PROPERTIES OF THE S-3 IRON-SULPHUR CENTRE OF SUCCINATE DEHYDROGENASE IN THE INTACT RESPIRATORY CHAIN OF BEEF HEART MITOCHONDRIA

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1. Introduction

A new mitochondrial iron-sulphur centre was recently reported by Ruzicka and Beinert [1]; this new species is paramagnetic in the oxidised form as are the bacterial high potential iron-sulphur proteins (Hipip [2]). This new iron-sulphur protein exhibits an EPR signal centred at g = 2.01 with a peak to peak width of approximately 25 gauss. A similar EPR signal was detected in the succinate-ubiquinone (UQ) reductase (Complex II [3]), in an equimolar concentration to that of flavin [4]. This signal was, however, not initially detected [4] in the succinate dehydrogenase (SDH) preparation, which contained the same content of non-haem iron and acid labile sulphide per flavin as Complex II, but was not reconstitutively active. Subsequently this Hipip-type iron-sulphur centre was found to become extremely labile towards oxygen upon removal of the dehydrogenase from the mitochondrial membrane. Thus, resonance absorbance of a Hipip-type iron-sulphur centre is detectable only in the reconstitutively active SDH preparation [5]. This Hipip-type iron-sulphur centre, present in the succinate dehydrogenase, was designated as Centre S-3, following Centres S-1 and S-2 (two ferredoxin type iron-sulphur centres previously identified in the succinate dehydrogenase [5]). More recently it has been shown that intact mitochondria contain two different 'Hipip'-type species (paramagnetic in the oxidised form); these species can be distinguished by their temperature and micropower saturation profiles and the absence of one of the species from sub-mitochondrial particle preparations [6]. Of the two 'Hipip' species only that which is present in submitochondrial particles is thought to be a component of the respiratory chain. It is Centre S-3, a constituent of submitochondrial particles and purified (active) succinate dehydrogenase, which is the subject of the present study.

It has been noted that under some conditions the EPR signal of a mitochondrial 'Hipip' is accompanied by partially overlapping signals, one on the high field side and one on the low field side of g = 2.01. These overlapping signals have been ascribed to a splitting of a Hipip signal caused by a spin-spin interaction between the Hipip in the oxidised state and ubiquinone in the free radical form, from the following lines of evidence [7]: I) Peaks at g = 2.04 and 1.99 are not seen in fully reduced or fully oxidised samples, but only in partially reduced preparations; II) Upon removal of ubiquinone, these peaks disappear and reincorporation of ubiquinone restores the peaks; III) The same magnetic field splitting of the Center S-3 signal was obtained in K-band and X-band EPR spectra. The present work reports observations which are contrary to a simple one to one spin–spin interaction, but which nonetheless strongly indicate some form of interaction between Centre S-3, and the semiquinone form of ubiquinone. The weakness of the interpretation of the data in terms of a simple spin–spin interaction stems from our observation that, under certain conditions, the Centre S-3 signal is the same approximate maximal size in the presence or absence of a 'spin-spin' interaction and Centre S-3 cannot, therefore, be contributing many electron spins to the additional signals caused by the 'interaction'. As suggested by Schepler et al. [17], the 'interaction' must be more complex than initially proposed [7].

2. Materials and methods

Heavy beef heart mitochondria and sub-mitochondrial particles were prepared according to the method of Löw and Vallin [8]. When ubiquinone-depleted mitochondria were required, the ubiquinone was removed by extracting twice with acetone as solvent at 0°C as described by Lester and Fleischer [9].

Redox titrations were conducted as described by Dutton [10] and samples for EPR were taken at intervals during a titration, according to Wilson et al. [11], frozen rapidly in an isopentane–cyclohexane freezing mixture (81°K) and stored in liquid nitrogen until assayed.

All spectra were obtained on a Varian E-4 ESR spectrometer. Temperature control was achieved by a variable temperature cryostat (Air Products Model LTD-3-110).

EPR sample tubes of quartz glass were calibrated with a standard copper sulphate-EDTA solution and values for signal heights were corrected correspondingly.

3. Results and discussion

Fig.1 shows EPR spectra obtained from beef heart submitochondrial particles at different electrode potentials, the samples being taken during a redox titration. The g = 2.01 signal of the S-3 centre can be readily discerned in the more oxidised samples. Additional signals, at g = 2.04 and g = 1.99, can be observed during the decline of the S-3 signal. In the most reduced spectrum shown in fig.1 (E_h = −25 mV), a signal peak at g = 1.93 can be seen; this is probably due to the partial reduction of Centre N-2, a non-haem iron centre which is paramagnetic in the reduced form [12].

The temperature dependence of the g = 2.01, 1.99 and 2.04 signals are plotted in fig.2 as a reciprocal of the absolute temperature plotted against signal height. The three signals show approximately similar temperature profiles. This similarity of profile suggests that the ubiquinone free radical signal is largely saturated throughout this temperature range because if there were a change in its degree of saturation, the high and low field splittings would change their relative intensities, although they would still be only observ-

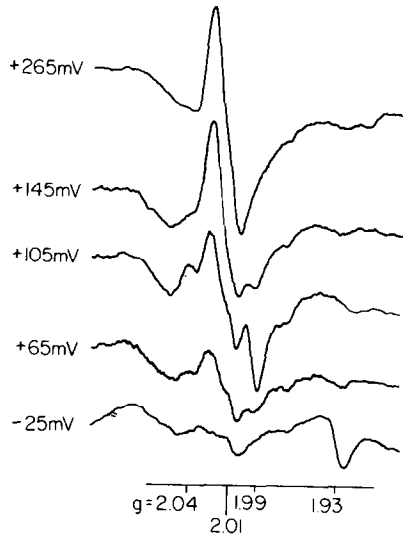

Fig.1. EPR spectra of sub-mitochondrial particles poised at the potentials shown. Spectra were recorded at 8° K at the following instrument settings: field modulation frequency, 100 kHz; microwave power, 5 mW; microwave frequency, 9.14 GHz; modulation amplitude, 10 gauss; time constant, 1 sec; scanning rate, 250 gauss/min. The sub-mitochondrial particles (13 mg protein/ml final concentration) were suspended in a medium containing 25 mM MOPS, 50 mM KCl, 100 mM sucrose, pH 7.5. 100 μM ferricyanide was added at the beginning of the reductive titration and 30 μM diaminodurol, phenazine methosulphate and pyocyanine were added at various potentials during the titration; the reductants were ascorbate, then NADH.

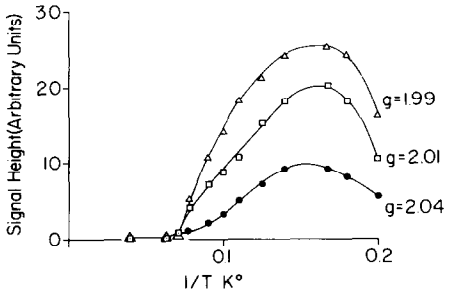

Fig.2. Temperature dependence of the g = 2.01, 1.99 and 2.04 signals from beef heart submitochondrial particles. The sample was poised at a potential of 105 mV as described in fig.1. The redox titration was carried out at pH 7.0; mitochondria (15 mg/ml protein) were used. The temperature at which spectra were recorded was varied. The instrument settings were: microwave power, 5 mW; frequency, 9.14 GHz; modulation amplitude, 10 gauss. The g = 1.99 and 2.04 signals were measured as described in fig.3.

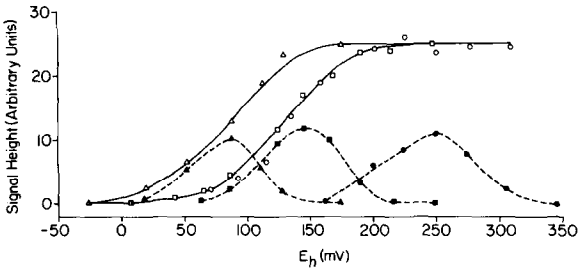

Fig.3. Variations with redox potential of the g = 2.01 (open markers) and g = 2.04 (closed markers) signals of the EPR spectrum. Spectra were recorded as described in fig.1. The submitochondrial particles, 13 mg per ml protein, were suspended in the following buffers: 25 mM maleate, pH 6.0; 25 mM HEPES, pH 7.0; 25 mM Tris–Cl, pH 8.5; with 50 mM KCl and 100 mM sucrose. Signal amplitudes were corrected for variations in tube diameter as described in the Methods section. The g = 2.04 signal amplitude was measured from an extrapolation of the g = 2.01 signal line shape as observed in the fully oxidised sample. Triangles, pH 8.5; squares, pH 7.0; circles, pH 6.0.

ed in the presence of an S-3 signal. At the microwave power used Centre S-3 does not saturate until below approximately 7°K.

Data from reductive redox titrations is represented in fig.3; results from oxidative titrations were similar. The midpoint potential of Centre S-3 was found to be approximately +120 mV between pH 6.0 and pH 7.0, and approximately +80 mV at pH 8.5. The 'spin–spin' interaction, however, is pH-dependent between pH 6.0 and pH 7.0 but less so between pH 7.5 and pH 8.5. The pH-dependence of midpoint potentials, indicating the involvement of protons in a redox reaction, is discussed in an extensive review by Dutton and Wilson [13]. Although the g = 1.99 signal is not included in fig.3 for the sake of clarity, when plotted it shows the same properties as the g = 2.04 signal, except that it is larger in amplitude.

Redox titrations with ubiquinone-depleted mitochondria confirmed that no extra EPR absorptions occur at g = 2.04 and g = 1.99 in the absence of ubiquinone. A Nernst plot of the electrode potential against the signal height of the g = 2.01 signal gave a midpoint potential (n = 1) for Centre S-3 in the absence of ubiquinone of approximately +100 mV; this value is pH-independent between pH 7.0 and pH 8.5.

The similarity in temperature profile of Centre S-3 and the 'interaction', the fact that no conditions have been found in which the 'wings' at g = 1.99 and g = 2.04 can be observed without the presence of a Centre S-3 absorption, and the apparent interaction of the species to constrain redox changes in each other, all point to an interaction between Centre S-3 and the semiquinone (UQ̇H). This is so because only a paramagnetic species is likely to be able to cause such a large 'splitting' of an EPR absorption [14–16].

The distance between the two interacting species has been calculated as described by Leigh [14] and a value of approximately 6 Å obtained from the values observed for the field splitting of approximately 90 gauss, this value represents a maximum distance for the interaction.

EPR spectra of beef heart mitochondria quick-frozen at various times after the addition of oxygen are shown in fig.4. The experimental incubation was carried out at −7°C. The EPR (8°K) absorptions at g = 1.93 and g = 1.94 are the partly saturated signals from Centres N-2 and N-1 plus S-1 respectively.

The kinetics of oxidation of Centre S-3 and Centre S-1 are not significantly different from each other.

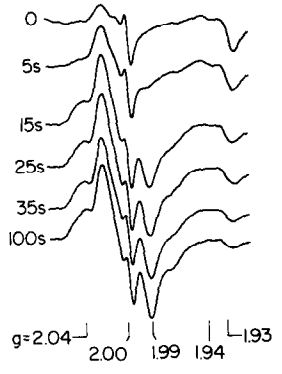

Fig.4. EPR spectra of beef heart mitochondrial samples, quick frozen at various time intervals after addition of oxygen at −7°C. Beef heart mitochondria, 22 mg protein/ml were suspended in a medium containing 25 percent ethylene glycol, 20 mM KCl, 50 mM sucrose and 20 mM HEPES, pH 7.0. The uncoupler CCCP, 6 μM was also added. Samples were incubated at −7°C, mixed with oxygen and rapid-frozen after various time intervals. EPR conditions were similar to those used in fig.1.

The rate of the rise of the spin-spin interactions, however, lags behind the rise of the Centre S-3 signal. The difference in the rise time of the 'interactions' may not necessarily indicate that Centre S-3 is oxidised before the fully reduced quinone; it could be that several of the quinones need to be oxidised to the semiquinone before an interaction occurs. In other words, the interactions could be between Centre S-3 and several semiquinones, thus explaining the apparently excessive number of electron spins which can be detected when the interaction is maximal.

It can be noted from the time course of the appearance of the 'spin–spin' interaction that although reduced ubiquinone becomes partially oxidised to the semiquinone, imparting the magnetic interaction, the interaction does not then disappear. This indicates that the semiquinone is not extensively further oxidised to UQ.

It has been noted that the partial 'splitting' of the g = 2.01 signal occurs in samples taken in the presence of oxygen at 30°C in the presence of uncoupler as long as some endogenous substrate is present, indicating partial reduction of UQ (fig.5). This is so even when all the other components of the respiratory chain in whole mitochondria seem to be oxidised. In fig.5 oxidised signals from Centre S-3 and an overlapping copper signal can be seen; the only indication of a reduced component is the 'spin–spin' interaction indicating the presence of the semiquinone. It could be suggested therefore that, under physiological conditions, the semiquinone is the oxidised form and only becomes fully oxidised under highly oxidised conditions (i.e. the normal steady state reaction is $UQ^{\cdot}H + H^{+} + e^{-} \rightleftarrows UQH_2$).

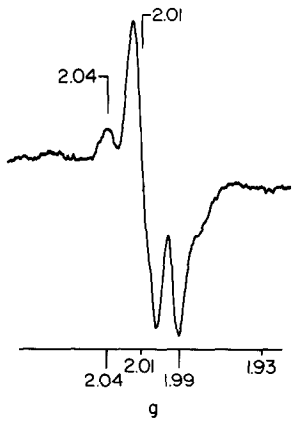

Fig.5. An EPR spectrum of mitochondria in the absence of added substrate and the presence of uncoupler and oxygen. Pigeon heart mitochondria were suspended in 0.225 M mannitol, 0.075 M sucrose, 0.5 mM EDTA and 0.05 M MOPS (pH 7.2) to a concentration of 12 mg/ml protein. 0.35 nmol FCCP/mg protein was added and the presence of oxygen was checked by an oxygen electrode. Conditions of EPR measurement were: time constant, 0.3 sec; scan rate, 200 gauss/min modulation amplitude, 10 gauss; field modulation frequency, 100 KHz, receiver gain, 1600; microwave power, 5 mW; microwave frequency, 9.14 GHz; temperature 8° K.

If, as suggested by the work of Beinert [7] and by the present work, there is a functional interaction between Centre S-3 and the semiquinone form of ubiquinone which may lead to the transfer of reducing equivalents between these two species and that UQ is involved as an n = 1 species, there are some interesting considerations concerning the respiratory chain, its arrangement and catalysis of proton transport. These observations indicate that UQ exists as a semiquinone in the mitochondrial membrane and is not completely disproportionated into a diamagnetic form, as is probable on extraction with organic solvent.

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