Resolution and Functional Characterization of Two Mitochondrial Iron–Sulphur Centres of the ‘HiPIP’ Type

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Two distinct iron–sulphur centres of the ‘HiPIP’ (high-potential iron–protein) type are distinguished in both pigeon heart and ox heart mitochondria. These two species, although both are paramagnetic in the oxidized state, exhibit signals which differ in their detailed line shape, field position, and temperature- and power-dependence. They also exhibit different thermodynamic and kinetic behaviour and are located on opposite sides of the mitochondrial coupling membrane. One of these centres corresponds to Centre S-3. The other ‘HiPIP’-type centre is removed readily from the mitochondrial membrane and its physiological function is not known.

A relatively symmetric highly temperature-sensitive e.p.r.* signal centred around $g = 2.01$ has been detected in various mitochondrial-membrane preparations (Ohnishi *et al.*, 1971; Orme-Johnson *et al.*, 1971) in the oxidized state. However, it has received little attention because originally it was considered to be due to ferric iron in a cubic environment and, in addition, the kinetic behaviour of this component was reported to be too slow (reduced only within seconds) for a component of the respiratory chain (Orme-Johnson *et al.*, 1971). However, purification of a protein exhibiting a ‘HiPIP’-type signal has revealed that the signal arises from a protein containing an equivalent concentration of iron and acid-labile sulphur (Ruzicka & Beinert, 1974). This iron–sulphur protein was found to be paramagnetic in the oxidized state, analogous to bacterial high-potential iron–protein (Bartsch, 1963), and thus classified as a ‘HiPIP’-type iron–sulphur centre, in contrast with ferredoxin-type iron–sulphur centres which are paramagnetic in the reduced form. This nomenclature (‘HiPIP’-type) no longer refers to the redox potential of the component. A similar ‘HiPIP’-type centre was found in succinate–ubiquinone reductase [Complex II (Hatefi *et al.*, 1962a)] at a concentration equivalent to that of flavin (Beinert *et al.*, 1974). However, this species was not detected in significant amounts in isolated succinate dehydrogenase preparations (Beinert *et al.*, 1974). Subsequently it was demonstrated that this ‘HiPIP’-type centre in the succinate–ubiquinone reductase segment becomes extremely labile on removal from the membrane, and the unmodified form was preserved only in the reconstitutively active succinate dehydro-

genase preparations and was designated as Centre S-3, following the format of Centres S-1 (Beinert & Sands, 1960; Ohnishi *et al.*, 1973) and S-2 (Ohnishi *et al.*, 1973, 1974b), two ferredoxin-type iron–sulphur centres previously identified in the isolated succinate dehydrogenase.

The present investigation provides evidence for the existence of at least one additional ‘HiPIP’-type iron–sulphur centre in mitochondria, and this centre exhibits e.p.r. and thermodynamic characteristics distinct from those of Centre S-3. The newly characterized ‘HiPIP’-type centre is provisionally named Centre *bc*-3, indicating that it is the third iron–sulphur centre which may be associated with the cytochrome *b*–cytochrome *c* region of the respiratory chain, after Centre *bc*-1 [Rieske’s iron–sulphur centre (Rieske *et al.*, 1964)] and Centre *bc*-2 [‘Centre 5’ (Ohnishi *et al.*, 1972)]. This nomenclature does not imply a functional role in the respiratory chain, nor does it imply a presence of the species in the isolated ubiquinone–cytochrome *c* reductase (Complex III) (Hatefi *et al.*, 1962b). The nomenclature will have to be revised as more becomes known about the roles of these centres.

The two ‘HiPIP’-type iron–sulphur centres are shown to be located anisotropically in the mitochondrial membrane, Centre S-3 being located on the matrix side and Centre *bc*-3 being located on the cytoplasmic side of the inner mitochondrial membrane. No function is known for Centre *bc*-3. This second ‘HiPIP’ species is detected only in intact mitochondria, and appears to be readily solubilized when mitochondria are disrupted to make sub-mitochondrial particles. Preliminary results of this investigation have been reported (Ohnishi *et al.*, 1975).

* Abbreviations: e.p.r., electron paramagnetic resonance; ‘HiPIP’, high-potential iron–protein.

Materials and Methods

Pigeon heart mitochondria and submitochondrial particles were prepared as previously described (Chance & Hagihara, 1963; Lindsay *et al.*, 1972). Heavy ox-heart mitochondria and submitochondrial particles were prepared by the method of Löw & Vallin (1963). E.p.r. samples of a 'HiPIP'-type iron-sulphur protein present in the supernatant obtained from disrupted mitochondrial suspension were prepared as follows. Pigeon heart mitochondria were suspended in a hypo-osmotic medium containing 1mM-succinate and 1mM-dithiothreitol (Ruzicka & Beinert, 1974) and disrupted by means of a Yeda press (Lindsay *et al.*, 1972). A clear supernatant was obtained by centrifugation of the disrupted mitochondrial suspension at 100000g for 40min and samples were oxidized with optimal concentrations of ferricyanide together with 50 μ M-phenazine methosulphate, and frozen quickly in an isopentane-methylcyclohexane (5:1, v/v) freezing mixture (81K). Redox titrations were conducted potentiometrically (Dutton, 1971) and samples for e.p.r. measurements were taken at intervals during a titration and frozen quickly as described above (Wilson *et al.*, 1970). All spectra were obtained on a Varian E-4 e.p.r. spectrometer. Temperature control was achieved by a variable temperature cryostat (Air Product model LTD-3-110). Temperature measurement and *g*-value corrections were conducted as previously described (Ohnishi, 1975).

Results

Spectral resolution of two mitochondrial 'HiPIP'-type centres

A multiplicity of 'HiPIP'-type iron-sulphur centres in mitochondria has been suggested from the redox titration of the $g = 2.02$ peak (Ohnishi *et al.*, 1972) and detection of similar signals in Complex II, Complex III (Orme-Johnson *et al.*, 1974) and in the high-speed supernatant of sonicated mitochondria (Ruzicka & Beinert, 1974). In order to resolve overlapping 'HiPIP' signals, the temperature-dependence of 'HiPIP' signals at a fixed microwave power, examined in pigeon heart mitochondria and submitochondrial particles was compared (Fig. 1). Iron-sulphur centres were oxidized with 3mM-potassium ferricyanide. Relative signal amplitude was determined as a peak height from the baseline in the lower magnetic field. As presented in Fig. 1(a), the 'HiPIP' signal in mitochondria can be detected over a wide range of temperature, namely at all temperatures obtainable below 25K at a power setting of 10mW. The temperature-dependence curve of mitochondrial 'HiPIP' signals indicates the existence of more than one species contributing to the 'HiPIP' signal in whole mitochondria. In contrast, a curve indicative of

a single species was obtained in submitochondrial particles (Fig. 1b) and this species was detectable only at temperatures below 15K.

As described below, the 'HiPIP' species detected in submitochondrial particles corresponds to Centre S-3. Assuming that signals measured in mitochondria below 7.3K arise only from Centre S-3, its contribution to the mitochondrial 'HiPIP' signals can be extrapolated as shown by the dashed line in Fig. 1(a), which is similar to the Centre S-3 temperature profile obtained from submitochondrial particles. Thus the

Fig. 1. Temperature profile of the e.p.r. signals arising from 'HiPIP'-type iron-sulphur centres present in pigeon heart mitochondria and submitochondrial particles

Pigeon heart mitochondria and submitochondrial particles were suspended in 0.20M-mannitol/0.05M-sucrose/30mM-morpholinopropanesulphonate buffer, pH 7.2, at protein concentrations of 36.8 and 36.0mg/ml respectively. Potassium ferricyanide at a final concentration of 3mM was added to both suspensions to oxidize the iron-sulphur centres. E.p.r. operating conditions were: magnetic field modulation, 100kHz; modulation amplitude 5×10^{-4} T [10^{-4} T (tesla) = 1 G (gauss)]; microwave power, 10mW; microwave frequency, 9.14GHz; time-constant 0.3s; scanning rate, 5×10^{-2} T/min. Peak heights from the low-field baseline were plotted as a function of sample temperature, with mitochondria (Fig. 1a) and submitochondrial particles (Fig. 1b). In Fig. 1a, the curve was resolved for contributions from two 'HiPIP' centres, Centres S-3 (----) and bc-3 (····). Arrows A and B indicate the sample temperatures for spectra (a) and (b) in Fig. 2.

Fig. 2. E.p.r. spectra obtained from pigeon heart mitochondria and submitochondrial particles under oxidizing conditions, taken at two different temperatures

The sample temperature for spectra (a) and (b) were 7K and 15K respectively (indicated by arrows A and B in Fig. 1). —, Spectrum obtained from mitochondria; ----, from submitochondrial particles. Other experimental conditions are the same as in Fig. 1.

temperature-dependence curve of mitochondrial 'HiPIP' signals was resolved into two species simply by subtraction of the dashed line from the overall curve. The two resolved curves indicate the presence of at least one additional 'HiPIP'-type iron-sulphur centre in mitochondria; this species is detectable at higher temperatures than the centre present in submitochondrial particles (Centre S-3).

Temperature profiles of 'HiPIP' signals in mitochondria and submitochondrial particles reveal that the second 'HiPIP' species is readily removed from the mitochondrial membrane during the disruption of mitochondria and subsequent isolation of the submitochondrial particles.

Figs. 2(a) and 2(b) show 'HiPIP' spectra of whole mitochondria (unbroken line) and submitochondrial particles (broken line), taken at 7K (illustrated with arrow A in Fig. 1) and 15K (arrow B in Fig. 1). The following observations can be made. (1) A 'HiPIP' spectrum obtained in submitochondrial particles is centred at $g = 2.01$ with a low field maximum of $g = 2.02$, which is the same as the Centre S-3 spectrum obtained with succinate-cytochrome *c* reductase (Ohnishi *et al.*, 1974c). In contrast, the e.p.r. spectrum of the second 'HiPIP' centre obtained in pigeon heart mitochondria at 15K is centred at $g = 2.02$ and has a (derivative) maximum at $g = 2.03$. These two centres also differ in their detailed line shapes. (2) No detectable quantities of the second 'HiPIP' species (Centre bc-3) remain in submitochondrial particles (Fig. 2b). Therefore in submitochondrial particles the e.p.r. spectrum of Centre S-3 was obtained without any overlap of the second 'HiPIP' signal at temperatures below 15K. In mitochondria at temperatures below 7K, the 'HiPIP' spectrum approaches that of

Fig. 3. Power-dependence of 'HiPIP'-type signals examined in pigeon heart mitochondria (●) and submitochondrial particles (○)

'HiPIP' peak heights relative to a baseline in the lower magnetic field are expressed as a function of the microwave power. The samples were the same as those used in Fig. 1. The e.p.r. sample temperature was kept constant at 11.4K. Other experimental conditions were the same as in Fig. 1.

Centre S-3; however, even at 6.5K, some resonance signal from the second 'HiPIP' contributes to the total 'HiPIP' signal, as indicated by the peak position being somewhat lower in magnetic field compared with the $g = 2.02$ peak of Centre S-3 obtained from submitochondrial particles (Fig. 2a). The small signals observed in mitochondria at $g = 1.99$ and 2.04 have been ascribed to the results of an interaction between Centre S-3 and a semiquinone form of ubiquinone (Beinert, 1974). (3) E.p.r. spectra obtained at 15K in submitochondrial particles (Fig. 2b, broken line) show a Cu^{2+} signal from the cytochrome oxidase which overlaps in its higher field positions with the second 'HiPIP' signal in the mitochondrial system, but no significant Cu^{2+} signal interference with the Centre S-3 spectrum was observed at lower temperature, owing to power saturation of the Cu^{2+} signal. The second 'HiPIP'-type centre is tentatively designated as Centre bc-3, because inhibitor studies indicate that this species appears to be associated with the cytochrome *b*-cytochrome *c* region of the respiratory chain.

The presence of at least two different 'HiPIP'-type iron-sulphur centres in mitochondria can also be detected from the power-saturation behaviour of the 'HiPIP' signal, which was examined at the given 'intermediate' temperature in both mitochondria and submitochondrial particles (Fig. 3). The Centre S-3 signal in submitochondrial particles is not saturated even with 100mW microwave power at 11.4K, whereas at the same temperature, saturation of the additional component (Centre bc-3) can be seen in whole mitochondria at microwave power in excess of 1mW. Representative e.p.r. spectra of Centres S-3 and bc-3 are presented in Fig. 4. The temperature and power profile of Centre bc-3 in the supernatant

Fig. 4. Typical e.p.r. spectra of two distinct 'HiPIP'-type iron-sulphur centres

The Centre S-3 spectrum, represented by a solid line, was obtained from submitochondrial particles. The Centre *bc*-3 spectrum (·····) was obtained from the supernatant of a mechanically disrupted mitochondrial suspension, centrifuged at 100000*g* for 40 min. Iron-sulphur centres were oxidized by 500 μ M-ferricyanide in the presence of 50 μ M-phenazine methosulphate. The protein content of the supernatant was 7.4 mg/ml. The protein concentration used for the Centre S-3 spectrum was 36.0 mg/ml of suspension. E.p.r. conditions: temperature, 15 K; microwave power, 1 mW for the spectrum of Centre *bc*-3. All other conditions were the same as those in Fig. 1. The gain on the vertical scale was greater by a factor of 1.3 for the Centre *bc*-3 spectrum than that for Centre S-3.

fraction (see the Materials and Methods section) is similar to that which is observed in intact mitochondria. It was found that the e.p.r. spectrum of Centre *bc*-3 of the (high-speed) supernatant fraction is readily destroyed by oxidizing agents such as oxygen or ferricyanide. This sensitivity to oxidizing agents is similar to that of the 'HiPIP'-type iron-sulphur protein purified by Ruzicka & Beinert (1974). The Centre *bc*-3 spectrum has a shoulder in the centre of the resonance absorbance. A similar central 'shoulder' was observed in spectra of whole mitochondria under the same non-saturating e.p.r. conditions (15 K, 1 mW).

Thermodynamic characterization of the 'HiPIP' centres

On the basis of separating the two 'HiPIP' centres by use of their different relaxation rates and the absence of Centre *bc*-3 from submitochondrial particles, midpoint potentials have been measured for the two species. A value of approx. +120 mV was obtained for the midpoint potential of Centre S-3 in submitochondrial particles (Ingledeew & Ohnishi,

Fig. 5. Plots of E_h against signal height for redox titrations of Centre *bc*-3 at pH 7.0 (Δ , $\blacktriangle$) and pH 8.5 ($\square$, $\blacksquare$)

Redox titrations were performed anaerobically under an argon atmosphere in a medium of 50 mM-KCl and 50 mM-morpholinopropanesulphonate buffer, pH 7.0, or 50 mM-Tris/HCl buffer, pH 8.5, containing pigeon heart mitochondria (20 mg of protein/ml). The redox dyes were added at concentrations between 20 μ M and 100 μ M. Duroquinone ($E_{m7.0} = -30$ mV), 2-hydroxynaphthoquinone ($E_{m7.0} = -145$ mV), pyocyanine ($E_{m7.0} = -34$ mV), phenazine ethosulphate ($E_{m7.0} = +35$ mV), phenazine methosulphate ($E_{m7.0} = +80$ mV), and diaminodurool ($E_{m7.0} = +240$ mV) were used as artificial redox mediators. Ascorbate ($E_{m7.0} = +60$ mV) and dithionite were used as reductants, and ferricyanide was used as oxidant. E.p.r. conditions are same as those in the legend of Fig. 1. Open and closed symbols represent separate experiments. Maximum signal heights in different sets of experiments were normalized as 100.

1975). The titration of the mitochondrial *bc*-3 centre proved difficult, ($n=1$ Nernst relationship was observed only for oxidative titrations). It appears that Centre *bc*-3 is readily modified by artificial oxidizing agents, thus leading to anomalous behaviour during reductive titrations. Fig. 5 shows oxidative titration curves of Centre *bc*-3 at pH 7.2 and 8.5. An approximate value of +160 mV was obtained for the midpoint potential of Centre *bc*-3; however, because of the irreversible nature of the titration, little reliance can be placed on the quantification of the midpoint potential. The values obtained for the midpoint potential of Centre *bc*-3 by oxidative titration were independent of pH.

Relationship of Centres S-3 and *bc*-3 to the various metabolic states of mitochondria and to respiratory-chain inhibitors

Fig. 6 presents e.p.r. spectra obtained with pigeon heart mitochondria in various respiratory states, recorded under e.p.r. conditions optimal for Centre S-3 spectra. To obtain a maximally oxidized state of Centre S-3, spectrum (a) was obtained with

Fig. 6. E.p.r. spectra of pigeon heart mitochondria in the different metabolic states, measured at 6.9K

Pigeon heart mitochondria were suspended to a final concentration of 18.2mg/ml in a reaction medium containing 0.2M-mannitol, 0.05M-sucrose, 0.2mM-EDTA and 50mM-morpholinopropanesulphonate, pH7.2, which was saturated with air at room temperature (23°C), in order to assure state 4 or uncoupled aerobic state long enough for e.p.r. sampling. (a) No substrate added; 5mM-ferricyanide was added to the uncoupled pigeon heart mitochondria (0.2nmol of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone/mg of protein) in order to obtain a fully oxidized sample. For the lower three spectra, 5mM-glutamate and 5mM-malate were added as substrates. (b) Aerobic state, uncoupled with carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. (c) State 4. (d) Uncoupled anaerobic-state mitochondria (10min after it became anaerobic). The different metabolic states were monitored by using a Clark oxygen electrode. E.p.r. conditions: microwave power, 10mW; modulation amplitude, 5×10^{-4} T; scanning rate, 2×10^{-2} T/min; time-constant, 0.3s; microwave frequency, 9.14GHz.

uncoupled mitochondria oxidized with 5mM-ferricyanide in the absence of added substrate. Spectra (b), (c), and (d) were obtained from mitochondria with added glutamate and malate as substrate. Spectrum (b) shows that Centre S-3 is largely oxidized (about 80%) in the uncoupled and aerobic state, as are other well-characterized respiratory-chain carriers in the region of loop 2 (Mitchell, 1968). In state 4 (controlled respiratory state) (Chance & Williams, 1956), Centre S-3 was approx. 60% reduced (spectrum c) and more than 90% was reduced in the uncoupled

anaerobic state. The effect of a possible spin-spin interaction between the semiquinone form of ubiquinone and Centre S-3 was not taken into account in the evaluation of the redox state of Centre S-3, as work has indicated that Centre S-3 does not give a significant contribution to the absorptions at $g = 2.04$ and 1.99 (Schepler *et al.*, 1975; Ingledew & Ohnishi, 1975; W. J. Ingledew & T. Ohnishi, unpublished work). These observations demonstrate that Centre S-3 alters its level of reduction according to the metabolic state of mitochondria, similarly to established respiratory-chain members. The redox potential of Centre S-3 in state-4 mitochondria seems to be within the E_h range of isopotential group II (Wilson & Erecinska, 1972). The following observations should be also pointed out. (1) In the uncoupled aerobic state, where no signals from reduced respiratory components were discerned, some of the ubiquinone located in the vicinity of Centre S-3 [possible electron donor to ubiquinone in the succinate-ubiquinone reductase segment of the respiratory chain (Ohnishi *et al.*, 1974c)] is in the semiquinone state (spectrum b), suggesting that the semiquinone form is a physiologically functional oxidized form of ubiquinone. (2) On addition of the artificial oxidant, ferricyanide, the $g = 2.04$ peak is greatly diminished, whereas the $g = 1.99$ peak showed less change. This observation may suggest that $g = 2.04$ and 1.99 signals in pigeon heart mitochondria may not be derived simply from spin-spin interactions. Fig. 7 presents e.p.r. spectra of the same mitochondrial samples as are shown in Fig. 6, measured under an optimal condition for the Centre bc-3 signal (15.2K, 10mW). Fig. 7 reveals that Centre bc-3 stays highly reduced (70% in this experiment, but often shows a higher degree of reduction in different batches of mitochondria) even in the uncoupled aerobic state (spectrum b) and seems to be fully oxidized only by ferricyanide (spectrum a), remaining largely uninfluenced by changes in the respiratory flux.

Inhibitor studies with antimycin A and cyanide were performed. Centre bc-3 was reduced by the addition of cyanide to mitochondria but found to be oxidized after the addition of antimycin A to respiring mitochondria (Fig. 8). In Fig. 8 the signals at $g = 2.06$ and $g = 1.92$ are due to Centre N-2, a centre associated with the NADH dehydrogenase, which is paramagnetic in the reduced form (in contrast with 'HiPIP' species). Centre S-3 is reduced by the addition of antimycin A and cyanide to respiring submitochondrial particles.

Kinetic behaviour of Centres S-3 and bc-3

The kinetic behaviour of Centre S-3 at low temperature (-7°C) on addition of oxygen to an anaerobic suspension of ox heart mitochondria suspended in an aqueous buffer containing 25%

Fig. 7. E.p.r. spectra of pigeon heart mitochondria in the different metabolic states, measured at 15.2K

Experimental conditions were the same as in Fig. 6, as were the e.p.r. conditions except for temperature.

(v/v) ethylene glycol is apparent in Fig. 9. The oxidation of Centre S-3 (increase in the signal at $g = 2.02$) is similar in its time-course to the oxidation of Centre S-1 (not shown), another component of succinate dehydrogenase (Beinert & Sands, 1960). In addition to Centre S-3, the additional signals that can be seen in Fig. 9 are: at $g = 2.04$ and 1.99, signals due to an interaction of two respiratory-chain components (Beinert, 1974; Ingledew & Ohnishi, 1975); at $g = 1.93$, the remnants of Centre N-2; at $g = 1.94$, the remnants of Centres N-1 and S-1 (Ohnishi, 1975). The redox state of Centre *bc*-3 in an anaerobic suspension varies considerably between the different preparations and does not change its oxidation state within the time-interval shown in Fig. 9.

Anisotropic arrangement of Centres S-3 and *bc*-3 in mitochondria

Evidence for a 'sided' arrangement of the 'HiPIP' centres in mitochondrial preparations was sought by use of bathophenanthroline sulphonate, an iron chelator of Fe^{2+} ions, which has been reported not

Fig. 8. Effect of (a) antimycin A and (b) cyanide on the redox state of Centre *bc*-3

Pigeon heart mitochondria (18.3mg/ml of protein) were incubated in a medium consisting of 0.2M-mannitol, 0.05M-sucrose and 50mM-morpholinopropanesulphonate buffer at pH7.2 and incubated with 5mM-glutamate and 5mM-malate as substrates in an oxygen electrode. Samples were taken for e.p.r. after the addition of 2nmol of antimycin A/mg of protein (a) or 4mM-KCN (b) and quickly frozen. E.p.r. conditions: temperature, 15.2°K; modulation amplitude, 5×10^{-4} T; microwave power, 10mW; scanning rate, 2×10^{-2} T/min; time-constant, 0.3s; microwave frequency, 9.14GHz.

to permeate mitochondrial membranes and to have a differential effect on succinate oxidation in whole mitochondria compared with submitochondrial particles (Tyler & Newton, 1970; Harmon & Crane, 1974). Spectra obtained from mitochondria and submitochondrial particles which were oxidized with ferricyanide after an overnight incubation with bathophenanthrolinesulphonate, are shown in Figs. 10 and 11. Fig. 10 shows that the Centre *bc*-3 signal was diminished in mitochondria treated with bathophenanthrolinesulphonate (spectra taken at 15K). Control samples were also incubated in the same way without the addition of bathophenanthrolinesulphonate. Centre *bc*-3 is not present in particles, whereas Centre S-3 is. A differential effect of bathophenanthrolinesulphonate on Centre S-3 can be seen in mitochondria and submitochondrial particles (Fig. 11). The Centre S-3 signal is depleted in the submitochondrial-particle preparation, but not in whole mitochondria.

Fig. 9. E.p.r. spectra of ox heart mitochondria quickly frozen at various time-intervals after addition of oxygen to anaerobic suspension at -7°C

Ox heart mitochondria (22mg of protein/ml) were suspended in a medium containing 25% (v/v) ethylene glycol, 20mM-KCl, 50mM-sucrose, 0.5mM-malate and 20mM-2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid buffer (pH 7.0). The uncoupler carbonyl cyanide *m*-chlorophenylhydrazine (0.27nmol/mg of protein) was also added. Portions of mitochondrial suspension in e.p.r. tubes were incubated at -7°C for about 5 min to anaerobiosis, then mixed with air and rapid-frozen after various time-intervals as indicated in the Figure. Spectra were recorded at 8.0K. E.p.r. conditions: microwave power, 5mW; modulation amplitude, 10^{-3}T ; time-constant, 1s; scanning rate, $2.5 \times 10^{-2}\text{T/min}$; microwave frequency, 9.14GHz.

A time-course of the bathophenanthrolinesulphonate effect on the two 'HiPIP' signals in mitochondria and submitochondrial particles is shown in Fig. 12. Long incubations were required for maximal loss of both Centre *bc*-3 and Centre S-3 signals in mitochondria and submitochondrial particles respectively. Similar results were obtained when bathophenanthrolinesulphonate was removed, after the incubation, by washing twice with fresh suspension medium before the addition of ferricyanide. It was observed that mitochondrial respiration both of succinate and of glutamate plus malate were almost completely inhibited in pigeon heart mitochondria within 1 min of incubation with bathophenanthrolinesulphonate at a concentration of $1\mu\text{mol/mg}$ of protein. However, succinate respiration in submitochondrial particles was mostly

Fig. 10. Effect of bathophenanthrolinesulphonate on the Centre *bc*-3 e.p.r. signal obtained from pigeon heart mitochondria

Mitochondrial suspension (50mg of protein/ml) containing 0.2M-mannitol, 0.05M-sucrose and 50mM-morpholinopropanesulphonate, pH 7.2, was incubated with 50mM-bathophenanthrolinesulphonate (potassium salt) ($1\mu\text{mol/mg}$ of protein) for 3 h at room temperature and then overnight at 4°C . Iron-sulphur centres were oxidized with 5mM-ferricyanide. Control samples were treated the same way except that buffer was added instead of bathophenanthrolinesulphonate. Protein concentrations of both (a) and (b) samples were 36.8mg/ml. E.p.r. conditions were the same as in Fig. 6, except that temperature was 16.1K.

Fig. 11. Effect of bathophenanthrolinesulphonate on the Centre S-3 signal present in pigeon heart submitochondrial particles (a) and mitochondria (b)

(a) and (b), controls; (a') and (b'), bathophenanthrolinesulphonate-treated. Pigeon heart submitochondrial particles and mitochondria were treated with bathophenanthrolinesulphonate as described in Fig. 10. E.p.r. measurements were performed at 7.0K. Other experimental and e.p.r. conditions are the same as for Fig. 6.

Fig. 12. Time-course of the bathophenanthroline-sulphonate effect on the e.p.r. signals of the two different 'HiPIP' centres in mitochondria and submitochondrial particles

Conditions were similar to those used in Figs. 10 and 11. (a) shows the effect of bathophenanthroline-sulphonate on Centre S-3 in whole mitochondria (○) and submitochondrial particles (■). (b) shows the effect of bathophenanthroline-sulphonate on Centre bc-3 in whole mitochondria (▲) (this centre is not detected in the submitochondrial-particle preparation). The spectra of Centre S-3 were taken at 6.9K, those of Centre bc-3 at 15.2K. Microwave power, 10mW; modulation amplitude, 5×10^{-4} T; scanning rate, 2×10^{-2} T/min; time-constant, 0.3s; microwave frequency, 9.14GHz.

insensitive to bathophenanthroline-sulphonate within this time-interval. NADH oxidation in submitochondrial particles was sensitive to bathophenanthroline-sulphonate inhibition ($1 \mu\text{mol/mg}$ of protein), but is significantly less sensitive than the mitochondrial NAD-linked substrate oxidation (maximum 70% inhibition). These results are somewhat at variance with the results obtained in ox heart systems (Harmon & Crane, 1974). The effect of bathophenanthroline-sulphonate on mitochondria is probably due to the reaction of this agent on the mitochondrial dicarboxylate carrier (Tyler & Newton, 1970; Passarella *et al.*, 1973). It was found that within the time-range required to inhibit substrate oxidation, no significant effect of bathophenanthroline-sulphonate on e.p.r.

signals of any iron-sulphur centres was detected. Prolonged incubation of submitochondrial particles with bathophenanthroline-sulphonate, as presented in Fig. 12, totally inhibited succinate oxidation. No e.p.r. signals of ferredoxin-type iron-sulphur centres in mitochondria and submitochondrial particles were affected, even by long-term treatment, with bathophenanthroline-sulphonate either in mitochondria or in submitochondrial particles.

Discussion

Since the application of sub-liquid-nitrogen temperatures to e.p.r. spectrometry, the number of iron-sulphur centres detected in mitochondrial preparations has greatly increased. The identification of Centre bc-3 as a species distinct from Centre S-3 brings the number of reported mitochondrial iron-sulphur centres to 13. Not all of these have, however, been conclusively shown to be essential respiratory-chain components (Ohnishi *et al.*, 1974b; Ohnishi, 1975). The recognition of mitochondrial e.p.r. absorption characteristics of a 'HiPIP'-type centre (i.e. an iron-sulphur centre which is paramagnetic in the oxidized state) is a fairly recent discovery (Ruzicka & Beinert, 1974). More specifically, a 'HiPIP'-type centre has been shown to be present in the succinate-ubiquinone reductase (Beinert *et al.*, 1974) and in the purified reconstitutively active succinate dehydrogenase (Ohnishi *et al.*, 1974c).

In the present study the properties of Centre S-3 were further investigated and a second 'HiPIP' Centre bc-3 was characterized. A preliminary report of the resolution of two 'HiPIP'-type mitochondrial iron-sulphur proteins has been published (Ohnishi *et al.*, 1975). The two 'HiPIP' centres differ in their detailed line-shape, temperature profile and signal g values. A value for the midpoint potential of Centre S-3 has been reported as $E_{m7.4} = +60\text{mV}$ (Ohnishi *et al.*, 1974c) and this value is lower than that reported in later studies (Inglede & Ohnishi, 1975; $E_{m7.2} = +120\text{mV}$). The former value, however, was obtained from succinate-cytochrome *c* reductase, which is a particulate preparation purified in the presence of detergent, and the latter from an intact mitochondrial-membrane system. Similarly, Centre N-2 in the purified particulate preparation, Complex I, was found to show a lower midpoint potential than that in the intact mitochondrial-membrane system (Ohnishi *et al.*, 1974a).

The midpoint potential measured for Centre S-3 in the intact membrane is rather high for a component of succinate dehydrogenase, although it does fill a potential gap between cytochromes *b* and cytochromes *c*. The sequential relationship of Centre S-3 to the cytochrome *b* in the respiratory chain is of great importance; their relative midpoint potentials suggest that Centre S-3 ought to function on the

oxygen side of the cytochromes *b* ($E_{m7.0} = -30$ and $+40$ mV) (Wilson & Dutton, 1970). Centre S-3, however, is a component of the isolated succinate dehydrogenase and as such ought to be on the substrate side of the *b* cytochromes, if the *b* cytochromes are on the main pathway of succinate oxidation.

Centre *bc*-3 does not titrate potentiometrically in a satisfactory manner; this is thought to be due to a modification caused by highly oxidizing conditions.

To establish whether or not Centres S-3 and *bc*-3 function in the respiratory chain, their redox responses to various metabolic conditions and their kinetics of oxidation after an anaerobic-aerobic transition were studied. The kinetic assays were performed manually in aqueous ethylene glycol mixtures at temperatures below 0°C. Low-temperature kinetics, developed by Strother & Ackerman (1961) and applied to mitochondrial systems by Erecinska & Chance (1972), is a method which has the advantage, especially in the preparation of samples for e.p.r., of slowing the reaction rates. Centre S-3 was oxidized with a similar time-course to that obtained for Centre S-1; this is taken to indicate a close association of these two components with each other. The apparent kinetic incompetence of Centre *bc*-3 and its redox behaviour in various metabolic states of mitochondria argue against a role for Centre *bc*-3 in the main electron-transport pathway. However, the oxidation of Centre *bc*-3 by the addition of antimycin A does indicate that Centre *bc*-3 is equilibrating with the respiratory chain on the oxygen side of the antimycin A block, although this equilibration is probably slow. The effect of various respiratory-chain inhibitors on the oxidation state of Centre S-3 indicates that the centre is located on the oxygen side of the rotenone block and on the substrate side of the antimycin A and cyanide blocks. The redox state of Centre S-3 responds to the 'energy state' of the mitochondria in classical fashion for a respiratory-chain component (Chance & Williams, 1956), except that in the presence of uncoupler, when all of the components ought to be highly oxidized (Klingenberg, 1968), some of the signals which have been attributed to an interaction involving the semiquinone form of ubiquinone (Beinert, 1974; Ruzicka & Beinert, 1975a; Ingledew & Ohnishi, 1975) remain, indicating that some semiquinone is still present.

Harmon & Crane (1974) have suggested that at least one of the iron-sulphur centres of the succinate dehydrogenase is located on the cytosolic side of the inner mitochondrial membrane, indicating that the succinate dehydrogenase enzyme goes through the membrane from matrix (where succinate is oxidized) to cytosolic side. This conclusion is based on their observation that succinate oxidation was inhibited by the reportedly impermeant iron chelator bathophenanthrolinesulphonate in whole mitochondria,

but not in the submitochondrial particles, which are inverted relative to the intact mitochondria. In the present study an effect of bathophenanthrolinesulphonate on an iron-sulphur centre was observed, but only after long incubation, whereas the effects reported by Harmon & Crane (1974) were rapid. It is apparent that the results of Harmon & Crane (1974) are due solely to the previously reported inhibition of the mitochondrial dicarboxylate carrier by bathophenanthrolinesulphonate (Tyler & Newton, 1970; Passarella *et al.*, 1973).

A 3-4 h preincubation of the mitochondrial membrane preparation with a high concentration of bathophenanthrolinesulphonate (50 mM) is required for an effect to be observed on the e.p.r. spectrum of either 'HiPIP' iron-sulphur centre. This time-course is consistent with the reported observations that even purified iron-sulphur proteins react very slowly with iron chelators in the absence of added denaturing agents (Malkin, 1973). After a long incubation with bathophenanthrolinesulphonate, succinate oxidation in submitochondrial particles was also inhibited, concomitant with the loss of Centre S-3. The use of bathophenanthrolinesulphonate as a marker for anisotropy of the mitochondrial membrane is open to criticism, especially in view of the long preincubations used in the present study; however, the reagent does have differential effects on the two mitochondrial 'HiPIP'-type iron-sulphur centres. In intact mitochondria only Centre *bc*-3 was lost after prolonged treatment with bathophenanthrolinesulphonate, whereas in submitochondrial particles (where Centre *bc*-3 is absent), Centre S-3 was lost; these observations are consistent with the proposed impermeability of the chelator.

The present investigation resolves the mitochondrial 'HiPIP' signal into two separate components. Evidence is presented which shows that the first of these two centres (Centre S-3) is a full member of the respiratory chain, between succinate and oxygen, but that the second component (Centre *bc*-3) is not a full member. It is suggested that Centre S-3 is localized on the matrix (inner) side of the mitochondrial coupling membrane, but that the non-participating Centre *bc*-3 is located on the cytosolic side of the inner mitochondrial membrane. It is implicit in this assignment of Centre S-3 [which has been suggested to be a respiratory-chain component which transfers electrons to ubiquinone (Ohnishi *et al.* 1974c)] that the whole of the succinate dehydrogenase molecule is located on the matrix side of the inner mitochondrial membrane.

Note Added in Proof (Received 28 October 1975)

Recently the ferredoxin-type iron-sulphur centre 'Centre 5' was reported to play a role as an electron carrier in the fatty acyl dehydrogenation pathway (Ruzicka & Beinert, 1975b).

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