INTER-RELATIONSHIPS BETWEEN MITOCHONDRIAL ENERGY CONSERVATION AT SITE I, PIERICIDIN A SENSITIVITY, AND EPR SPECTRA IN TORULOPSIS UTILIS

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1. Introduction

Mitochondrial energy conservation at site 1 [1–5] and sensitivity towards the respiratory inhibitors rotenone [2, 3, 5] and piericidin A [5] are absent from *Sacharromyces cerevisiae* and *S. carlsbergensis* but present in *T. utilis* [6, 7]. The association between inhibitor sensitivity and site 1 energy conservation has been thought to indicate that in some way these features are interdependent [3,6,8]. Further support for such an interdependence was provided by our finding that iron-limited growth of *T. utilis* resulted in the loss of both site 1 energy conservation and of sensitivity towards piericidin A. An involvment of non-heme iron at site 1 [9] is also indicated by the association of a g = 1.94 EPR signal with the NADH dehydrogenase system of *T. utilis* [10, 11] but not *S. cerevisiae* [8].

In this communication we describe (a) growth conditions producing *T. utilis* cells with mitochondria that possesss site 1 energy conservation but lack piericidin A sensitivity, (b) the effects of sulphate-limited growth on the respiratory chain of *T. utilis*, and (c) EPR studies on submitochondrial particles from *T. utilis* that had been grown under various conditions of iron or sulphate limitation.

2. Methods

Continuous culture conditions were as described previously [7] except where indicated below. The iron content of the growth medium was assayed colorimetrically [12] and sufficient carrier free Fe^{59} citrate was added to bring the specific activity to 1μC:/μg atom Fe. The non-heme iron content of mitochondria was calculated from their radioactivity, the specific activity of Fe^{59} in the growth medium, and the mithochondrial content of heme iron assayed spectrophotometrically as cytochromes [7]. P/O ratios were assayed polarographically. In all instances the P/O ratio with glycerol-3-phosphate as substrate was between 1.6 and 2.0, and sites II and III were considered to be present. A similar ratio with pyruvate and malate as substrates was taken to indicate the absence of site 1, whereas ratios of 2.6–3.0 showed its presence. Sulphate limited growth was achieved by reducing the sulphate concentration of the growth medium [7] to 100 μM and adding 10 mM KCl to maintain the potassium concentration. $MnCl_2$ was omitted from the sulphate limited medium and also from the iron limited medium in those instances specified below. This was necessary to prevent the appearance of manganese lines in the EPR spectra which can obscur those attributed to non-heme iron proteins. Submitochondrial particles

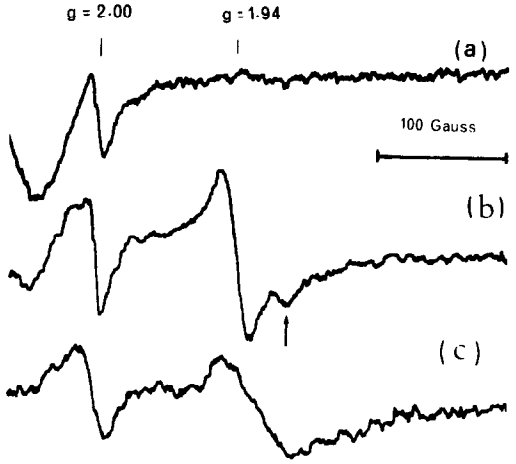

Fig. 1. EPR spectra obtained on addition of NADH to submitochondrial particles from *T. utilis* grown in media of differing iron contents. The media were as described [7] except for the following: (a) 0.47 μg atom Fe/L, 130 mM glycerol, no added $MnCl_2$. (b) 1.2 μg atom Fe/L, 55 mM glycerol, no added $MnCl_2$. (c) 0.37 μg atom Fe/L, 55 mM glycerol, no added $MnCl_2$.

Samples were frozen in 3 mm internal diameter quartz tubes 10 min after the addition of 1 μmole of NADH at 0–4°C. EPR spectra (9.3 G Hz) were obtained at about 98°K and 10 milliwatt power. Instrument settings were identical and protein concentrations were all about 50 mg/ml. The features in the region of $g = 2.0$ and above arise largely from traces of cupric ion and free radical in the samples.

were prepared by ultrasonic disruption of mitochondria [7] followed by differential centrifugation.

3. Results

The iron concentration of the growth medium in the absence of added iron was about 0.2 μg atom/L, and this more accurate value is preferred to our previous estimate of 0.7 μg atom/L [7]. Under these conditions the cell yield in the chemostat was 2.9 mg dry wt/ml, the glycerol concentration in the chemostat 60 mM, and both site I energy conservation and sensitivity to 0.5 μM piericidin A were absent from the mitochondria. When the iron concentration in the growth medium was increased to 0.54 μg atom/L the culture changed over 24 hr to a new steady state characterised by a cell yield of 5.6 mg dry wt/ml, complete utilisation of glycerol, a continued absence of site I energy conservation and piericidin A sensitivity and a mitochondrial non-heme iron content of 0.27 natom/mg protein. A further increase of the growth medium iron concentration to 0.65 μg atom/L increased the cell yield to 7.9 mg dry wt/ml, increased to P/O ratio with pyruvate and malate to 2.4–2.9, increased the mitochondrial non-heme iron content to 0.33 natom/mg protein, and did not affect the insensitivity to piericidin A (0.5 μM at a mitochondrial protein concentration of 0.2 mg/ml i.e., a twenty five field or greater excess over the usual 50–100 μmole piericidin A/mg protein needed for maximum inhibition of pyruvate and malate oxidation [7]). The return of energy conservation at site I was also demonstrated by the energy dependent reduction of NAD or low potential flavoproteins by glycerol-3-phosphate oxidation [7]. These observations demonstrate that under suitable growth conditions site I energy conservation and piericidin A sensitivity can be dissociated from each other in *T. utilis*. Dissociation can also be achieved by the addition of inhibitors of protein synthesis to iron-limited *T. utilis* cells during their non-growing recovery [7, 13].

Mitochondria prepared from cells grown under sulphate-limited conditions were similar to those from iron-limited growth in that both piericidin A sensitivity and site I energy conservation were absent. Increasing the sulphate concentration to 300 μM resulted in the parallel return of both properties. For EPR measurements, cells were grown on a medium containing no added $MnCl_2$. Growth was unaffected except at high cell densities (6–7 mg dry at/ml) when manganese limitation occurred. Sub-mitochondrial particles prepared from cells grown under iron limited conditions (0.2 to 0.5 μgatoms Fe/1, no added $MnCl_2$, 130 mM glycerol, dilution rate 0.22 hr^{-1}) did not exhibit any detectable NADH or glycerol-3-phosphate reducible EPR signals in the $g = 1.9$ region (fig. 1a). It should be noted that oxidation rates of these substrates were not affected by iron limited growth (1.2 μgatoms Fe/1, no added $MnCl_2$, 55 mM glycerol, dilution rate 0.24 hr^{-1}), both site I phosphorylation and piericidin A sensitivity were present, and the EPR spectra of the sub-mitochondrial particles were generally similar to those described by others [10, 11, 14]. For the signal obtained on adding NADH (fig. 1b), the main derivative type peak centred close to $g = 1.94$, had a

line width of about 25 gauss, and appears to be the signal attributed to the iron of NADH dehydrogenase [15]. The peak at higher field (arrowed) probably corresponds to succinate dehydrogenase or glycerol-3-phosphate dehydrogenase iron [14, 16]. With glycerol-3-phosphate in place of NADH this feature was unchanged but the main derivative peak had a rather lower amplitude. Addition of excess sodium dithionite produced an increase in the amplitude of the high field peak without apparently affecting the NADH dehydrogenase peak. Altering the growth conditions to those appropriate for the presence of site I phosphorylation and the absence of piericidin A sensitivity (0.37 μgatoms Fe/l, no added $MnCl_2$, 55 mM glycerol, dilution rate 0.24 hr^{-1}) resulted in submitochondrial particles with modified EPR spectra (fig. 1c). The rather broad unresolved derivative type peak (line width 50 gauss) was centered at about $g = 1.93$ and was obtained either with NADH or with glycerol-3-phosphate. There appeared to be little contribution from NADH dehydrogenase but a substantial one from other non-heme iron proteins. Again this peak increased in intensity after addition of sodium dithionite to the sample.

4. Discussion

Results from the experiments utilizing small changes in iron concentration in the region where iron limitation gives way to glycerol limitation clearly show that site I energy conservation can occur in the absence of sensitivity towards piericidin A, in contrast to the conclusions of Ohnishi and Schleyer [17]. The separation of these two properties by manipulation of the iron concentration suggests a dual involvement of non-heme iron in the segment of the respiratory chain between $NADH_2$ and the cytochromes. One involvement is in piericidin A sensitivity and the other in site I energy conservation.

The EPR spectroscopy demonstrates that the $g = 1.94$ signal associated with the NADH dehydrogenase system need not be present in detectable amounts for the occurence of site I energy conservation. This is in agreement with the findings of Imai et al. [18] on the effects of iron limited growth on the respiratory chain of *Micrococcus denitrificans*. However, there does appear to be a closer correlation between this signal and piericidin A sensitivity, although on the basis of experiments on batch cultures of *T. utilis*, Ohnishi et al. [14] reported little correlation between the signal, piericidin A sensitivity and chemically assayed non-heme iron. However, in their system, which used batch culture, two further variables were present. Firstly, the rate of NADH oxidation varied with iron concentration in the medium, and secondly, during batch culture, the available iron concentration varies inversely with cell growth. In addition, they report levels of non-heme iron about 10 fold higher than those reported here.

Each atom of iron is commonly associated with an atom of "labile" sulphur [15] and the effects of sulphate limited growth in abolishing site I energy conservation and piericidin A sensitivity are consistent with a role for iron-sulphur protein(s) in this region. Further support for this conclusion is provided by the complete absence of substrate reducible EPR signals in the region of $g = 1.9$ in submitochondrial particles from sulphate limited cells.

The absence of any detectable APR signals in the $g = 1.9$ region in preparations from iron or sulphate limited cells indicates that the presence of these signals is not obligatory for fully active NADH or glycerol-3-phosphate oxidase.

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