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An asymmetric low-spin EPR signal of cytochrome b_6 in the cytochrome b_6f-complex from spinach chloroplasts

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Cytochrome b_6 from the chloroplast cytochrome b_6f-complex shows an asymmetric $g_z = 3.85$ low-spin EPR signal in addition to a signal at $g_z = 3.6$–3.7, similar to cytochrome b in mitochondrial or bacterial cytochrome bc_1-complexes. However, the signal is more unstable and probably represents the highly strained conformation of only part of the low-potential heme in cytochrome b_6.

Cytochrome b_6, Heme; EPR, Chloroplast

1. INTRODUCTION

High-spin (g = 6; [1,2]) and low-spin EPR signals (g = 3.5–3.6; [3,4]) have been attributed to cytochrome b_6 of chloroplasts. Recently we documented that the high-spin form originates from the low-spin form upon inactivation of the cytochrome b_6f-complex by loss of a His-ligand [5]. We concluded that physiological cytochrome b_6 is low spin, with a broad symmetrical signal at g = 3.5–3.6, lacking the second, asymmetric low-spin signal at g = 3.8 known for mitochondrial cytochrome b in the cytochrome bc_1-complex [6].

Here we show, however, that an asymmetric signal at g = 3.85 in addition to the symmetric signal can be observed at low T also with the cytochrome b_6f-complex, if it is freshly prepared. This provides a further analogy between cytochromes b of the b_6f- and the bc_1-type [7].

2. MATERIALS AND METHODS

The cytochrome b_6f-complex was prepared using the detergent Nonanoyl-*N*-methylglucamide (MEGA-9) [8]. To obtain high concentrations the procedure was modified by omission of the final sucrose density gradient. A second ammonium sulfate precipitation in the presence of 0.25% cholate resulted in almost the same purity [9] and much higher final concentrations (this modification was suggested to us by Dr P. Rich, Bodmin, England). EPR measurements were performed immediately. X-band spectra were recorded on a Bruker ER 220 D equipped with an Oxford Instruments helium cryosystem.

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3. RESULTS AND DISCUSSION

At T below 7 K a signal at g = 3.85 can be detected in the isolated cytochrome b_6f-complex (fig.1), which resembles the g_z = 3.8 EPR signal of low-potential cytochrome b in the cytochrome bc_1-complexes [6,10]. However, the signal is small compared to the broad, symmetric signal at g = 3.6 previously attributed to cytochrome b_6 [3–5]. The signal disappears upon reduction of the complex with dithionite, and is most easily seen in ascorbate-reduced samples, because the intensity of the g = 4.3 signal of rhombic Fe diminishes upon reduction allowing better examination of the g = 3.7–4.0 region. In this spectrum, which lacks the g_z = 3.54 signal of cytochrome f, the signal at

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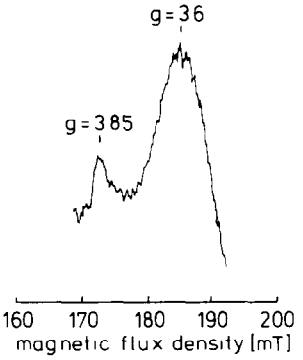

Fig.1. Low T EPR-spectrum of ascorbate-reduced cytochrome b_6f-complex in the g = 3–4 region. The cytochrome b_6f-complex was 12 μM taken directly from the sucrose density gradient [8]. A grain of ascorbate was added before freezing. EPR conditions: T, 3.6 K; microwave frequency, 9.31 GHz; microwave power, 32 mW; modulation amplitude, 0.1 mT.

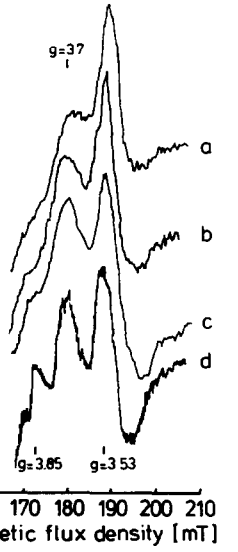

Fig.2. Low T EPR-spectra of oxidized cytochrome b_6f-complex in the g = 3–4 region. The cytochrome b_6f-complex was 70 μM taken after a second ammonium sulfate precipitation in the presence of cholate [9]. Cytochrome b_6 was fully, cytochrome f was 95% oxidized, as determined by optical difference spectroscopy. EPR conditions: T was 6.5 K for spectra a–c, 3.9 K for spectrum d; microwave frequency, 9.32 GHz; microwave power was 20, 63, 200 and 200 mW for the spectra a, b, c and d, respectively; modulation amplitude, 0.1 mT.

g = 3.85 is clearly asymmetric. This signal can also be detected in fully oxidized samples at higher concentrations and low T (fig.2d). However the asymmetry is obscured by its position on the upward deflection of the g = 4.3 Fe signal and the higher noise level. At 6.5 K the signal is seen only as a shoulder (fig.2a–c).

At low T (below 7 K) and high microwave power (200 mW), the symmetric g_z peak of cytochrome b_6 in fig.2, previously observed as a broad feature at g = 3.5–3.6 [3–5], becomes narrower and clearly separated from the signal of cytochrome f (g = 3.54) (fig.2a–d). Its position is at g = 3.7 in fig.2, but at g = 3.6 in fig.1. This difference could be explained by the assumption that the g = 3.85 species can change to the one with g = 3.6, as is known for mitochondrial cytochrome b [11]. The broadness and variation of the peak around g = 3.6, especially when no g = 3.85 signal can be detected, suggests that this signal is composed of two different components. Varying contributions of the components might cause the varying peak of the composite signal.

Aging of the complex, even by storage at −30°C, leads to loss of the signal at g = 3.85, suggesting that it arises from a rather labile configuration of the heme. This loss is accompanied by the appearance of the high-spin signal at g = 6 [1,2,5]. However it cannot be decided at present whether this conversion is direct or occurs via intermediate states [6], including possibly the one with the symmetric g = 3.6 signal. Since the appearance of the high-spin signal at g = 6 is correlated with the loss of plastoquinol-plastocyanin oxidoreductase activity of the cytochrome b_6f-complex, but loss of the g = 3.85 signal upon storage in the freezer is not, we favour the second possibility, in which both low-spin signals would come from physiologically active cytochrome b_6 hemes. One even might speculate that the interconversion of the two forms might have functional significance during the reaction cycle of the proton pumping cytochrome b_6f-complex [7].

Taking into account the corrections for different g factors proposed by De Vries and Albracht [12], we calculated the ratio of the spins present in the signals at g = 3.7 and 3.54 for cytochrome b_6 and f, respectively, in fig.2. This ratio comes very close to 2 at T = 3.9 K and P = 200 mW. Therefore we conclude, that the g = 3.85 signal represents only

a minor fraction of the cytochrome b_6 complement, different from the situation in the mitochondrial cytochrome bc_1-complex, where a stoichiometry of 1 : 1 is found for the two cytochrome *b* signals [6]. By analogy to mitochondrial cytochrome *b* [6] we conclude, that the $g = 3.85$ signal belongs to the low-potential heme of cytochrome b_6. The *g* factor is a little bit higher (3.85 vs 3.80) but the asymmetric line shape is similar to the signal of mitochondrial low-potential cytochrome *b*. This line shape has been explained by the dependence of the *g* factor on crystal field parameters [6]. According to these considerations the abnormally high *g* factor and the asymmetry of the signal reflect a highly strained configuration of the heme-ligands. The relative instability of cytochrome b_6 compared to cytochrome *b* in bc_1-complexes, causing the easy loss of the signal at $g = 3.85$, might be explained by a difference in primary structure of these two types of proteins [13]. Hydropathy analysis suggests for both types that the two hemes are bound by transmembrane helices 2 and 5, each containing 2 histidines close to opposite surfaces of the membrane. However, cytochrome b_6 contains 14 instead of 13 amino acid residues between the two histidines in helix 5. This might exert an additional, helical strain on the His-ligation of the hemes [13].

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