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Electron spin resonance of cytochrome b_2 and of cytochrome b_2 core

The ESR spectra of several haem proteins have been measured and information concerning their structures has been obtained[1–3]. Very recently, EHRENBERG AND BOIS POLTORATSKY[4,5] have made a detailed study, by this method, of cytochrome b_5 of liver microsomes at various pH. They have found, at neutrality, a main low-spin component with g values of 3.03, 2.23 and 1.43 and a small portion of a high-spin signal at $g = 6.2$. In alkaline medium (pH 12) they observed a narrowing of the low-spin absorption with limiting g values of 2.82, 2.28, 1.68 simultaneously with a considerable increase of the high-spin absorption.

It is known that cytochrome b_2 (L-lactate cytochrome c oxidoreductase, EC 1.1.2.3), first crystallized from yeast by APPLEBY AND MORTON[6], has a molecular weight of 183000 according to the method of ultracentrifugation diffusion[7] and contains two moles of haem and two moles of flavin mononucleotide. A low molecular weight derivative, the cytochrome b_2 core (noyau cytochromique b_2, *cf.* ref. 8) has been obtained either following trypsic hydrolysis ("t" type) or in the supernatant upon recrystallisation of cytochrome b_2 in the oxidised form ("s" type)[9]. These types, which appear to be very similar, were purified and it was confirmed that they have a molecular weight of about 11000 and carry one haem group[8,9] but no flavin. In many respects, this cytochrome b_2 core very closely resembles cytochrome b_5[10,5]: it has the same molecular weight, the same positions for the haem peaks in the absorption spectrum, the same absorbance coefficients and about the same redox potential.

The ESR study of cytochrome b_2 and of the cytochrome b_2 core was therefore performed to facilitate a comparison with cytochrome b_5 and also to elucidate whether the presence of flavin modifies the spectra of the former; by changing the pH, the dissociation of protons which control the state of the haem can be studied.

The cytochrome b_2 used in this experiment was crystallised by the method of APPLEBY AND MORTON[6]. Cytochrome b_2 core ("s" type) was prepared by the method of IWATSUBO *et al.*[9,11] and lyophilised. For the ESR measurements, crystalline cytochrome b_2 was packed in an ESR quartz tube (diameter, 3 mm) by centrifugation, and lyophilised cytochrome b_2 core was dissolved in 0.1 ml of 0.1 M phosphate buffer (pH 7.0). The pH was measured by a Beckman one-drop glass electrode after addition of acid or alkali. A Varian V-4502 EPR spectrometer was used at liquid-nitrogen temperature for the ESR measurements.

As shown in Figs. 1 and 2a, the ESR spectra of cytochrome b_2 and of cytochrome b_2 core at neutral pH are virtually identical and the g values of these two

species are estimated to be 2.99, 2.28 and 1.49. These signals are due to a low-spin state of the ferric ion. Thus, haem iron in both these cytochromes has a strong ligand field, which means that the energy separation between the $d\varepsilon$ and $d\gamma$ orbitals is quite large and that these ligand fields are due to fifth and/or sixth coordination. From the above results, one can conclude that the flavin moiety does not affect the haem molecule, as has been suggested previously by spectral and oxidation–reduction observations[12].

When the pH was increased, two different kinds of low-spin signals appeared successively in alkaline medium for the cytochrome b_2 core, as shown in Figs. 2b and 2c: one at pH 11.5 whose g values are 2.44, 2.16 and 1.91 and the other at pH 12.1 with the g values 2.70, 2.24 and 1.75. Comparison of the latter values with those for the alkaline kind of cytochrome b_5 (low-spin state), seems to reveal a great similarity ($g = 2.82$, 2.28 and 1.68 according to EHRENBERG AND BOIS POLTORATSKY).

Furthermore, the spectrum at pH 11.5 showed the high-spin signal in the $g = 6$ region analogous to that observed by the same authors for cytochrome b_5; this signal increased at pH 12.5. As the ESR of free haematin in alkaline medium represents a high-spin state, the high-spin signal observed at high pH could be due to free haem.

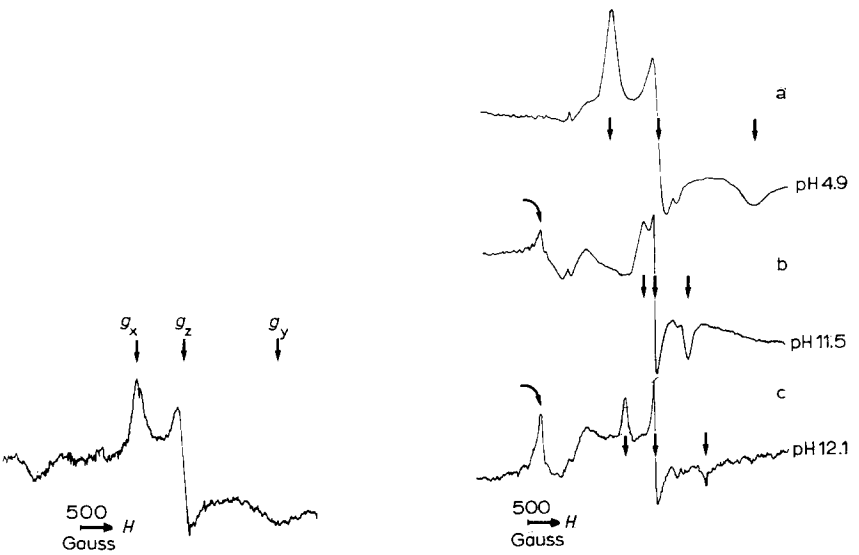

Fig. 1. ESR spectrum of cytochrome b_2. Crystalline cytochrome b_2 was packed into a quartz tube at a concentration of about 30 mg/ml. ESR measurement was performed at liquid-nitrogen temperature and at 1 mW power. The calibration of the magnetic field was measured with diphenyldipicrylhydrazyl. Microwave frequency = $9.2 \cdot 10^9$ cycles/sec.

Fig. 2. ESR spectrum of cytochrome b_2 core at various pH values. The concentration of the cytochrome b_2 core was about 100 mg/ml. The experimental conditions are the same as those of Fig. 1. The curved arrow indicates the high-spin signal $g = 6$; the straight arrows indicate the low-spin signal.

In view of the similarity between the b_5 and b_2 core cytochromes it would be worthwile to examine whether the differences observed in the number of low-spin states (two for b_5, three for b_2) as a function of pH can be confirmed.

A possible explanation for the two successive low-spin states with increasing pH could be the successive breakage of the two iron–protein bonds associated with

ionization of the ligands; the pK values could be estimated as 10.7 and 12.1, respectively. However, the latter is so high that it is likely to represent an indirect effect correlated with protein denaturation.

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Oxygen evolution by isolated chloroplasts with carbon dioxide as the hydrogen acceptor. A requirement for orthophosphate or pyrophosphate

Chloroplasts isolated in sugar media and incubated under aerobic conditions in the presence of bicarbonate will evolve oxygen in the light at rates comparable to those obtained in the presence of artificial hydrogen acceptors[1]. Simultaneous measurements of oxygen and carbon dioxide gave parallel progress curves, each with an initial lag, and values in agreement with the fixation of one molecule of carbon dioxide for each molecule of oxygen produced. In the absence of added bicarbonate, oxygen evolution started but fell off and ceased as the endogenous carbon dioxide became exhausted. At this point evolution could be restarted by the addition of bicarbonate[1]. Fig. 1 shows a comparable requirement for P_i by chloroplasts prepared and incubated in phosphate-free media. Again, oxygen evolution started after an initial lag but then fell off, presumably as the endogenous phosphate was utilised. When net evolution