AN EPR STUDY OF CYTOCHROMES FROM SPINACH CHLOROPLASTS

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Received 25 October 1979

1. Introduction

Optical spectroscopy has been the most important technique for the study of cytochromes in photosynthetic systems (reviewed [1,2]). However, interpretation of spectral changes is made difficult by the great extent of overlap of absorbance bands. Thus, in chloroplasts, 3–4 different cytochromes are present and these have absorption maxima within 10 nm of each other with spectral widths of the same magnitude. Low-temperature absorbance spectra yield higher resolution but give more problems with quantitation. Electron paramagnetic resonance (EPR) spectroscopy has been applied to the study of cytochromes in bacterial systems [3,4] and clearly offers well resolved spectra with the possibility of direct concentration determinations. However, chloroplast cytochromes have not yet been studied by EPR with the exception of one brief report [5], probably because the cytochrome EPR signals are extremely weak, at least 10 times weaker than the signals from other paramagnetic centers such as iron–sulfur centers.

The EPR characteristics of cytochromes from spinach chloroplasts are reported here. Cytochromes *f* and b_{559} show well resolved peaks with a variation in the latter depending on sample state and method of oxidation. No signal from cytochrome b_6 has been detected.

2. Materials and methods

Broken spinach chloroplasts and photosystem II-enriched subchloroplast fragments were prepared as in [6,7]. A cytochrome complex containing cytochromes b_6 and *f* was prepared by a procedure modified from that in [8]; the properties of this preparation will be described in a subsequent publication. A purified preparation of chloroplast cytochrome *f* was obtained by the procedure in [9].

Since most of the chloroplast samples were in the form of pastes, the concentrations given are only approximate. Oxidation of chloroplast samples was performed by suspension of the sample (20 mg chl) in 40 ml 2 mM potassium ferricyanide, followed by centrifugation and resuspension in buffer (50 mM Tricine (pH 8.0) plus 10 mM NaCl).

Disturbing broad EPR signals, presumably due to oxygen, were observed in many chloroplast samples and, therefore, the samples were made semi-anaerobic by the addition of 20 mM glucose to the last suspension medium, followed by the addition of glucose oxidase (0.2 mg/ml) and catalase (0.03 mg/ml) to the sample in the EPR tubes. Unless stated otherwise, the chloroplast samples were kept in the dark for at least 10 min before the spectrum was recorded.

EPR spectra were recorded at 18 K on a Bruker Model ER 200 TT X-band spectrometer equipped with a Nicolet model 535 signal averager and a tape recorder or on a modified JEOL X-band spectrometer. Some spectra were corrected for a sloping baseline. At the microwave power used, there was no saturation of the cytochrome EPR signals. Quantitation was made as in [10,11], using cytochrome *c* as a standard.

3. Results

Many chloroplast samples showed peaks in the *g* 6 region that conceivably could arise from heme compounds containing high-spin Fe (III). However, the concentration of such paramagnetic centers was

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estimated to be <20% of that of the reaction center (P700) and in this paper only low-spin Fe(III) signals are considered.

As isolated, the cytochrome b_6–f complex had no significant low-spin signal (fig.1A). The slope below 0.19 T is due to non-heme iron. After oxidation with ferricyanide, a signal typical of low-spin Fe(III) appeared with a g_z-value of 3.5 (fig.1B). The same peak was observed in the spectrum of an oxidized sample of a partially purified cytochrome f preparation with an intensity roughly corresponding to that determined optically. Photosystem II subchloroplast fragments gave an entirely different signal (fig.1C) with a g_z-value of 2.9. The corresponding g_y-value was 2.26, but the g_x-peak could not be detected. The signal disappeared on reduction; on irradiation at low temperature, a new signal irreversibly appeared (fig.1D) that was not present in the sample before reduction.

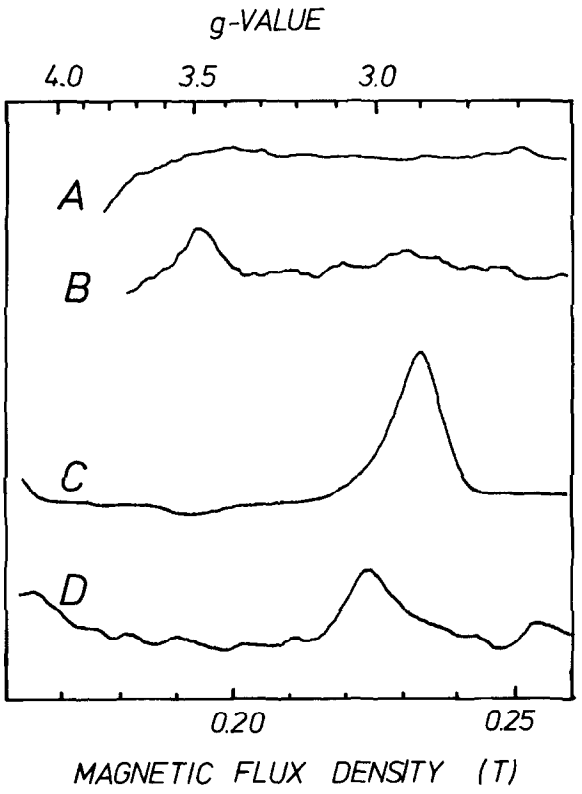

Fig.1. EPR spectra of a chloroplast cytochrome complex and photosystem II fragments recorded in the g_z region of low-spin Fe(III) heme. (A) Cytochrome b_6–f complex containing 10 μM cytochrome b_6. (B) The same sample as in (A) but after the addition of 0.5 mM $K_3Fe(CN)_6$. The spectrum was recorded at the same gain as in (A). Spectrum A and the spectrum of a sample with a large excess of $K_3Fe(CN)_6$, scaled to minimize the ferricyanide signal, have been subtracted. (C) Photosystem II subchloroplast fragments with a cytochrome b_{559} at ~30 μM. (D) Photosystem II subchloroplast fragments (cytochrome b_{559} at 10 μM) reduced with solid ascorbate and irradiated for 10 min at 77 K with white light from a 200 W tungsten lamp. The spectrum of the sample before irradiation has been subtracted. EPR conditions: microwave frequency, 9490 MHz; microwave power, 6 mW; field modulation, 3.2 mT; sweep rate, 0.1 T/min; and sample temperature, 18 K. The spectra are the average of 8 scans in A and B and of 2 scans in C and D.

Ferricyanide-washed broken chloroplasts were used to obtain samples in which the cytochromes were oxidized in the dark (see section 2). The main features observed were peaks at g 3.5 and g 3.0 with a shoulder at g 2.9 (fig.2A). Ferricyanide-washing did

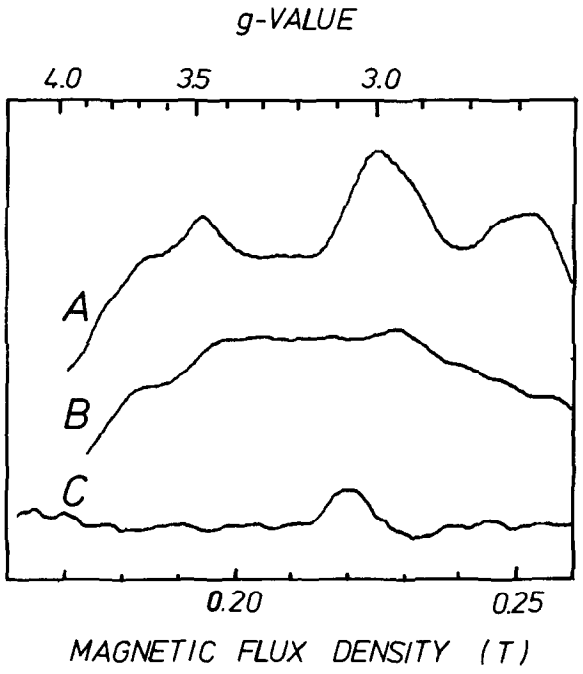

Fig.2. EPR spectra of cytochromes in broken spinach chloroplasts (~8 mg chl/ml) recorded as in fig.1. (A) Chloroplasts washed with 2 mM $K_3Fe(CN)_6$ and with 0.2 mM $K_3Fe(CN)_6$ added at 0°C 20 min before freezing. The spectrum of ferricyanide has been subtracted as in fig.1B. The signal at g 2.6–2.8 is due to incomplete cancellation of the ferricyanide signal. (B) The same ferricyanide-washed preparation as in (A) but with the addition of 10 mM hydroquinone instead of ferricyanide. (C) Difference spectrum (light minus dark) as in fig.1D (irradiation at 18 K) of chloroplasts with no additions. All spectra are the average of 4 scans and are recorded with the same spectrometer gain.

not always produce maximal signal intensity, particularly for the g 3.0 peak, and therefore, more ferricyanide was added to the sample after the washing in this particular sample. Figure 2A shows the resulting spectrum after subtraction of a small fraction of the spectrum of a sample containing a large excess of ferricyanide. The features in the g 2.6–2.8 region are due to the remaining ferricyanide signal.

The intensity of the g 3.5 peak roughly corresponds to the concentration of the reaction centers (~1 spin/500 chl molecules), and the g 2.9–3.0 peak is due to low-spin Fe(III) heme at a concentration 2–3-times higher. The g 3.5 and 3.0 peaks are associated with the highest oxidation–reduction potential in that they are reduced by 10 mM hydroquinone (fig.2B). The g 2.9 peak was more variable from one sample to another but seems to be at least partially reducible by hydroquinone (fig.2B).

Intact or broken chloroplasts with no addition generally showed only weak signals. On irradiation at low temperature, a signal appeared (fig.2C) similar but not identical to that obtained in the photosystem II preparation (see fig.1D).

4. Discussion

The high-spin Fe(III) heme signals observed here and reported in [5] represent only a small fraction of the chloroplast cytochromes. However, it cannot be excluded that these cytochromes exist in an equilibrium between high- and low-spin forms with the major form being low-spin and giving rise to the signals discussed in the following.

The low-spin signals are weak in intensity due to the large g-value anisotropy. Thus, the amplitudes are of the order of 10–50-times lower than those of the bound iron–sulfur centers. It should also be noted that the area under the g_z-peak of a low-spin signal is strongly dependent on the g-value. A peak at g_z 3.0 is ~2-times as strong as one at 3.5 and when g_z approaches 4 the intensity decreases further [11]. This could conceivably be the reason why no signal from cytochrome b_6 has been observed even though it is oxidized in the sample of fig.1A. Alternatively, an interaction with another electron acceptor, suggested by the $n = 2$ dependence in redox titrations [12], might result in extensive signal broadening.

The peak at g 3.5 arises from cytochrome f ($E'_0 \approx 350$ mV) since it appears at high oxidation–reduction potentials only (fig.1B, 2A) and is present in preparations which have no cytochrome b_{559} (fig.1B). The position and shape show no variation with different samples in contrast to cytochrome b_{559} (see below). The g_z-value is higher than that of cytochrome c at neutral pH (g 3.0) and more like that of cytochrome c at high pH [13], where the axial methionine ligand is thought to be replaced by lysine. This suggests that sulfur is not an axial ligand of the iron in cytochrome f. In fact both the optical [14] and EPR [15] spectra of cytochrome c_1 are more similar to those of cytochrome f than are the spectra of cytochrome c.

The signal at g 2.9 from photosystem II subchloroplast fragments (fig.1C) is undoubtedly due to cytochrome b_{559}, the only cytochrome present in this preparation [7]. Optical studies have revealed that in chloroplasts, cytochrome b_{559} occurs in two forms with different oxidation–reduction potentials. A high-potential form has an $E_m \approx 380$ while a lower-potential form has an $E_m \approx 80$ [16–18]. In the EPR spectra, there are also two components with different oxidation–reduction behaviour in the g 2.9–3.0 region (fig.2A). The g 3.0 peak seems to have the highest oxidation–reduction potential, since it is the last feature to be oxidized by ferricyanide and it is fully reduced by hydroquinone. Thus, it can be assigned to the high-potential form of cytochrome b_{559}.

It would be tempting to associate the peak at g 2.9 with the low-potential form of cytochrome b_{559}. It has a g-value close to that of the cytochrome in the photosystem II preparation (fig.1C), which is known to contain cytochrome b_{559} in its low-potential form [7,19]. The fact that most of this peak can be reduced by hydroquinone ($E'_0 = 260$ mV) casts some doubt on this identification. However, as pointed out in [20], a range of oxidation–reduction potentials of this cytochrome have been observed so that the so-called low-potential form of cytochrome b_{559} is not a well defined species. This could explain the variability of the amplitude of this peak which was observed in our studies.

Optical studies have shown that irradiation at low temperature of reduced samples of chloroplasts induces an oxidation of the high-potential form of cytochrome b_{559} [1,2,20]. Oxidation is also observed with photosystem II preparations although all the cytochrome b_{559} is in the low-potential form. As shown in fig.1D, 2C, photooxidation can be followed by EPR as well. The shift in the peak position relative

to that of the chemically oxidized sample could be a reflection of the difference in the redox state of neighboring electron acceptors which are produced as a result of the photoreaction (see [4]). Alternatively, the heme environment in the photooxidized sample might be different from that of the chemically oxidized sample since in the former case the conformation of the reduced protein would be 'frozen in'. It should be noted that all differences observed between cytochrome b_{559} signals could be due to rather subtle changes in the iron environment and do not suggest an exchange of ligands.

There is general agreement in this work between the intensity of the EPR signals and the optically determined concentrations of the cytochromes. Specifically, the ratio of the peaks in fig.2A is in accordance with [1,2] and the assignment given above. The total amount of cytochrome b_{559} (g 2.9–3.0) is known to be 2–3-times that of cytochrome f (g 3.5) with the high-potential form of cytochrome b_{559} (g 3.0) having a higher concentration than that of the low-potential form (g 2.9).

Acknowledgements

This work has been supported in part by grants from the National Science Foundation and the National Institutes of Health to R. M. and from the Knut and Alice Wallenberg Foundation and the Swedish Natural Science Research Council to T. V. We would like to thank Eva-Mari Aro for preparation of the cytochrome b_6–f complex and Richard Chain for many helpful suggestions and discussions.

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