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Three-iron-sulfur centers of pea mitochondria

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EPR signals of three distinct types of three-iron-sulfur center were observed in pea mitochondria: the signal of Center S-3 (low-field peak at $g = 2.016$), the signal of Center ISP-1 (low-field peak at $g = 2.024$) and the signal of the axial Center ISP-2 with two maxima, at $g = 2.027$ and 2.016. Succinate increases the signal amplitude of Center ISP-1 and diminishes that of Center ISP-2; malate has an opposite effect. Membrane damage enhances the effect of malate and decreases that of succinate.

Introduction

Higher plant mitochondria show a number of EPR signals at cryogenic temperatures due to iron-sulfur centers. The complement of iron-sulfur centers, paramagnetic in their reduced forms (N-1a, N-1b, N-2, N-3, N-4, S-1) is generally analogous to that of animal systems. [1–4]. However, except for Center S-3 of succinate dehydrogenase [5], few data are presently available concerning centers, paramagnetic in oxidized form. Rich and Bonner [5] have found that as the temperature of an aerobic sample of *Sauromatum guttatum* spadix mitochondria was raised, a peak at a g value of approx. 2.03 appeared alongside the Center S-3 peak. The $g \approx 2.03$ feature was absent from submitochondrial particles. The concentration of the center in *S. guttatum* mitochondria was no more than 25% that of Center S-3. In mitochondria of other plant species the center was present, even in lower concentrations, or was absent.

Cammack and Palmer observed a signal with a peak at g 2.02 in *Arum maculatum* spadix submitochondrial particles. This signal, in contrast to the signal of Center S-3, was detectable at 30 K [3].

In 1982 [6], we reported the results which provided evidence for the existence of at least three different types of iron-sulfur center paramagnetic in the oxidized state in pea mitochondria. A multiplicity of centers had been suggested from temperature dependence of the signals. The centers were found at comparable concentrations [6]. Subsequently we resolved mitochondrial signals at $g \approx$ 2.00–2.03 into three separate components: the signals of Center S-3 and the Centers designated as ISP-1 and ISP-2 [7]. ISP-1 was found to be membrane-bound and ISP-2 was found to be located in matrix [8]. We have shown that the signals arise from the oxidized forms of ISP-1 and ISP-2 (dithionite removed the signals). On the basis of their EPR characteristics we concluded that these centers are three-iron clusters [9].

Recently, Brouquisse et al. [10] have observed a signal similar to ISP-2 in potato mitochondria.

In the present study, the properties of ISP-1 and ISP-2 were investigated in more detail; the effects of respiratory substrates on the EPR signals of these centers were demonstrated. A preliminary report of part of this work has already been published [9].

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Experimental procedure

Pea seedlings were grown in the dark for 10–15 days. Mitochondria were isolated as described by Kalachjova et al. [11] (see also Ref. 4). Oxygen uptake was measured polarographically at 25 °C in a Clark-type electrode. The respiratory control ratio was approx. 2.0–2.5. Submitochondrial particles were prepared as described by Grubmeyer et al. [12]. EPR measurements were carried out with an EPR-2V spectrometer (Institute of Chemical Physics) equipped with a helium flow transfer line.

Results

The EPR spectrum of pea mitochondria as isolated (Figs. 1a and 3a) in the $g \approx 2.01$ region is presumably composed of several signals. This spectrum has a low-field maximum at $g \approx 2.03$ and a shoulder in the center of the resonance absorbance. The sharp signal observed in the $g = 2.00$ region arises from free radicals. We shall not discuss it further.

Use of certain treatments of mitochondria and submitochondrial particles made it possible to obtain representative spectra and temperature dependences of three distinct centers. For example, in oxygenated submitochondrial particles in the $g \approx 2.01$ region at 15 K (Fig. 1b) the only detectable signal of an iron-sulphur center is that of Center S-3. An overlapping split signal, which has been attributed to an interacting pair of ubisemiquinone radicals [5], is also seen in Fig. 1b. The Center S-3 signal is centered at $g \approx 2.01$, has a low-field peak at $g = 2.016$ and has a peak-to-peak width $\Delta H = 27$ G. The temperature dependence of the signal amplitude is shown in Fig. 2a. These properties are rather similar to the analogous plant and animal components [5].

Rich and Bonner noted that a large proportion of Center S-3 was reduced only sluggishly or not at all under steady-state conditions, and even upon anaerobiosis only about 80% of the center was succinate- and NADH-reducible [5]. In order to activate succinate dehydrogenase and to achieve full reduction of the Center S-3, we used long-term (5–7 min) incubation of mitochondria with succinate at high concentration (0.08 M). One could expect that in such samples only the signals of the

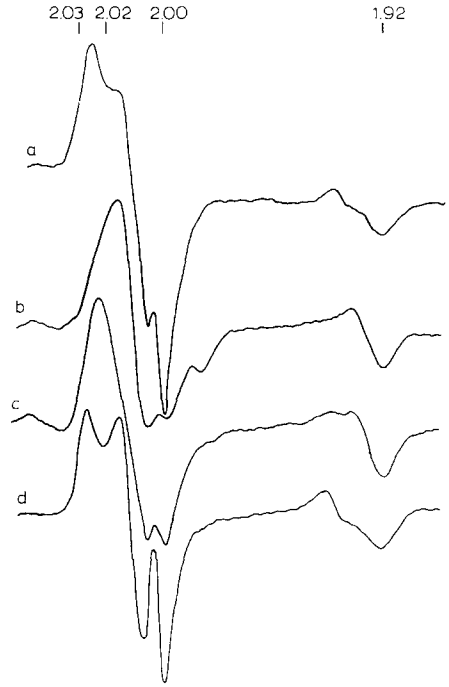

Fig. 1. (a) EPR spectrum of pea mitochondria as isolated, temperature 25 K; traces b–d in the $g \approx 2.02$–2.03 region show the representative spectra of three-iron-sulfur centers: (b) Center S-3 (submitochondrial particles, temperature 15 K; (c) Center ISP-1 (mitochondria in the presence of 0.08 M succinate, temperature 22 K); (d) Center ISP-2 (frozen-thawed mitochondria in the presence of 0.02 M malate, temperature 27 K). The temperatures were chosen such that the optimum conditions were obtained recording representative spectra of 3Fe centers. The samples except (b) were incubated anaerobically. The concentrations of protein in the samples are not comparable. EPR conditions: microwave frequency, 9440 MHz; modulation frequency, 100 kHz; microwave power, 50 mW; modulation amplitude, 3 G; time constant, 1 s.

centers, that are paramagnetic in the reduced state, and the spectrum shown in Fig. 3a would be observed. However, a relatively symmetric signal centered at $g \approx 2.01$, with a low-field peak at $g = 2.024$ was present (Fig. 1c, 3b). The signal had a peak-to-peak width of 38 G. The temperature dependence of the signal is shown in Fig. 2b. We designated the Center exhibiting this signal as ISP-1.

At 25 K the signal of Center S-3 is practically undetectable due to the relaxation broadening. The spectrum of mitochondria, as isolated at this temperature, is shown in Fig. 3a. On addition of malate, or glutamate plus malate, the maximum at $g \approx 2.03$ and the shoulder in the center of the

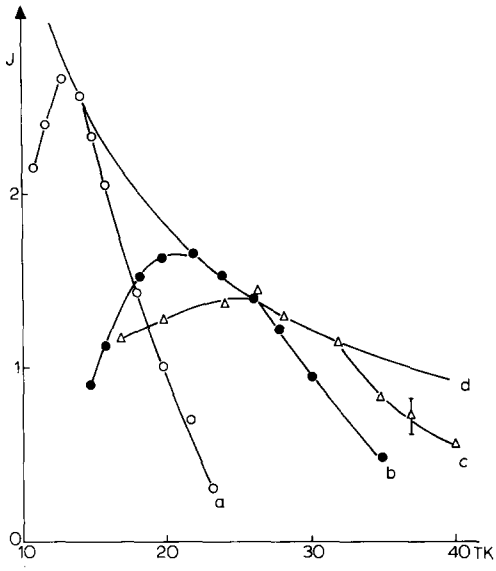

Fig. 2. Temperature-dependence curves of three-iron-sulfur centers of pea mitochondria. (a) Center S-3 (submitochondrial particles); (b) Center ISP-1 (mitochondria in the presence of 0.08 M succinate); (c) Center ISP-2 (maximum at $g = 2.027$, frozen-thawed mitochondria in the presence of 0.08 M succinate, 0.02 M malate, 0.01 M glutamate); (d) curve corresponding to Curie's law. The ordinate is relative signal intensity (arbitrary units). EPR operating conditions were the same as in Fig. 1.

resonance absorbance became a little more prominent. Freezing-thawing without substrates also caused only slight changes (data not shown). However, freezing-thawing in a medium containing malate induced drastic changes of spectra (Fig. 1d). A spectrum with two maxima, at $g = 2.027$ and $g = 2.016$, was recorded at temperatures above 25 K (Fig. 1d). The addition of succinate to this system resulted in full reduction of Center S-3. Use was made of this to obtain the spectrum with two maxima (Fig. 3e) without overlap of the other signals over a wide range of temperature (15–40 K). The temperature dependence of the maximum at $g = 2.027$ is shown in Fig. 2c. The temperature dependence of the second maximum is very similar (not shown). This fact led to conclusion that both maxima can be attributed to one center with axial symmetry. This center has been called Center ISP-2.

In this experiment, freezing-thawing in the presence of malate and succinate was used as a means of obtaining a representative spectrum and a temperature profile of Center ISP-2. At the same time, the spectra obtained in native mitochondria incubated with malate plus succinate seem to be of particular interest. In mitochondria which were thus incubated, the ISP-1 signal emerged but, in contrast to mitochondria incubated with succinate only, the ISP-2 signal was also present. Use of different substrate concentrations makes it possible to obtain different ISP-1 : ISP-2 signal-intensity ratios (Fig. 3c, d). The increase of malate concentration in the samples of native mitochondria containing succinate increases the intensity of ISP-2 signal and diminishes that of ISP-1. Comparison of the spectra presented in Fig. 3d and e shows that freezing-thawing of the sample containing malate and succinate resulted in the disappearance of ISP-1 signal.

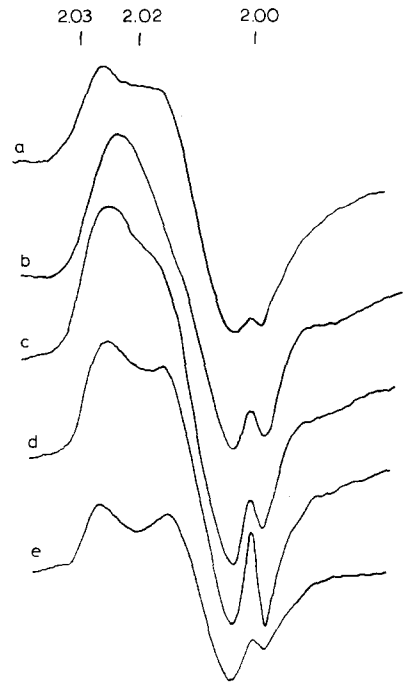

Fig. 3. The effects of respiratory substrates on EPR spectra of three-iron-sulfur centers of pea mitochondria. The protein concentration is the same in all samples. Registration temperature 25 K. (a) Mitochondria as isolated; (b) mitochondria in the presence of 0.08 M succinate; (c) mitochondria in the presence of 0.08 M succinate, 0.002 M malate, 0.001 M glutamate; (d) mitochondria in the presence of 0.08 M succinate, 0.02 M malate, 0.01 M glutamate; (e) the same sample as (d) after freezing-thawing. EPR conditions were the same as in Fig. 1. The receiver gain was the same for all samples.

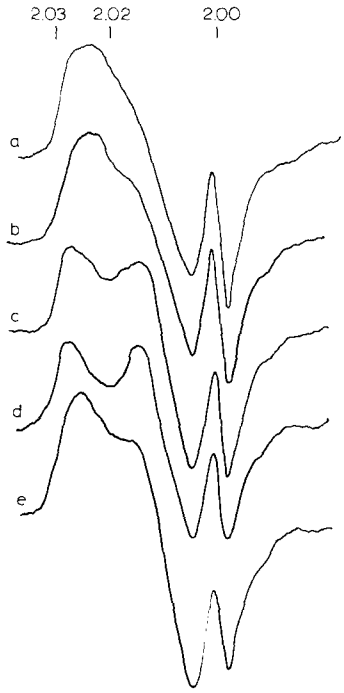

Fig. 4. The effect of membrane damage on EPR spectra of pea mitochondria in the presence of 0.08 M succinate at 25 K. Several cycles of freezing-thawing were carried out: (a) one; (b) two; (c) four; (d) five; (e) dimethylsulphoxide (2% v/v) was added (without freezing-thawing). EPR conditions were the same as in Fig. 3.

Contrary to this finding, the spectrum of succinate-treated mitochondria in the absence of malate is modified only slightly upon freezing-thawing. Several cycles of freezing-thawing were required to achieve the disappearance of ISP-1 and the development of ISP-2 (Fig. 4a–d). The effect of dimethylsulphoxide on succinate-treated mitochondria was analogous to that of freezing-thawing (cf. Fig. 4b and e).

The results above show that succinate tends to increase the signal of ISP-1 and to diminish that of ISP-2; malate has an opposite effect, membrane damage enhances the effect of malate and decreases that of succinate.

The results of double integration on corresponding signals show that the 3Fe centers are present in pea mitochondria at comparable concentrations. The ratio of S-3, ISP-1 and ISP-2 was found to be approx. 1 : 1.4 : 1.3.

In contrast to the behavior of Center S-3 and the centers paramagnetic in the reduced state, the ISP-1 and ISP-2 signal amplitudes did not depend on aerobic or anaerobic states.

In Fig. 1, the peaks at $g < 2$ arise from the reduced iron-sulfur centers of Complexes I and II. These centers in mitochondria, as isolated, are not fully reduced due to substrate depletion, which in some cases also causes the appearance of a cytochrome oxidase Cu^{2+} signal in the region $g \approx 2.00$. It is apparent from Fig. 1 that, even in the presence of the substrates, the signals of the reduced centers are considerably weaker than the signals of ISP-1 and ISP-2. Obviously, the components of the signals of the reduced centers at $g \approx 2.02$–2.03 cannot be responsible for the spectral changes in this region upon substrate addition.

Discussion

The peaks in the $g \approx 2.02$–2.03 region disappear upon reduction of mitochondria by dithionite with methylviologen as mediator [9]. This fact indicates that the signals of ISP-1 and ISP-2 arise from the oxidized forms of the centers. The g values ($g_{av} \approx 2.01$), lineshapes and temperature dependences of ISP-1 and ISP-2 signals are very similar to those arising from 3Fe clusters which have been obtained from other biological systems [13]. Such properties are known to be a strong indication of this type of center [13]. On this basis we suggested that ISP-1 and ISP-2 are of the 3Fe-type of center [9]. It should be noted that the same assignment was made in Ref. 10 for the axial signal in potato mitochondria.

In our recent study [8], the location of the iron-sulfur centers was investigated. In agreement with earlier reports [3,5], the signal of Center S-3 was observed in submitochondrial particles. The peak at $g = 2.027$ detected in mitochondria was absent from submitochondrial particles. This points to the ISP-2 matrix location. To determine the ISP-1 location the spectra of succinate-treated mitochondria and submitochondrial particles were compared. The signal of ISP-1 was observed in the latter. The amount of Center ISP-1 in the submitochondrial particles was about 50% of that obtained in mitochondria [8]. These results provided evidence for ISP-1 location in the inner mitochondrial membrane. The removal of part of

the species from the mitochondrial membrane during the disruption of the mitochondria points to the fact that ISP-1 is not particularly stable.

Bearing in mind these data, we can now compare ISP-1 and ISP-2 with the centers observed by other authors in plant mitochondria. The properties of ISP-1, namely its location in the inner mitochondrial membrane, signal-shape and the fact that the signal is detectable at 25–30 K, are similar to the properties of the center present in *Arum maculatum* spadix submitochondrial particles [3]. Cammack and Palmer assumed that the signal arises from an artificial 'superoxidized' form of the iron-sulfur center, though they left open the possibility of its being assigned to a biologically significant center.

The properties of ISP-2 are analogous to those of the center observed by Rich and Bonner in *S. guttatum* spadix mitochondria [5]. The center is detectable at 30 K, exhibits a peak at $g \approx 2.03$ and is located in matrix. However, the center was found at low concentrations, whereas we obtained the signal intensity comparable to that of Center S-3 and without overlap of other centers [7]. The features characterizing the ISP-2 signal are similar to those of a signal observed recently in the matrix of potato mitochondria [10]. The ISP-2 signal also resembles the signal of beef liver cytoplasmic aconitase [13]. In Refs. 5 and 10 the signals with a peak at $g \approx 2.03$ were assigned to aconitase. In our early work [7] we made the same suggestion. However, in view of our present knowledge, this assignment is difficult to reconcile with certain results. For example, from this standpoint it is difficult to explain why succinate should remove the signal. That is why we are now inclined to assume that ISP-2 is not aconitase and we suggest that the signal arises from some iron-sulfur protein having a regulatory role (see below), although, at this time, the question cannot be definitely answered and one must bear in mind the alternative interpretation.

The observation of ISP-1 and ISP-2 signals in intact mitochondria and the fact that these signals respond to substrate addition rule out the possibility that they are artificial 'superoxidized' forms. At present, the problem of biological significance of 3Fe clusters remains unresolved [13]. There are certain observations which are in favor of the possible meaning of $3Fe \rightleftarrows 4Fe$ conversions in physiological control mechanisms of aconitase and *Desulfovibrio gigas* ferredoxin [13–15]. The present investigation provides evidence for the biological significance of 3Fe centers in plant mitochondria. From this standpoint, assuming that 4Fe forms are EPR-silent, $3Fe \rightleftarrows 4Fe$ conversions can be considered as the regulation mechanism of the electron flux from different substrates. We think that this hypothesis offers a reasonable picture of events, although of course its validity remains a matter of further exploration.

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