THE DEVELOPMENT OF MITOCHONDRIAL IRON-SULFUR PROTEINS DURING RESPIRATORY ADAPTATION OF *SACCHAROMYCES CARLSBERGENSIS*

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1. Introduction

The process of respiratory adaptation, which occurs when suspensions of anaerobically-grown yeast cells are aerated [1], provides a convenient system for studies of the biosynthesis of respiratory components and their integration into functional mitochondrial electron transport chains [2–4]. The development of the respiratory chain and of other mitochondrial enzyme activities, as well as formation of highly organized mitochondria, has been studied in detail in *Saccharomyces carlsbergensis* [5,6]; we now present data on the time-course of appearance of iron-sulfur centers in this system as detected in EPR spectra.

Submitochondrial particles from aerobically-grown *S. cerevisiae* [7,8] or *S. carlsbergensis* [9] show no EPR signals associated with the NADH dehydrogenase segment of the respiratory chain, either at 77°K or at temperatures below 30°K, i.e. identical spectra were obtained when either NADH or succinate plus glycerol 1-phosphate were used as reductants [10]. The absence of the NADH dehydrogenase iron-sulfur centers simplifies the interpretation of EPR signals from other iron-sulfur centers which appear as respiratory adaptation progresses. In mammalian or avian systems, three different iron-sulfur centers [designated as Centers S-1, S-2 and S-3 (11-13)] in the succinate dehydrogenase segment and two (Rieske's iron-sulfur center and 'Center 5') in the cytochrome b-c_1 region of the respiratory chain have so far been identified [10].

2. Materials and methods

S. carlsbergensis (N.C.Y.C. 74S) was grown under strictly anaerobic conditions in a complex growth medium in the presence of 10% glucose, Tween 80 and ergosterol [14]. Respiratory adaptation was carried out in the absence of net cell growth [5,6]. Cells were harvested and sphaeroplasts prepared as previously described [15,16]. Preparation of submitochondrial particles from these, and from aerobically-grown *S. cerevisiae* cells, was by the method of Ohnishi et al. [9].

EPR spectra were obtained with a Varian E4 spectrometer at temperatures below 77°K, which were obtained by cooling samples with a stream of cold helium gas derived from boiling liquid helium. Temperature measurement was performed with a thermocouple (gold-cobalt alloy-versus-platinum) placed just below samples. All preparations were kept frozen in liquid nitrogen and thawed anaerobically before use. The half-reduction potentials of iron-sulfur centers were measured potentiometrically [17,18] using the chamber designed to permit anaerobic transfer of aliquots directly into EPR sample tubes which were then rapidly frozen in liquid isopentane at 113°K. Oxidation–reduction mediators employed were diaminodurol, phenazine methosulphate, phenazine ethosulfate, duroquinone, pyocyanine, resorufin, 2-OH-naphthoquinone, phenosafranine, benzyl viologen and methyl viologen.

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3. Results and discussion

In order to present EPR spectra of individual iron-sulfur centers detectable in *saccharomyces* mitochondrial membrane, the redox potential of an anaerobic suspension of *S. cerevisiae* submitochondrial particles was adjusted to appropriate values in the presence of redox mediators, using freshly prepared dilute solution of dithionite or 0.1 M ferricyanide. As presented in fig.1, EPR signals arising from various ferredoxin-type iron-sulfur centers such as Rieske's iron-sulfur center (g_z = 2.03, g_y = 1.90 and g_x = 1.80) (Spectrum A); Center S-1 (g_z = 2.03, g_y = 1.93, and g_x = 1.91) [11] and 'Center 5' (g = 2.08 and 1.89) [10] (Spectrum B); and Center S-2 (g_z = 2.03, g_y = 1.93 and g_x = 1.91) [11]; are clearly distinguished. The two distinct ferredoxin type iron-sulfur centers of succinate dehydrogenase, Centers S-1 and S-2, have previously been characterized in particulate succinate-cytochrome *c* reductase or succinate-UQ reductase and in purified soluble succinate dehydrogenase preparations from beef-heart [11–13]. Thus the classical 'g = 1.94 signal' arising from Center S-1 with a midpoint potential of 0 ± 15 mV is seen in the preparation poised at E_h of −197 mV, whereas a low potential iron-sulfur center, Center S-2, with a midpoint potential of −245 ± 15 mV gives additional EPR signals at the same field position, but with different line-shape at −455 mV. As shown in fig.2, NADH can reduce Center S-1, whereas Center S-2 can be reduced only by dithionite addition. At extremely low temperatures, Center S-1 signals are highly saturated (fig.2, spectrum B), while the Center S-2 signals are detectable only at temperatures below 20°K [11]. Thus, EPR spectra of Centers S-1 and S-2 in *saccharomyces* submitochondrial particles reduced with dithionite can be resolved by simply adjusting the temperature of EPR measurement (Spectra C and D, respectively). In submitochondrial particles of *S. carlsbergensis*, EPR signals arising from

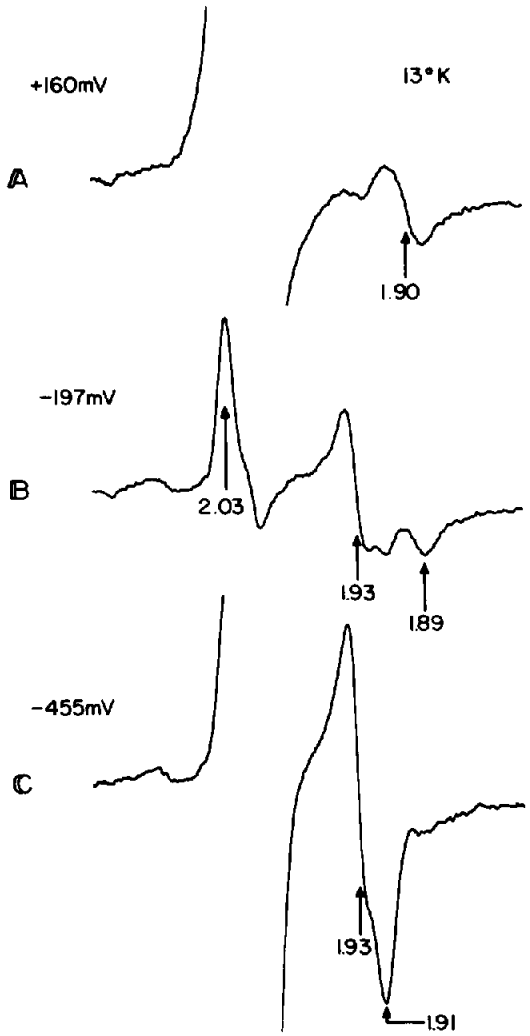

Fig.1. EPR spectra of iron-sulfur centers in *S. cerevisiae* submitochondrial particles poised at three different redox potentials, measured at 13°K. Submitochondrial particles were suspended at approx. 31 mg of protein per ml in a medium containing 0.3 M mannitol, 5 mM K-phosphate buffer, pH 7.2, 1 mM $MgCl_2$ and 50 mM morpholinopropane sulphuric acid buffer, pH 7.2. Oxidation–reduction potentials were measured potentiometrically as described in the text. Oxidation–reduction mediators added were 38 μM diaminodurol, 63 μM phenazine methosulfate, 63 μM phenazine ethosulfate, 25 μM duroquinone, 9 μM pyrocyanine, 8 μM resorufin, 78 μM phenosafranine, 92 μM benzylviologen, 167 μM methylviologen and 13 μM 2-OH-naphthoquinone. The oxidation-reduction potential of the system was lowered by stepwise additions of small aliquots of freshly prepared dilute solution of dithionite. Oxidation–reduction potentials shown in the figure are relative to the standard hydrogen electrode. The EPR operating conditions were: modulation frequency, 100 kHz; modulation amplitude, 125 gauss; microwave power, 20 mW; microwave frequency, 9.1 GHz; time constant, 0.3 sec; scanning rate, 500 gauss per min; temperature, 13°K. The ordinate is the first derivative of the microwave absorption in an arbitrary unit.

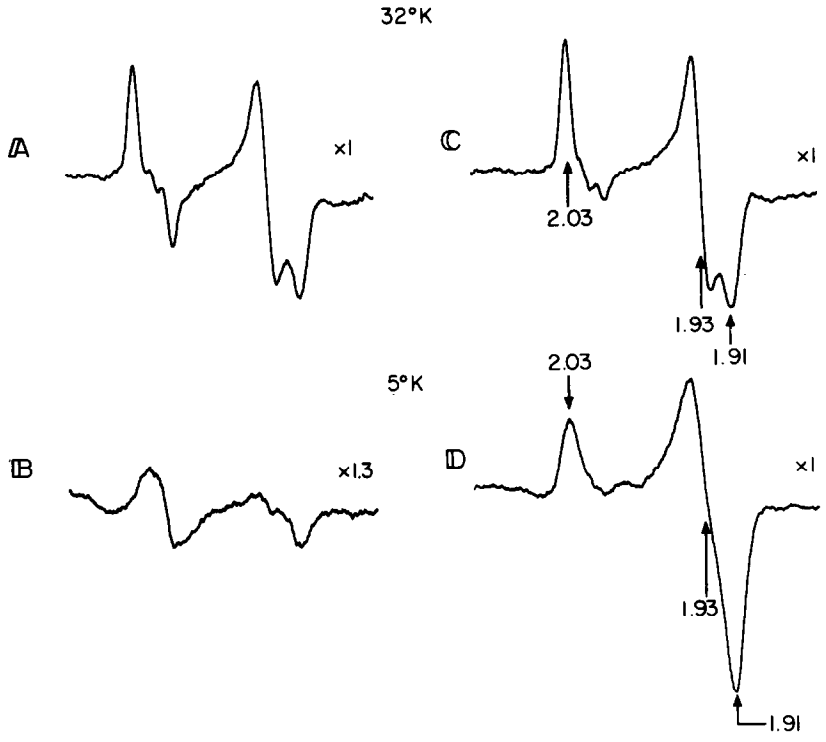

Fig.2. EPR spectra of *S. cerevisiae* submitochondrial particles reduced with NADH or dithionite, measured at two different temperatures. Protein concentration of submitochondrial particles was 62 mg prot/ml. Spectra A and B were obtained with particles reduced with 2 mM NADH, while spectra C and D with particles reduced with an excess of dithionite power. EPR conditions are same as those in fig.1, except temperature settings as illustrated in the figure.

various iron-sulfur centers can be similarly resolved. Fig.3 shows the progress of development of iron-sulfur centers during respiratory adaptation of *S. carlsbergensis*. Particles from the anaerobically grown yeast show no EPR spectra from iron-sulfur centers. The development of detectable iron-sulfur center S-1 (spectrum C) and S-2 (spectrum C′) occurs after the first 30 min of adaptation and both species give maximum signals only after 2 hr (fig.3). Although spectra are not presented, development of Rieske's iron-sulfur center and 'Center 5' show similar time courses as Centers S-1 and S-2.

In addition to these ferredoxin-type iron-sulfur centers (paramagnetic in the reduced state), a Hipip-type iron-sulfur center (paramagnetic in the oxidized form) with a resonance absorbance centered around g = 2.01 can also be seen (fig.1A). This Hipip-type iron-sulfur center is similar to Center S-3 which has been recently identified in succinate-UQ reductase [12] or in purified soluble succinate dehydrogenase [13] isolated from beef heart mitochondria. Detailed studies on the development of Hipip-type iron-sulfur center(s) during respiratory adaptation are currently in progress.

Other characteristics of the respiratory system observed after 2 hr of respiratory adaptation include the development of maximum antimycin A- and cyanide-sensitivity of oxygen uptake [6] and the presence of rapidly oxidizable cytochromes $a + a_3$ and c ($t_{1/2}$ at 24°C = 20 and 30 ms respectively) [5]. Only after 3 hr of respiratory adaptation was cytochrome c_1 detectable. We therefore conclude that the development of the iron-sulfur proteins, a characteristic of the fully functional respiratory chain, proceeds alongside that of the cytochromes during the early phases of mitochondrial biogenesis induced

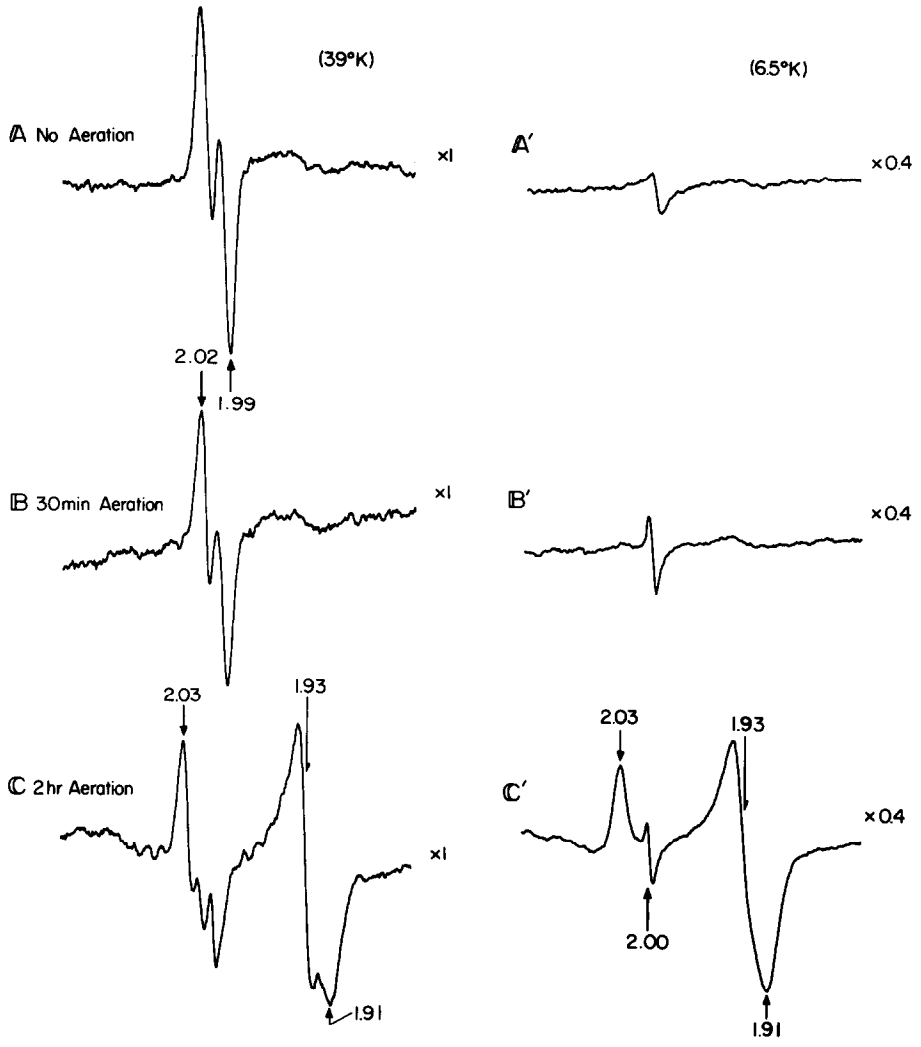

Fig.3. EPR spectra of dithionite-reduced submitochondrial particles prepared from anaerobically-grown *S. carlsbergensis* and after different periods of respiratory adaptation measured at 39°K and 6.5°K. EPR operating conditions were as in fig.1. Protein concentrations were 91, 62, and 100 mg/ml respectively in A, B and C.

by aeration of anaerobically-grown yeasts.

It is interesting to point out that unidentified signals (fig.3, spectrum A) with peaks at g = 2.02 and 1.99 can be detected at relatively higher temperatures in the anaerobically grown yeast system. The disappearance of the component(s) responsible for these signals occurs concurrently with the appearance of iron-sulfur centers and cytochromes in the process of the respiratory adaptation (Spectra B and C).

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