Electron-Paramagnetic-Resonance Spectroscopy Studies of Iron–Sulphur Centres of Submitochondrial Particles from Iron- and Sulphur-Deficient *Candida utilis*

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1. Measurements were made at 12°K of the electron-paramagnetic-resonance (e.p.r.) spectra of submitochondrial particles from *Candida utilis* cells grown under conditions that alter the amount of the mitochondrial NADH dehydrogenase (EC 1.6.99.3). 2. Iron-limited growth decreases the extent of iron–sulphur e.p.r. signals to undetectable values that are less than 1% of those normally found with glycerol-limited growth. 3. Small but significant signals attributable to the NADH dehydrogenase were detected in submitochondrial particles from sulphate-limited cells. 4. Measurements made on submitochondrial particles prepared from these and other phenotypically modified cells lead us to conclude that the presence of low-temperature e.p.r.-detectable iron–sulphur centres attributable to the NADH dehydrogenase are necessary but not sufficient for the coupling of ATP synthesis to the NADH dehydrogenase reaction in the mitochondrial membrane of *C. utilis*. 6. The amplitude of the $g=2.01$ signal observed in non-reduced submitochondrial particles is approximately tenfold diminished by iron limitation but not significantly altered by sulphate limitation.

Sands & Beinert (1960) first applied e.p.r.* spectroscopy to isolated mitochondria and submitochondrial particles. Among the signals detected at 77°K was one elicited only by reduction with sodium dithionite or NADH, and centred at $g=1.94$. This signal was attributed to the mitochondrial respiratory-chain NADH dehydrogenase, which is the membrane-bound enzyme containing both FMN and iron–sulphur centres and is normally responsible in mammalian mitochondria for the oxidation of intramitochondrial NADH by the respiratory-chain cytochromes (Singer & Gutman, 1971). The physiological acceptor for membrane-bound NADH dehydrogenase is probably endogenous ubiquinone, and the associated synthesis from ADP and phosphate of an ATP molecule for each molecule of ubiquinone reduced is referred to as site-1 phosphorylation, thereby distinguishing it from oxidative phosphorylation associated with the remainder of the respiratory chain (Lehninger, 1966).

A number of studies have explored a possible correlation between the presence of an NADH-elicited $g=1.94$ signal in submitochondrial particles

and the capacity for site-1 phosphorylation. These have been summarized by Ohnishi (1973). At first they provided an undisputed correlation, for it was observed that whereas ox heart mitochondrial particles exhibited both an NADH-elicited $g=1.94$ signal and site-1 phosphorylation, these features were absent from *Saccharomyces cerevisiae* (Schatz & Racker, 1966; Schatz *et al.*, 1966). Subsequently this simple situation has been complicated by the discovery that e.p.r. spectroscopy at very low temperatures (below 30°K) revealed multiple iron–sulphur centres in the NADH dehydrogenase (Ohnishi *et al.*, 1970, 1971; Orme-Johnson *et al.*, 1971), and that the presence or absence of site-1 phosphorylation in yeast mitochondria was dependent not only on the species studied, but also on the particular growth conditions for that species (Light *et al.*, 1968; Clegg *et al.*, 1969; Ohnishi *et al.*, 1969; Ohnishi, 1970, 1972; Mackler & Haynes, 1973; Ghosh & Bhattacharyya, 1971).

In the present paper we describe measurements made below 30°K of the e.p.r. spectra of submitochondrial particles derived from *Candida utilis* cells that have been grown in continuous culture under conditions of glycerol-, iron- or sulphate-limited

* Abbreviation: e.p.r., electron paramagnetic resonance.

growth, with or without various further treatments of the cells that can result in the recovery of previously depleted mitochondrial non-haem iron or acid-labile sulphide (Clegg & Garland, 1971; Haddock & Garland, 1971).

Materials and Methods

Growth of cells

C. utilis (strain no. 193; National Collection of Yeast Cultures, Nutfield, Surrey, U.K.) was grown in continuous culture as described by Light & Garland (1971) at a dilution rate of 0.2 h^{-1} . All media contained 133 mM-glycerol, and manganese salts were omitted. The glycerol-limited media contained $2\text{ }\mu\text{M}$ added iron (as Fe^{3+}) which was omitted for iron-limited growth (Clegg & Garland, 1971). Sulphate-limited growth (Haddock & Garland, 1971) was achieved with a medium containing 133 mM-glycerol, 12.8 mM- NaH_2PO_4 , 2.8 mM- Na_2HPO_4 , 75 mM- NH_4Cl , 10 mM-KCl, 1 mM-citric acid, $4\text{ }\mu\text{g}$ of biotin/litre, $100\text{ }\mu\text{M}$ - K_2SO_4 and 1 ml of the following salts solution per litre of medium: $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1.25 M; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1 M; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.5 mM; ZnO, 5 mM; CuCl_2 , 0.05 mM, in 3 M-HCl. Antifoam (type GF from Midland Silicones Ltd., Barry, Glam., U.K.) was added to the final medium (0.24 ml/litre).

Preparation of submitochondrial particles

Cells were harvested, converted into spheroplasts and used for the preparation of mitochondria and thence submitochondrial particles as described by Clegg & Garland (1971).

Verification of iron- or sulphate-limited growth

The concentration of both glycerol and cells in the chemostat effluent was periodically measured, and the dry-weight yield of cells produced per mol of glycerol utilized (i.e. the Y_{glycerol}) was calculated (Clegg & Light, 1971). Y_{glycerol} values of greater than $50\text{ g}\cdot\text{mol}^{-1}$ indicated glycerol limitation and the presence of all three oxidative-phosphorylation sites in association with the respiratory chain, whereas Y_{glycerol} values of approx. $40\text{ g}\cdot\text{mol}^{-1}$ indicated iron or sulphate limitation with loss of ATP synthesis coupled to the NADH-ubiquinone oxidoreductase segment of the respiratory chain (Clegg & Light, 1971). Further confirmation of iron limitation was obtained by measuring the sensitivity of the NADH oxidase activity of submitochondrial particles to the respiratory inhibitor piericidin A; glycerol-limited cells yield submitochondrial particles that were sensitive to piericidin A, whereas those from iron- or sulphate-limited cells were insensitive (Ragan & Garland, 1971).

Recovery procedures

Aeration of washed non-growing sulphate- or iron-limited cells for 12–14 h at 30°C in water was carried out as described by Clegg & Garland (1971). This so-called recovery procedure results in the return of site-1 phosphorylation, and the cells are referred to as sulphate-recovered or iron-recovered. When cycloheximide ($100\text{ }\mu\text{g/ml}$) was added to the recovery medium, the resulting cells are referred to as sulphate-recovered (cycloheximide) and iron-recovered (cycloheximide) cells.

Measurements of electron paramagnetic resonance

These were made with a Varian E9 spectrometer operating at 9 GHz (Varian Associates Ltd., Walton-on-Thames, Surrey, U.K.), with temperature control and measurement as described by Lowe *et al.* (1972). Spectra were generally measured at a sample temperature of $12 \pm 0.2^\circ\text{K}$ and at 20 mW microwave power. More detailed measurement conditions are described in the legends to the Figures. Suspensions of submitochondrial particles were placed in e.p.r. sample tubes of 3 mm internal diameter (Downie & Garland, 1973), with or without various treatments such as the addition of NADH as indicated in the legends to the Figures. After preparation the samples were frozen and stored in liquid N_2 . When it was wished to investigate the effect on the e.p.r. spectrum of further reduction of the sample by dithionite, the sample was thawed under a stream of purified argon (Swann & Bray, 1972) and $5\text{ }\mu\text{l}$ of 1 M-sodium dithionite in 0.01 M-NaOH was added with a microsyringe fitted with a 25 cm-long needle. The sample was then mixed with a length of stiff nichrome wire ending in a bead slightly smaller than the bore of the sample tube and made of epoxy resin. After mixing with dithionite the sample was immediately refrozen in liquid N_2 .

Terminology for iron-sulphur centres of submitochondrial particles showing e.p.r. resonances in the range $g=1.86$ to 2.10

We have adopted the terminology of Orme-Johnson *et al.* (1971), who resolved the low-temperature spectrum of the NADH dehydrogenase of ox heart submitochondrial particles into four components of differing oxidoreduction potentials, temperature sensitivities and microwave power-saturation characteristics. Fig. 1 indicates the contribution of each centre to the complete spectrum. The approximate field positions of the prominent peaks for the iron-sulphur centres in the NADH dehydrogenase segment of the respiratory chain of *C. utilis* submitochondrial particles reported by Ohnishi *et al.* (1972a) are: Centre 1 (signals q, r and s), $g=2.02$,

Fig. 1. Components of the low-temperature e.p.r. spectrum of submitochondrial particles from *C. utilis*

The resonances due to each centre (Ohnishi *et al.*, 1970) are represented by upward and downward bars to indicate peaks and troughs respectively, and are plotted against a g -value scale. Dotted bars are components normally obscured by other features of the spectrum. A spectrum of particles from glycerol-limited cells, reduced with NADH, has been aligned with the g -value scale to show the contribution from each centre to the whole spectrum. The bar diagrams represent 'cubic' iron (a), Centre 1 (b), Centre 2 (c) and Centres 3 and 4 (d). The conditions for measurement were: sample temperature, 12°K; modulation amplitude 10^{-3} T; microwave frequency, 9.1 GHz; microwave power, 20 mW; protein concentration, 42 mg/ml; and receiver gain 6.3×10^2 .

$g=1.94$ and $g=1.92$ respectively; Centre 2 (o, p), $g=2.05$ and $g=1.92$; Centres 3 and 4 (l, m, n), $g=2.10$, $g=1.88$ and $g=1.85$. Of these centres, Centre 1 is detected as a signal at 77°K, whereas the other centres are not all detected except at about 20°K or less, as was shown by Ohnishi *et al.* (1970, 1971) and Orme-Johnson *et al.* (1971). Our e.p.r. spectra are presented in the Figures as the first derivatives in arbitrary units of the microwave-power absorption,

plotted against the magnetic field. We refer to maxima and minima in the first-derivative spectrum as peaks (upward) and troughs (i.e. downward peaks) respectively, for that is how they appear in the Figures. We give g values to the nearest second decimal place.

Results

Spectra of submitochondrial particles from glycerol-limited cells

Fig. 2 shows spectra of submitochondrial particles of glycerol-limited cells, measured at 12°K in the unreduced state, reduced with NADH alone, or NADH and dithionite together. The main features are similar to those described by Ohnishi *et al.* (1971) and Cobley *et al.* (1973) for stationary-phase batch-cultured *C. utilis*, and show in the oxidized state (Fig. 2, trace a) a prominent signal with a peak at $g=2.02$ and trough (downward peak) at $g=2.00$, which we will refer to as the $g=2.01$ signal (Orme-Johnson *et al.*, 1971). A much smaller peak at $g=2.05$ and the trough at $g=1.92$ are probably due to a partial reduction of Centre 2 even in the absence of an added reductant. Reduction of the respiratory chain with NADH caused the appearance of a spectrum (Fig. 2, trace b) similar to that described under comparable conditions by Ohnishi *et al.* (1971), with distinct peaks at $g=2.10$ (l of Centres 3+4), $g=2.05$ (o of Centre 2), $g=1.95$ (Centre 1) with a shoulder at $g=1.94$ (r of Centre 1) and troughs at $g=1.92$ (s of Centre 1 with p of Centre 2), $g=1.89$ and $g=1.86$ (m and n of Centres 3+4). The $g=2.02$ peak of high-spin ferric iron obscures the $g=2.02$ peak (q of Centre 1) in NADH-reduced submitochondrial particles. Trace (c) of Fig. 2 shows the spectrum after further reduction with dithionite. The $g=2.01$ signal is diminished in height, presumably owing to reduction of the centre responsible for this signal in the oxidized state. These results confirm those of Ohnishi *et al.* (1971), and serve as a point of reference for describing the effects of iron and sulphate limitation on the e.p.r. spectra. None of the low-temperature e.p.r. spectroscopic features caused by reduction with NADH arose when glycerol 3-phosphate was the reductant (Ohnishi *et al.*, 1971), and the features elicited with NADH can be attributed to the NADH dehydrogenase.

Under the conditions used for these measurements (12°K, 20 mW microwave power) some of the signals are partially saturated, with amplitudes decreased to about 50% of those observed under non-saturating conditions. Nevertheless, all the peaks described by Orme-Johnson *et al.* (1971) and due to NADH dehydrogenase are readily detected and quantified under our measurement conditions. Increase of sample temperature caused by microwave heating was not a problem at 12°K with our helium cooling system, since normalized intensity of the cubic iron signal remained constant up to at least 100 mW power.

Fig. 2. E.p.r. spectra of submitochondrial particles from glycerol-limited cells of *C. utilis*

Trace (a) was obtained from particles oxidized by the addition of oxygenated buffer just before freezing. Trace (b) was obtained from particles reduced with NADH and trace (c) from particles further reduced by the addition of sodium dithionite as described in the Materials and Methods section. The conditions for measurement were as described in the legend to Fig. 1. The protein concentration in the sample and the receiver gain were: for trace (a), 80 mg/ml and 5×10^2 ; for trace (b), 42 mg/ml and 6.3×10^2 ; and for trace (c), 73 mg/ml and 8×10^2 . The labelling on trace (c) refers to (a) and (b) as well.

Spectra of submitochondrial particles from sulphate-limited and sulphate-recovered cells

Spectrum (a) of Fig. 3 for NADH-treated submitochondrial particles from sulphate-limited cells

exhibits a signal at $g=2.01$. There is also a distinct signal between $g=1.95$ and $g=1.92$, but present in much lower amounts than in submitochondrial particles from glycerol-limited cells. The middle spectrum shows that there is a return of all four iron-sulphur centres in the sulphate-recovered cells. This is matched by the return of site-1 phosphorylation during sulphate recovery. The inclusion of cycloheximide in the recovery medium does not prevent the return of site-1 phosphorylation (Haddock & Garland, 1971), nor does it prevent the return of e.p.r.-detectable iron-sulphur signals (Fig. 3c). However, the intensity of these signals is diminished by about threefold relative to the sample recovered without cycloheximide, and only the features in the $g=1.92$ – 1.95 region are unequivocally discernible.

Spectra of submitochondrial particles from iron-limited and iron-recovered cells

The spectrum of NADH-reduced submitochondrial particles from iron-limited cells shown in the trace (a) of Fig. 4 is clearly devoid of any of the signals characteristic of the iron-sulphur centres of the respiratory chain. By contrast, the spectrum obtained after the iron-recovery procedures (Fig. 4b) shows an obvious peak at $g=1.95$ and troughs at $g=1.92$, $g=1.89$ and $g=1.86$, with a peak at $g=2.10$ and a shoulder at $g=2.05$. If the iron-recovery procedure was carried out in the presence of cycloheximide, the e.p.r. spectrum was not significantly different from that of particles from unrecovered iron-limited cells (compare traces a and c of Fig. 3).

Relative peak heights of iron-sulphur e.p.r. signals

The spectra shown above were obtained under a variety of protein concentrations and instrumental gain settings. In order to facilitate comparisons the heights of the peaks of interest have been corrected to a standard protein concentration and gain setting, and are listed in Table 1.

Effect of iron and sulphate limitation on the e.p.r. signal between $g=2.02$ and $g=2.00$ detected in non-reduced submitochondrial particles

The prominent e.p.r. signal with a peak at $g=2.02$ and a trough at $g=2.00$, seen in submitochondrial particles in the absence of reductant (i.e. the oxidized state), is similar to the comparable signal in ox heart submitochondrial particles and attributed by Orme-Johnson *et al.* (1971) to high-spin ferric iron in a cubic environment. Ruzicka & Beinert (1974) have identified signals of this type with that arising from a high-potential iron-sulphur protein isolated from ox heart mitochondria. Further, Der Vartanian *et al.* (1974) demonstrated by ^{57}Fe isotopic substitution

Fig. 3. E.p.r. spectra of submitochondrial particles from sulphate-limited and sulphate-recovered cells of *C. utilis*

The particles were reduced with NADH as described in the Materials and Methods section. The protein concentration in each sample was 40 mg/ml and the conditions for measurement were as described in the legend to Fig. 1. Trace (a) was obtained from particles from sulphate-limited cells at a receiver gain of 5×10^2 ; trace (b) was from particles from sulphate-recovered cells at a receiver gain of 4×10^2 ; and trace (c) was from particles from sulphate-recovered (cycloheximide) cells at a receiver gain of 6.3×10^2 .

Fig. 4. E.p.r. spectra of submitochondrial particles from iron-limited and iron-recovered cells of *C. utilis*

The particles were reduced with NADH as described in the Materials and Methods section. Conditions for measurement were as described in the legend to Fig. 1, and the receiver gain in each trace was 2×10^3 . Trace (a) was obtained from particles from iron-limited cells at a protein concentration of 79 mg/ml. Trace (b) was obtained from particles from iron-recovered cells at a protein concentration of 62 mg/ml, and trace (c) was from particles from iron-recovered (cycloheximide) cells at a protein concentration of 39 mg/ml.

that the similar signal in cell membrane fragments of *Azotobacter vinelandii* is due to an iron-containing structure. In view of these reports we compared the amplitude of the $g=2.01$ signal, measured from $g=2.02$ to 2.00, in the 12°K spectra of non-reduced submitochondrial particles prepared from the various yeast phenotypes. After correcting for protein concentration and running conditions, the amplitudes of the signal from $g=2.02$ to 2.00 were not significantly

different for glycerol-limited, iron-recovered, sulphate-limited, sulphate-recovered and sulphate-recovered (cycloheximide) samples, whereas the iron-limited and iron-recovered (cycloheximide) samples had the amplitude of this signal decreased to 10% or less of the other samples. These results provide independent evidence for attributing the $g=2.01$ signal to an iron-containing structure. If the iron-containing structure exhibiting the $g=2.01$

Table 1. *Amplitudes of signals in e.p.r. spectra of submitochondrial particles*

The experimental conditions are those given in the legends of Figs. 1–4. Amplitudes were measured, either from the baseline to the peak or trough (downward peak) in the region of the g value listed, or as the difference in amplitudes at two different g values ($g=1.94$ refers to measurements from the shoulder in this region). In all instances the reductant was NADH. Amplitudes of the $g=1.95$ – 1.92 signal were normalized (to an instrument gain setting of 10^3 and a protein concentration of 40 mg/ml). Amplitudes of other features are reported relative to this, and the identity of the centre corresponding to the spectral features is given in parentheses.

	Normalized amplitude at $g=$ 1.95–1.92 (1+2)	Ratio of amplitudes at listed g values				
		2.10 1.95–1.92 (3+4)	2.05 1.95–1.92 (2)	1.95–1.94 1.95–1.92 (1)	1.89 1.95–1.92 (3+4)	1.86 1.95–1.92 (3+4)
Glycerol-limited	126	0.07	0.12	0.53	0.19	0.10
Iron-limited	<1					
Sulphate-limited	2.3			Approx. 0.5		
Sulphate-recovered	50	0.11	0.18	0.46	0.21	0.18
Sulphate-recovered (Cycloheximide)	5.5			Approx. 0.5		
Iron-recovered	6	0.15	0.18	0.64	0.33	0.21
Iron-recovered (Cycloheximide)	<2					

signal in *C. utilis* preparations involves acid-labile sulphide, as is the case for ox heart mitochondria (Ruzicka & Beinert, 1974), then the response of its synthesis or structure to sulphate deficiency is clearly different from that of the iron–sulphur centres of the NADH dehydrogenase.

Discussion

Magnitude of e.p.r. signals from iron–sulphur centres and the supply of iron or sulphate in the growth medium

It is clear from Table 1 that even the larger e.p.r. signals at about $g=1.94$ seen in spectra of submitochondrial particles from glycerol-limited cells are not detected in particles from iron-limited cells. The limit of detection corresponds to about 1% of the amplitude between $g=1.95$ and $g=1.92$ observed in particles from glycerol-limited cells. It should be noted that even the glycerol-limited cells are grown at relatively low entering iron concentrations ($2\mu\text{M}$), and supplementation of the glycerol-limited medium with higher iron concentrations increases the concentration of iron–sulphur centres in submitochondrial particles (Ohnishi *et al.*, 1969). There is a small but easily detectable e.p.r. signal in the $g=1.94$ region of spectra of particles from sulphate-deficient cells, its amplitude being about 3% of that in glycerol limitation.

Relatively small increases of e.p.r.-detectable iron–sulphur centres occur during recovery procedures,

less than 10% of the normal signal intensities returning during iron recovery and less than 40% during sulphate recovery.

Effects of cycloheximide on the recovery procedure

Exposure of sulphate-limited cells to cycloheximide during the recovery procedure abolishes the reappearance of mitochondrial sensitivity to the respiratory inhibitor piericidin A, but does not inhibit the return of site-1 phosphorylation (Clegg & Garland, 1971; Haddock & Garland, 1971). The evidence for the presence of site-1 phosphorylation in mitochondria from sulphate-recovered (cycloheximide) cells consists of convincing polarographic measurements of the number of respiratory-chain phosphorylation sites, and the demonstration of energy-dependent reversal of electron flow at site 1 (Haddock & Garland, 1971). Nor is there any ambiguity about the reappearance of at least some of the iron–sulphur centres attributable to the NADH dehydrogenase (Fig. 3, Table 1), there being clearly marked e.p.r. signals corresponding to Centres 1+2 (peak at $g=1.95$, trough at $g=1.92$ and a shoulder at $g=1.94$), with possibly the presence of Centres 3+4. The shape of the signal in the $g=1.95$ – 1.92 region indicates that both Centres 1 and 2 are present in proportions that are unchanged from the glycerol-limited state. The small peak at $g=2.05$ is only just discernible among background fluctuations of the baseline, but provides further evidence, not available in an earlier report (Garland *et al.*, 1972), for the

presence of Centre 2 in sulphate-recovered (cycloheximide) cells.

The evidence on site 1 in mitochondria from iron-recovered (cycloheximide) cells is less convincing (Clegg & Garland, 1971). The polarographic measurements of mitochondrial respiration indicated that the addition of cycloheximide to the iron-recovery medium lowered the P/O ratio (i.e. the ratio of the number of mol of ATP synthesized by oxidative phosphorylation to the number of g-atoms of oxygen utilized) from a mean of 2.77 (eight mitochondrial preparations, S.E.M. ± 0.35) to a mean of 2.33 (three preparations, S.E.M. ± 0.15) (Clegg & Garland, 1971). Nor was reversal of electron transport at site 1 demonstrated in mitochondria from iron-recovered (cycloheximide) cells. Ohnishi *et al.* (1972b) concluded that cycloheximide inhibited the recovery of site 1 in iron-deficient cells and also the return of e.p.r.-detectable iron-sulphur. Our own low-temperature spectra also show that there are no definite e.p.r. signals from iron-sulphur proteins in the $g=1.95$ – 1.92 region in submitochondrial particles from iron-recovered (cycloheximide) cells (Fig. 4c).

Do the growth conditions modify the relative proportions of the iron-sulphur centres of the NADH dehydrogenase?

Orme-Johnson *et al.* (1971) concluded that probably equimolar amounts of Centres 1 to 4 were present in the NADH dehydrogenase of ox heart mitochondria. Our own data (Table 1) indicate particularly for the iron-recovered samples that, as the growth conditions were varied, the concentration of Centres 3+4 detected increased up to twofold relative to that of Centres 1+2. However, the possibility of the presence of additional iron-sulphur proteins in our samples (cf. Cobley *et al.*, 1973) is not excluded by our data.

Is there a correlation between the presence of e.p.r.-detectable iron-sulphur proteins in the NADH dehydrogenase segment of the respiratory chain and the presence of site-1 phosphorylation?

Two variables may be said to be correlated when the presence of one implies the presence of the other. Imai *et al.* (1968), Clegg *et al.* (1969) and Ohnishi (1970) described situations where site-1 phosphorylation existed in the absence of e.p.r. signals characteristic of the NADH dehydrogenase iron-sulphur. The absence of e.p.r. signals could have been due to their low value and lack of instrumental sensitivity; in the instance of submitochondrial particles derived from sulphate-limited cells recovered in the presence of cycloheximide, iron-sulphur centres undetected at 98°K (Clegg *et al.*, 1969) are apparent at 12°K (Table 1, Fig. 3). Thus from the data available it can be

concluded that when e.p.r. spectra are measured at about 12°K rather than 77°K or higher, there is as yet no instance of site 1 occurring in *C. utilis* without some concomitant e.p.r. signals arising from the NADH dehydrogenase. The reverse is not true, as is shown by the presence detectable e.p.r. signals resembling part at least those of the NADH dehydrogenase under conditions when site 1 is undetectable, as for example, in submitochondrial particles from sulphate-limited cells (Fig. 3a) or from exponential-phase batch-cultured cells (Cobley *et al.*, 1973). Even if a correlation cannot be supported, it seems true that the e.p.r. signals of the iron-sulphur centres of the NADH dehydrogenase are necessary but not sufficient for the presence of site-1 phosphorylation in *C. utilis*.

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