydrogenase ($g_{max}$ 2.02) could readily be discerned, although additional signals on the high and low field side were also detectable. Above 10 K, the signal of centre S-3 broadened out and the additional spectral features became more prominent. Signals were observed centred at $g$ 2.04, $g$ 2.004, $g$ 1.99 and $g$ 1.97. It is noteworthy that both the $g$ 2.004 and $g$ 1.97 features showed a similar temperature dependence. In the temperature range 17–19 K the $g$ 2.004 feature which is prominent at lower temperatures is apparently replaced by a second component. At present it is widely accepted that these complex spectral features can be attributed to ubisemiquinone or flavin species [2,4]. Thus, the main contribution to the $g$ 2.04, $g$ 1.99 and $g$ 1.97 signals is the split spectrum of a ubisemiquinone pair interacting with centre S-3. In addition, the $g$ 2.004 feature can be attributed to an unsplit ubisemiquinone or flavin [12]. Since a free radical is expected to be strongly saturated below

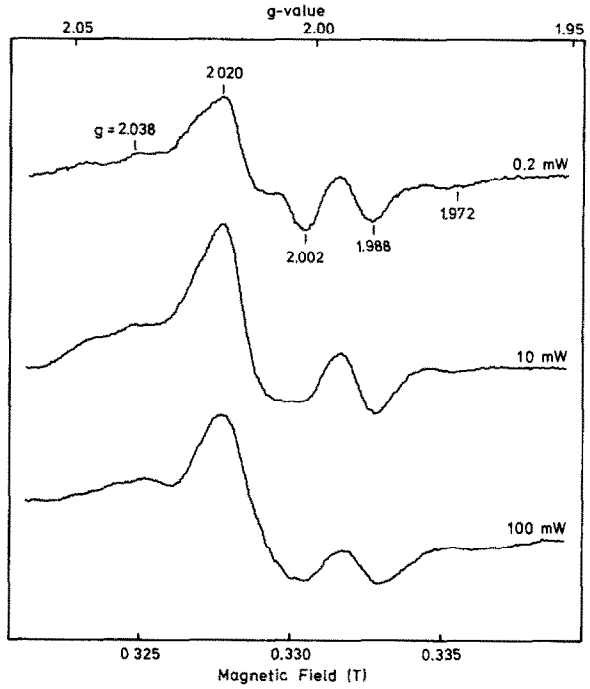


Fig.2. EPR spectra of aerobic mung bean mitochondria at 10.5 K measured at different microwave powers. Mitochondrial protein concentration as in fig.1. EPR conditions as in fig.1.

77 K (due to a small spin-orbit coupling) we have to assume that $g$ 2.004 component is interacting with another paramagnetic species of a high relaxation rate. Centre S-3 or the relaxed ubisemiquinone species are likely candidates. However, this interaction does apparently not result in a splitting. As this signal was found to be of a heterogeneous nature [13] a further contribution from ferric ion in a cubic environment cannot be ruled out.

The temperature-dependent features of the complex overlapped spectra can be rationalized by assuming a higher relaxation rate for centre S-3 compared to the split and unsplit semiquinone species. Thus, in the temperature range of 7–10 K, the S-3 signal would predominate in the $g$ 2.00 region and at higher temperatures the split and unsplit semiquinone or flavin species. These conclusions were corroborated by microwave power saturation of the EPR spectra at 10.5 K (fig.2). Spectra features which represent a major contribution of centre S-3 (namely $g$ 2.02) are expected to become more prominent at high microwave power, since the split and unsplit semiquinone or flavin species are more saturated. Figure 2 shows that this is the case since in the spectra recorded at 100 mW, the signal of centre S-3 predominated, whereas at 0.2 mW all species were detectable without any appreciable saturation. If we assume that the microwave magnetic field at 0.2 mW was too low to allow a differentiation between the faster and slower relaxing species, we can explain why temperature-dependent spectral changes could not be observed in [5]. (Note, that the nominal value of the microwave power refers to the microwave power $P_0$ incident on the cavity but does not give the microwave magnetic field at the sample, which depends on $P_0Q$, where $Q$ is dependent on the characteristics of the cavity and of the cryostat.) From a plot of $\log S/\sqrt{P}$ against $\log P$ [14] of the different features of the complex spectrum at 13 K we find similar properties (half-saturation power $P_{1/2}$ = 6–7 mW) for the $g$ 2.04, $g$ 1.99 and $g$ 1.97 species, whereas the $g$ 2.02 feature exhibits $P_{1/2}$ 25 mW. Thus, centre S-3 represents the species with the fastest relaxation and the semiquinones or flavins exhibit slower relaxation.

A further complexity of the spectra arose from the apparent shift of the $g$ 2.02 component (at 8.6 K) to lower magnetic field ($\sim g$ 2.03) at higher temperature (19.3 K) (fig.1). This shift has been shown to be diagnostic for the presence of a second HiPIP-type centre, namely the bc-3 or Ruzicka centre [15,16]. The saturation behaviour of the $g$ 2.02 component represents also to a minor extent the bc-3 centre which is, however, more saturated than centre S-3 [16]. The difference between the S-3 signal alone and the split semiquinone signals would be, therefore, more pronounced. Since both, the signals of the interacting semiquinones and of centre bc-3 are lost in plant submitochondrial particles [5] it is not possible to obtain spectra without a contribution of centre bc-3. In an attempt to resolve the complex spectrum into its major components we studied the effect of SHAM and ethanol on the EPR properties.

### 3.2. *Effect of SHAM*

SHAM is a potent inhibitor of cyanide- and antimycin-A-insensitive alternative oxidase in plant mitochondria [18] and its addition to aerobic mitochondria results in disappearance of the split ubisemiquinone signals. In an attempt to resolve the perturbation of SHAM, difference EPR spectra were calculated (fig.3). It is noteworthy that by subtracting the spectra of the SHAM-treated sample from the aerobic sample (fig.3a) a spectrum was obtained which is very similar to the calculated spectrum for an interacting ubisemiquinone pair [2,6]. The separation of the $g$ 2.04 and $g$ 1.97 features is 10.8 mT, and 8.1 mT for the $g$ 2.04 and $g$ 1.99 features. Both are in close agreement with the EPR properties of two dipolar coupled semiquinone molecules assuming $g_\parallel$ 2.066 $g_\perp$ 2.004, where the separation $L_\parallel$ is 8.2 mT and $L_\perp$ + 11.5 mT at 9.2 GHz [2]. We conclude that SHAM interferes only with the spectrum of the dipolar coupled ubisemiquinone pair and not with the signals of centre S-3 or the unsplit semiquinone or flavin ($g$ 2.004) species.

A similar difference spectrum was observed for beef heart mitochondria in the presence of SHAM (not shown). In contrast to mung bean mitochondria, the unsplit $g$ 2.004 signal was not detected (however, see [3,6]). The separation between the $g$ 2.04 and the $g$ 1.97 features is 10.8 mT and 8.0 mT for the $g$ 2.04 and $g$ 1.99 components in good agreement with the splittings observed for mung bean mitochondria. The mechanism of interaction between the two semiquinones is therefore similar in both species.

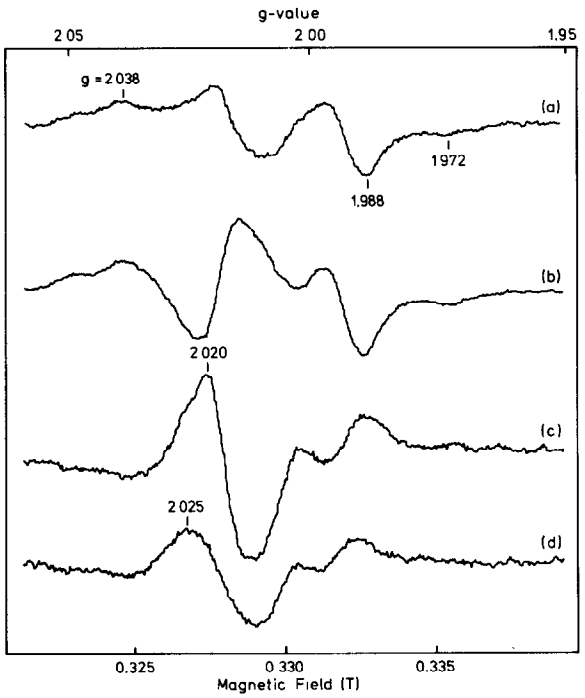


Fig.3. Difference EPR spectra of mung bean mitochondria. Mung bean mitochondria were suspended in medium A to final conc. 15 mg/ml. Difference spectra were derived as indicated in section 2. (a) Aerobic sample minus aerobic sample in the presence of 2 mM SHAM at 10.5 K. (b) Aerobic sample minus aerobic sample in the presence of 0.1% (v/v) ethanol at 10.5 K. (c) Difference EPR spectrum according to (a) but at 8.6 K minus difference EPR spectrum according to (b) but at 8.6 K. (d) Difference EPR spectrum according to (a) but at 19.3 K minus difference EPR spectrum according to (b) but at 19.3 K.

### 3.3. *Effect of alcohols*

Ethanol and propanol show a more complex reactivity than SHAM. In addition to the interference with the split signals they apparently also effect the signal of the HiPIP centres. The *g* 2.02 component is increased by 40% in the presence of ethanol. The corresponding difference EPR spectrum (fig.3b; aerobic sample minus aerobic sample in the presence of 0.1% (v/v) ethanol) exhibits spectral features which cannot be accounted solely by a semiquinone pair as in fig.3a. By subtracting the difference EPR spectrum at 8.6 K (fig.3b; aerobic minus aerobic in the presence of ethanol) from the difference spectrum (fig.3a; aerobic minus aerobic in the presence of SHAM we actually see (fig.3c) that the additional features are due to a signal of centre S-3 (peak at *g* 2.02). At higher temperature (19.3 K), where S-3 is expected not to contribute to the spectra [16] the difference spectrum (aerobic minus aerobic in the presence of ethanol) still showed the extra components (similar to fig.3b). However, the peak and trough portions were shifted to the low field side (peak at *g* 2.03) as expected for a bc-3 centre [16]. The spectrum corresponding to fig.3c, however calculated for spectra at 19.3 K, exhibited a signal clearly attributable to centre bc-3.

## 4. Discussion

The small but definite differences in the temperature dependence of centre S-3 and the split and unsplit signals strongly suggest that the split signal is actually due to a semiquinone pair and not to centre S-3 interacting with a semiquinone [2]. The microwave power saturation behaviour indicates that centre S-3 has the fastest relaxation and the unsplit and split semiquinones a slower relaxation rate. The close agreement between the experimentally determined separation of the *g* 2.04 and *g* 1.97 and *g* 1.99 features observed in this support and the EPR properties of a simulated spectrum of a ubisemiquinone pair [2,6] is strong evidence to assign the splitting to a dipolar coupled semiquinone pair. It would therefore seem that in mung bean mitochondria the complex spectrum can be at least resolved into two HiPIP type species (namely centre S-3 and centre bc-3), two dipolar coupled semiquinone species and an unsplit semiquinone or flavin species.

Ethanol removes the splitting of the semiquinone signals and apparently increases the S-3 and bc-3 signals at a concentration where electron transport is not effected according to measurements with the oxygen electrode [5]. Similar results were obtained using ethanol saturated with air or argon. This apparent increase may be due to an oxidation of S-3 with the concomitant reduction of semiquinones. On the other hand, the concentration of SHAM required for removing the splitting is considerably higher than that

necessary for inhibiting the alternative pathway [5]. A possible mode of action of SHAM is most probably the perturbation of the environment around Site II thus resulting in a breakdown of the dipolar interaction between the semiquinones. If SHAM merely perturbs the interaction of the semiquinone pair with centre S-3, then we would expect to detect the signal at higher temperatures. However, no split signal was observed $< 77$ K. It has been suggested that hydroxamates competitively inhibit the reduced hydroquinone substrate for its binding site [17] possibly by polyfunctional hydrogen bonding [18]. Since SHAM does not affect the signal size or shape or power saturation of centre S-3 (this report, [12]) it would argue against a direct role of this centre in alternative respiratory oxidations. The specific interaction of SHAM with the semiquinone pair would seem to suggest that the most likely candidate, at present, for the alternative oxidation mechanism are quinones in a hydrophobic protein environment. A quinone binding protein which can serve as an electron (hydrogen) carrier from succinate to ubiquinone has been described [19].

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