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# EPR STUDIES ON THE RESPIRATORY CHAIN OF WILD-TYPE *SACCHAROMYCES CEREVISIAE* AND MUTANTS WITH A DEFICIENCY IN SUCCINATE DEHYDROGENASE

J. DE KOK, J. L. M. MULLER and E. C. SLATER

*Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam, Plantage Muidergracht 12, Amsterdam (The Netherlands)*

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## SUMMARY

1. Three nuclear mutants of *Saccharomyces cerevisiae* deficient in succinate dehydrogenase have been isolated. Two of these mutants are allelic.

2. The amount of covalently bound flavin of submitochondrial particles of the two allelic mutants is about 14 % and that of the third mutant about 50 % of the amount in wild-type particles. The turnover number of succinate dehydrogenase of particles is decreased in all mutants. The turnover number of fumarate reductase is increased in the two allelic mutants, but decreased in the third mutant.

3. EPR spectra, measured at 82 °K, show that the amplitude of the $g = 1.93$ signal in particles of the two allelic mutants is less than 10 % of that in wild-type particles. It is concluded that iron-sulphur centres other than those of succinate dehydrogenase make only a negligible contribution to the line at $g = 1.93$ in wild-type particles.

4. EPR measurements below 20 °K show that the amplitude of the signal at $g = 2.01$ detected in oxidized particles is decreased in particles of the two allelic mutants.

5. A signal with lines at $g = 2.027$ and $g = 1.933$ is detected at low temperatures in all particle preparations, even in those from a cytoplasmic petite mutant. It is suggested that this signal is derived from a contaminant and not from the inner membrane.

## INTRODUCTION

The mitochondrial respiratory chain of *Saccharomyces cerevisiae* differs from that of mammalian mitochondria mainly in the region of the NADH dehydrogenase. The iron-sulphur centres that are associated with the NADH dehydrogenase of other organisms are absent from *S. cerevisiae* [1–3]. This yeast species thus allows EPR investigations to be made on iron-sulphur centres of other segments of the respiratory chain without interference by those belonging to NADH dehydrogenase. The respiratory chain of *S. cerevisiae* can, moreover, be modified by inducing genetic defects.

If a genetic defect concerns a paramagnetic centre, this centre could, in principle, be allocated to a particular segment of the respiratory chain by correlating abnormalities in the EPR spectrum with the functional defect. The present investigation probes the usefulness of this approach by comparing EPR spectra of wild-type submitochondrial particles with those of mutants deficient in succinate dehydrogenase.

A preliminary account of part of this work has been presented [4].

## METHODS AND MATERIALS

Strain $DW_3$ (a, leu, his, ade) was obtained from Dr D. Wilkie. Strain 17 ($\alpha$, ura, ade) and strain $DP_1$-1B ($\alpha$, his, try) were obtained from Dr H. Jakob and Dr P. P. Slonimski, respectively. Strain $F_7$ ($\alpha$/a, $\rho^-$) was a gift of Dr L. A. Grivell. Strain $DW_3$ was mutagenized with $HNO_2$ [5], strain $DP_1$-1B with ethylmethane sulphonate [6]. Mutants were selected on the criterion that they should be unable to grow on non-fermentable carbon sources but able to stain with 2,3,5-triphenyltetrazolium chloride when grown on glucose medium [7, 8]. Mutant cells fulfilling this criterion were then plated on a medium containing 0.5 g glucose, 1 g yeast extract, 2 g peptone, 2 g agar and 3 mg bromocresol purple per 100 ml, brought to pH 7.0 with 1 M NaOH. When colonies of a mutant strain produced enough acid to change the colour of the pH indicator to yellow this was taken as an indication that the mutant was deficient in a Krebs-cycle enzyme [cf. ref. 9]. Submitochondrial particles of mutant strains A6 and A24, derived from the parent $DW_3$, and strain $X_3$, derived from the parent $DP_1$-1B, showed a low ratio of succinate oxidation to NADH oxidation (cf. Results). For use in biochemical experiments diploid strains 6H and 24C were constructed, which are diploid, prototrophic and homozygous for the mutant alleles of strains A6 and A24, respectively.

Methods of genetic analysis, culture of cells and preparation of submitochondrial particles were as described in ref. 8.

EPR measurements were performed with a Varian E3 apparatus. The samples were cooled with cold helium gas to obtain temperatures below 35 °K. Temperature was measured with a calibrated carbon resistor placed immediately below the sample. Temperatures above 77 °K were obtained with a Varian nitrogen-flow system with automatic temperature control. EPR samples were prepared as follows. Additions were made to EPR tubes containing the submitochondrial particles suspended in 0.25 M mannitol, 10 mM potassium phosphate (pH 7.5) and 1 mM EDTA. After mixing at 0 °C the tube was centrifuged for 30 s to remove air bubbles, incubated at 23° C for the times indicated and then immersed in liquid nitrogen.

## RESULTS

Genetic analysis showed that the defects of the succinate dehydrogenase-deficient mutants A6, A24 and $X_3$ segregated as expected for nuclear single-gene mutations. Tests for allelism showed that mutants A6 and A24 are allelic, but that mutant $X_3$ belongs to a different complementation group. None of the mutants showed an appreciable tendency to form cytoplasmic petites. The percentage of such double mutants in cultures was always less than 2 %.

Table I shows that, under the growth conditions employed, the cytochrome

TABLE I

CYTOCHROME CONTENT OF SUBMITOCHONDRIAL PARTICLES

The cytochrome content was obtained from difference spectra (dithionite-reduced minus oxidized), using the following absorbance coefficients: cytochrome $aa_3$ (605–630 nm): 24 $mM^{-1} \cdot cm^{-1}$ [10]; cytochrome $b$ (562–575): 25.6 $mM^{-1} \cdot cm^{-1}$ [11]; cytochrome $(c_1+c)$ (553–539): 20.1 $mM^{-1} \cdot cm^{-1}$ [11].

| Strain | Content of component (nmol/mg protein) | | |
|---|---|---|---|
| | cytochrome $aa_3$ | cytochrome $b$ | cytochrome $(c_1+c)$ |
| 17 (wild type) | 0.110 | 0.48 | 0.60 |
| 6H | 0.063 | 0.47 | 0.56 |
| 24C | 0.084 | 0.42 | 0.47 |
| X3 | 0.050 | 0.35 | 0.36 |

TABLE II

RESPIRATION OF SUBMITOCHONDRIAL PARTICLES WITH SUCCINATE AND NADH

Oxygen uptake was measured polarographically at 25 °C in a medium containing 50 mM potassium phosphate (pH 7.5), 1 mM EDTA and 10 $\mu$M bovine cytochrome $c$. Substrate concentrations were: 1 mM NADH or 10 mM succinate.

| Strain | Respiratory rate (natoms O per min per mg protein) | |
|---|---|---|
| | Succinate | NADH |
| 17 (wild type) | 346 | 1630 |
| 6H | 33 | 464 |
| 24C | 0 | 1425 |
| X3 | 44 | 350 |

TABLE III

TURNOVER NUMBERS OF SUCCINATE DEHYDROGENASE AND FUMARATE REDUCTASE WITH RESPECT TO COVALENTLY BOUND FLAVIN IN SUBMITOCHONDRIAL PARTICLES

Methods of determination of covalently bound flavin and succinate dehydrogenase activity (measured at 30 °C with phenazine methosulphate as primary electron acceptor) are given in ref. 8. Fumarate reductase activity was measured at 30 °C with $FMNH_2$ as electron donor as described in ref. 12. Activities were extrapolated to infinite dye concentration.

| Strain | Covalently bound flavin (nmol/mg protein) | Turnover number ($s^{-1}$) | |
|---|---|---|---|
| | | Succinate dehydrogenase | Fumarate reductase |
| 17 (wild type) | 0.14±0.03* | 187±20* | 5.3±0.5* |
| 6H | 0.02 | 49 | 15.5 |
| 24C | 0.02 | 0 | 9.8 |
| X3 | 0.07 | 59 | 2.9 |

* Means ±S.D. of three particle preparations

content of submitochondrial particles of the mutants is slightly decreased compared with wild-type particles.

Table II compares the rates of succinate and NADH oxidation of wild-type and mutant particles. Particles of strains 6H and $X_3$ catalyse a decreased, but still considerable, succinate oxidation. Further properties of these mutants assembled in Table III indicate, however, that they are affected in succinate dehydrogenase, since intrinsic properties of this enzyme are altered. Submitochondrial particles of the mutants contain a lower amount of covalently bound FAD than wild-type particles, and the turnover number of succinate dehydrogenase with respect to covalently bound flavin is decreased in the particles of all three mutant strains. The enzyme succinate dehydrogenase also catalyses the reduction of fumarate by certain dyes of low potential, such as $FMNH_2$ [12]. The turnover numbers of fumarate reductase are increased in particles of mutant strains 6H and 24C, but decreased in those of strain $X_3$. The turnover numbers of both reactions as catalysed by the enzyme of wild-type particles are in good agreement with the values published by Singer et al. [13].

The EPR spectrum of wild-type particles without further addition (Fig. 1), measured at 10 °K, shows a prominent signal with a peak at $g = 2.020$ and trough at $g = 2.008$. This signal has also been observed in oxidized particles of *Candida utilis* [2] and higher organisms [2, 14, 15] and has been ascribed to one or more centres of high-potential iron protein [16]. The signal copurifies in part with the succinate: ubiquinone oxidoreductase (Complex II) [17]. Fig. 1 shows smaller lines with apparent $g$ values at 2.044, 2.034, 1.989 and 1.971. These lines disappear both upon oxidation with ferricyanide and upon reduction with succinate or NADH. The lines at $g = 2.034$ and $g = 1.989$, but not those at $g = 2.044$ and $g = 1.971$, have been found in particles of higher organisms, with maximal intensity at intermediate

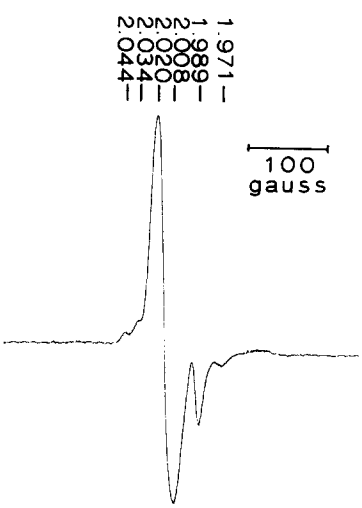


Fig. 1. EPR spectrum of wild-type (strain 17) particles without further addition. Protein concentration, 27 mg/ml. Conditions of EPR spectroscopy: temperature, 10.5 °K; microwave power, 2 mW; microwave frequency, 9.02 GHz; modulation amplitude, 12.5 G.

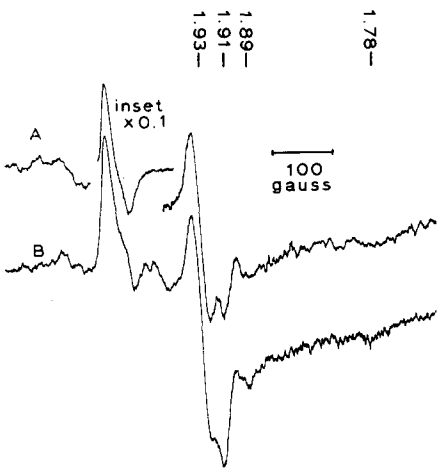


Fig. 2. EPR spectra at 13 °K of reduced wild-type (strain 17) particles. The same preparation of particles (27 mg protein per ml) was used as in Fig. 1. Particles were incubated 5 min with 20 mM succinate (Trace A) or 2 min with $Na_2S_2O_4$ (Trace B). Traces A and B were scanned at the same sensitivity of the instrument, except for the inset in trace A, which was scanned at 10 times lower sensitivity. Temperature 13 °K, other instrument settings as in Fig. 1.

stages of reduction [14]. The preparation of yeast particles used in this experiment probably contained reducing equivalents. These lines were not present in most other particle preparations.

Upon reduction of the particles with NADH or succinate (Fig. 2, trace A)

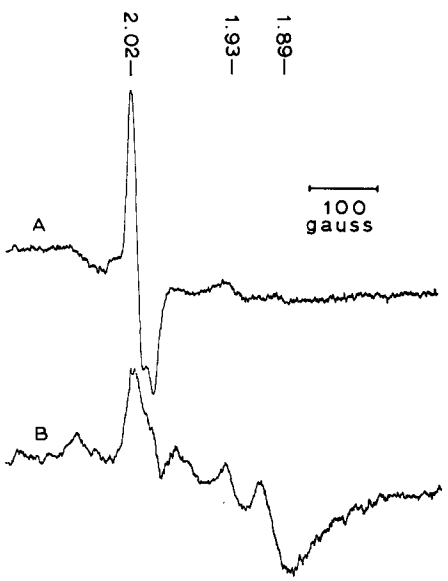


Fig. 3. EPR spectra at 13 °K of particles of mutant strain 24C. Protein concentration, 26 mg/ml. A, particles without further addition; B, particles reduced with $Na_2S_2O_4$. Trace B was scanned at 3 times higher sensitivity than trace A. Conditions of EPR spectroscopy were the same as in Fig. 2.

TABLE IV

INTENSITY OF THE SIGNAL AT $g = 2.01$ IN OXIDIZED PARTICLES AND OF THAT AT $g = 1.89$ IN REDUCED PARTICLES

Conditions of EPR spectroscopy were the same as in Fig. 2. The intensity of the signal at $g = 2.01$ was measured as the distance from top to baseline in the first-derivative spectrum. The intensity of the signal at $g = 1.89$ was measured as the distance from trough to baseline. Intensities were corrected for differences in protein concentration and tube diameter.

| Strain | Intensity (in % of wild-type signal) | |
|---|---|---|
| | $g = 2.01$ | $g = 1.89$ |
| 17 (wild type) | 100 | 100 |
| 6H | 11 | 145 |
| 24C | 24 | 136 |

only about one half of the signal with a peak at $g = 2.020$ and trough at $g = 2.008$ disappears. A similar spectrum has been published by Ohnishi et al. [2]. The signal disappears completely after reduction with dithionite (Fig. 2, trace B). The latter spectrum shows lines at $g = 2.025$, $g = 1.93$, $g = 1.91$, $g = 1.89$ and $g = 1.78$. These lines are most appropriately ascribed to the iron-sulphur centres of succinate dehydrogenase and the iron-sulphur centre present in the $bc_1$ complex described by Rieske [18]. The line at $g = 2.025$ receives contributions from the $g_z$ lines of both succinate dehydrogenase and Rieske's iron-sulphur centre. The lines at $g = 1.93$ and 1.91 correspond to $g_y$ and $g_x$ of succinate dehydrogenase, respectively. The line at $g = 1.89$ corresponds to the trough of the $g_y$ line of Rieske's iron-sulphur centre and the line at $g = 1.78$ to $g_x$ of this centre. In accordance with Ohnishi et al. [19] the signal

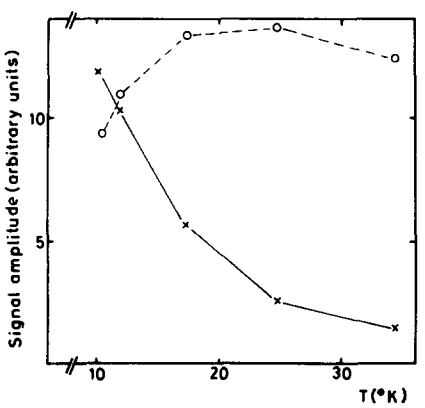


Fig. 4. Comparison of the effect of temperature on the amplitude of the signals at $g = 1.93$ in particles of wild-type strain 17 and mutant strain 6H. The amplitude of the signals was measured as the distance from top to trough in first-derivative spectra obtained at varying temperature. Instrument settings were as in Fig. 1. Particles of strain 17 (27 mg protein per ml) and strain 6H (27 mg protein per ml) were incubated with 4 mM NADH for 3 min. ○—○, particles of strain 17; ×—×, particles of strain 6H.

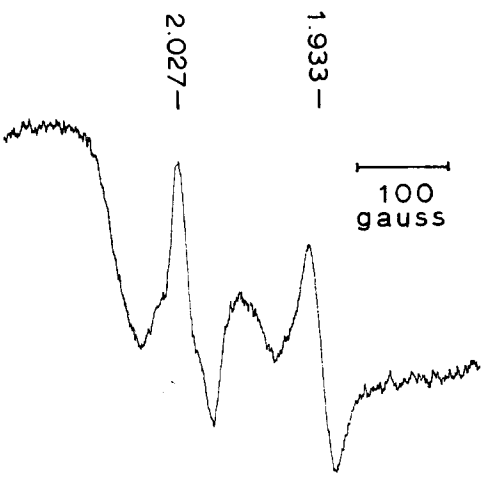


Fig. 5. EPR spectrum at 13 °K of particles of a cytoplasmic petite mutant (strain F7) without further addition. Protein concentration 27 mg/ml. Conditions of EPR spectroscopy were as in Fig. 2.

of succinate dehydrogenase is increased upon reduction with dithionite and changed in line shape, which is ascribed to the reduction of an additional iron-sulphur centre that exhibits EPR signals at the same magnetic field but with a different line shape.

EPR spectra of particles of mutant 24C (Fig. 3) show the signals of Rieske's iron-sulphur protein and of the high-potential iron protein. The shape of the latter signal is different from that in wild-type particles and it is less intense. Table IV compares the signal amplitudes of the high-potential iron protein and Rieske's iron-sulphur protein in particles of the wild-type strain and the two allelic mutants.

The spectra of Fig. 3 also show a line at $g = 1.93$. This line is presumably not derived from succinate dehydrogenase or modified succinate dehydrogenase, since: (1) the line at $g = 1.93$ is not accompanied by a line at $g = 1.91$; (2) the intensity of the line is not increased on reduction; (3) the line is much more temperature sensitive than the signal of succinate dehydrogenase under the conditions of measuring the spectra as shown in Fig. 4; and (4) the line is present in particles of both mutants and wild-type and even in particles of a petite mutant (Fig. 5). Because particles of petite mutants have only a very low succinate dehydrogenase activity and are deficient in most other constituents of the respiratory chain, it seems likely that this signal ($g_\perp = 1.933$, $g_\parallel = 2.027$) comes from a contaminant present in the inner-membrane preparation.

Fig. 6 shows the EPR spectra of NADH-reduced particles of wild-type and mutant 24C, measured at 82 °K. The amplitude of the line at $g = 1.93$ in mutant particles is less than 10 % that in wild-type particles. The same applies to particles of strain 6H (not shown). This finding suggests that at 82 °K iron-sulphur centres other than those of succinate dehydrogenase make only a negligible contribution to the line at $g = 1.93$ in wild-type particles. Particles of strain $X_3$ have a definite line at $g = 1.93$ (not shown). The amplitude of the line in strains $X_3$ and 17 correlates well with the succinate-dehydrogenase activity, in contrast to the concentration of covalently bound flavin (Table III).

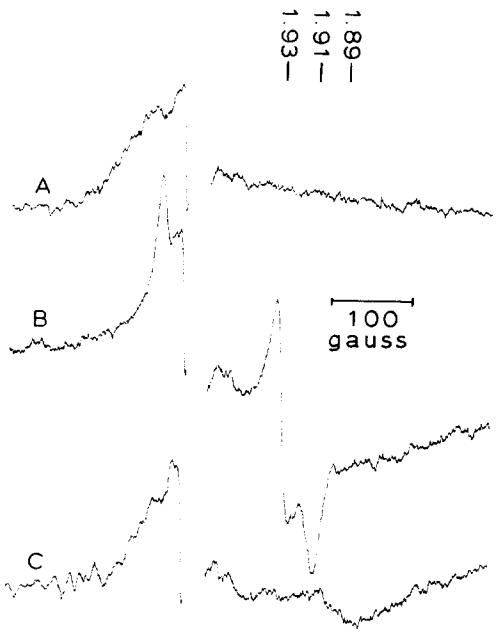


Fig. 6. EPR spectra at 82 °K of NADH-reduced particles of wild-type strain 17 and mutant strain 24C. The same preparations of particles of strain 17 (27 mg protein per ml) and strain 24C (26 mg protein per ml) were used as in Figs 1 and 3, respectively. Particles were incubated with 4 mM NADH for 3 min. Conditions of EPR spectroscopy: temperature, 82 °K; microwave power, 100 mW; modulation amplitude, 12.5 G. A, baseline recorded with water; B, particles of strain 17; C, particles of strain 24C. The traces were all recorded at the same sensitivity.

## DISCUSSION

Mutations that result in a deficiency in succinate dehydrogenase may be expected to occur in structural genes of this enzyme, in genes involved in the incorporation of the prosthetic groups and possibly also in genes involved in the regulation of the biogenesis of this enzyme and in genes of membrane proteins to which the enzyme may be bound. Such mutants could, in principle, be useful to investigate various aspects of the assembly of this enzyme. The sites of the primary defect in the mutants described in this paper are still obscure. Since, however, intrinsic properties of succinate dehydrogenase in mutants 6H and 24C are profoundly altered (Table III), it seems likely that these mutants are affected in one of the two subunits [20] of the enzyme proper. The amount of covalently bound flavin present in the particles of these mutants is very low. Although it might be thought that this flavin belongs to a different enzyme (cf. ref. 21), the fact that particles of these mutants catalyse a considerable fumarate reductase activity supports the notion that this flavin is largely a true constituent of (modified) succinate dehydrogenase.

Several possibilities may be considered as to the cause of the lowered amount of covalently bound flavin. One is that a modified enzyme is not easily incorporated into the membrane. A correlation may be drawn with the fact that subtle changes of soluble mammalian succinate dehydrogenase have a profound effect on its ability

to reconstitute with alkali-treated particles (for a recent review see ref. 22).

It is interesting that not only the amount of covalently bound flavin and the amplitude of the line at $g = 1.93$ are decreased in mutant particles but also that of the signal of high-potential iron protein. The latter is presumably not due to a general repression phenomenon, since the amplitude of the line at $g = 1.89$ is not lower in mutant particles than in wild-type particles (Table IV). Beinert et al. [17] found that the high-potential iron protein is concentrated in purified Complex II (succinate: ubiquinone oxidoreductase) of beef-heart mitochondria. It seems not unreasonable to suppose that the association of high-potential iron protein with the membrane is weakened in the mutants.

The present investigation further demonstrates the applicability of a biological approach to problems concerning the function, allocation and assembly of electron carriers of the respiratory chain.

## ADDENDUM

Since this paper was submitted for publication Ohnishi et al. [23] have reported that reconstitutively active soluble succinate dehydrogenase of beef heart contains an iron-sulphur centre similar to that of high-potential iron proteins. This finding could largely account for the fact that particles of mutants 6H and 24C show a decreased signal at $g = 2.01$ (Table IV), if these mutants contain smaller amounts of the succinate dehydrogenase subunits, as discussed above.

## ACKNOWLEDGEMENTS

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