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On the nature of the $g = 6$ EPR signal in isolated cytochrome $b_6 f$ complex from spinach chloroplasts

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Cytochrome b_6 in freshly prepared, active cytochrome $b_6 f$ complex from spinach chloroplasts shows a broad, low-spin EPR signal around $g_z = 3.6$. Maximally half of the hemes of cytochrome b_6 can be changed to high spin with a signal at $g = 6$ by inactivating treatments, or by isolating cytochrome b_6. In this state the heme reacts with NO. Reduction rates suggest that it is the low-potential heme which changes. The change is accompanied by the loss of the shift in the g_y signal of the Rieske FeS-center by quinone analogs.

Introduction

Photosynthetic electron transport in chloroplasts and cyanobacteria involves a cytochrome $b_6 f$ complex, which functions as a plastoquinol-plastocyanin oxidoreductase between the two photosystems. This complex has been isolated and was characterized in detail [1–3]. It is similar to the cytochrome bc_1 complexes from mitochondria and bacteria which act as ubiquinol-cytochrome *c* oxidoreductases [4]. In particular these complexes have the common redox-centre composition of one heme *c*, one high potential Fe_2S_2 centre named after Rieske, and two hemes *b* with different redox potentials.

EPR spectroscopy, in addition to optical spectroscopy, served to characterize these centres. However, there is considerable discrepancy concerning the assignment of EPR features to the hemes of cytochrome b_6. Because of its dithionite reducibility a signal at $g = 6$ ($S = 5/2$) was considered to reflect cytochrome b_6 [5,6]. This view was challenged on the basis of the small spin concentration present in the $g = 6$ signal as well as by the observation of a broad low-spin feature around $g = 3.6$ titrating like cytochrome b_6 [7,8].

Here we present some further evidence that even in the isolated cytochrome $b_6 f$ complex physiologically active b_6 is in a low-spin state, whereas the $g = 6$ species is very likely due to b_6 heme having lost its 6th ligand.

The high-spin signal can be induced by several treatments inactivating the complex. It is accompanied by changes of the Rieske-FeS centre which suggests a concerted action of the Rieske and the b_6 subunit in forming the quinol-oxidizing site (Q_0 site).

Materials and Methods

The cytochrome $b_6 f$ complex was prepared using the detergent MEGA-9 [3] instead of octylglucoside [2], yielding enzyme samples with

Abbreviations: DBMIB, 3,5-dibromomethylisopropyl-*p*-benzoquinone; MEGA-9, *N*-methyl-*N*-nonaoylglucamide; Mes, 4-morpholineethanesulphonic acid.

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turnover numbers of about 25 s^{-1} under standard assay conditions [3]. EPR experiments were performed on the cytochrome b_6f complex at concentrations of up to 50 μM cytochrome f taken out of the sucrose density gradient – the final purification step. These samples, which were also used for the optical experiments, contained 30 mM 4-morpholineethanesulphonic acid (Mes) (pH 6.9), about 20% sucrose and 0.1% soybean lecithin. Further concentration steps – ultrafiltration through Amicon XM-100 filters, dialysis against polyethylene glycol-20000 (SERVA) or pelleting by ultracentrifugation and resuspending – resulted in partial loss of the catalytic activity, and the appearance of the high-spin $g = 6$ EPR signal, probably caused by increased concentrations of, or prolonged exposure to detergent. Accordingly, samples displaying large high-spin $g = 6$ EPR signals were usually prepared by increasing the concentration of the detergent MEGA-9 to 60 mM.

Optical spectra were recorded with a Kontron Uvikon 860 dual-beam spectrophotometer interfaced to a Z80-based homebuilt microcomputer system.

Cytochrome b_6 reduction kinetics were measured in an Aminco DW 2 spectrophotometer equipped with an Aminco-Morrow stopped-flow apparatus.

EPR X-band-spectra were taken on a Bruker ER 420 (Dr. J. Hüttermann, Homburg/Saar, F.R.G.) equipped with a homebuilt helium-flow-system and a Bruker 200t (Service de Biophysique, CEN Saclay) with an Oxford Instruments helium cryosystem.

NO ligation was achieved by adding some grains of $NaNO_3$ to the oxidized samples followed by a very small amount of solid dithionite [9].

Results

By our method of isolating the cytochrome b_6f complex described in Refs. 2 and 3 we obtain samples of high purity in which the $g = 6$ peak is totally absent (Fig. 1a). This high-spin signal is inducible by aging (several hours at room temperature), by oxidation with excess ferricyanide, by high concentrations of detergent, or by pelleting via ultracentrifugation as described for the purification procedure [3], but in low sucrose (14%; Fig. 1b). Compared to the sample of Fig. 1a, the one of 1b was only 40% active. The region between g = 3 and 4 of both samples shows an overlap of an ascorbate-reducible signal at $g = 3.5$ attributed to cytochrome f, which is more than 90% oxidized in the samples of Fig. 1a and b, and a much broader peak centering at slightly lower field ($g = 3.58$), which can only be reduced by dithionite.

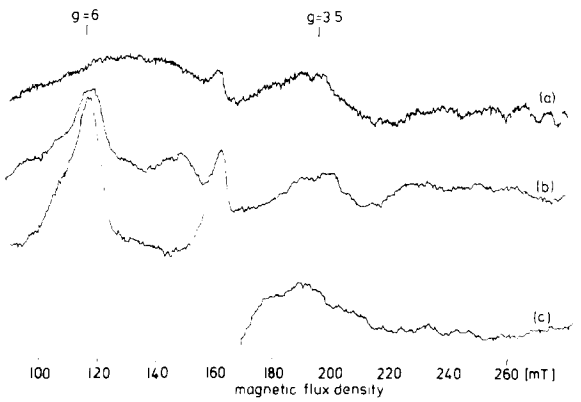

Fig. 1. EPR-spectra of cytochrome b_6 at various stages. (a) Active complex (5 μM cytochrome f). (b) Concentrated by ultracentrifugation in 14% sucrose medium [3] (40 μM cytochrome f). (c) Isolated cytochrome b_6 subunit [2] (50 μM cytochrome b_6). The spectra are differences of fully oxidized minus dithionite-reduced samples to correct for the cavity baseline. EPR conditions: temperature, 8 K; microwave frequency, 9.44 GHz; microwave pwoer, 20 mW; modulation amplitude, 3.2 mT; instrument gain, $5 \cdot 10^5$ for a; $5 \cdot 10^4$ for b; and $3.2 \cdot 10^4$ for c.

Although the possibility that high-spin b_6 heme plays a role in physiologically active b_6f cannot be fully excluded, these results strongly support the notion that both hemes of cytochrome b_6 are low-spin in the intact complex, whereas denaturing conditions increase the amount of high-spin heme.

Normally the high-spin state in cytochromes is linked to fivefold coordination of the iron [10], because a loss of the 6th ligand lowers the tetragonal crystal field splitting parameter to such an extent that spin maximation becomes favourable. To see if this applies to cytochrome b_6, we tested samples with low and high amounts of high-spin concentration for their ability to bind the nitroxy group as an exogenous ligand. Indeed samples with a large $g = 6$ EPR signal after ligation yielded large EPR features characteristic of NO-ligated

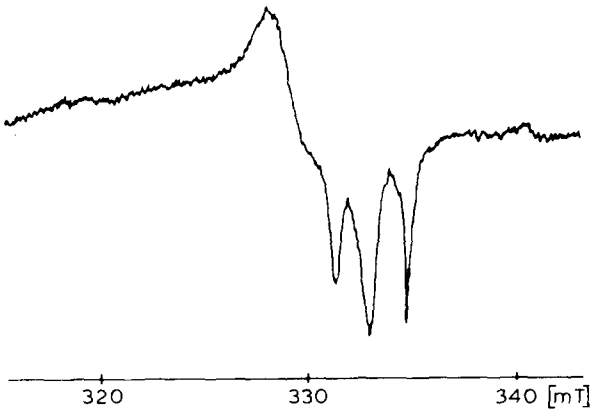

Fig. 2. EPR-spectrum of NO-treated cytochrome b_6f complex. The sample was 5 μM in cytochrome f and had been exposed to 60 mM MEGA-9. It showed a large high-spin density before the NO treatment. EPR conditions: temperature, 77 K; microwave frequency, 9.31 GHz; microwave power, 10 mW; and modulation amplitude, 0.32 mT.

hemoproteins [11] (Fig. 2), whereas preparations free of $g = 6$ EPR features just lost all cytochrome signals due to reduction (not shown). In addition, the optical difference spectrum between the NO-treated and the fully reduced cytochrome b_6f complex (Fig. 3) shows features similar to the difference spectrum of NO-treated minus untreated hemoglobin, which is high-spin and fivefold

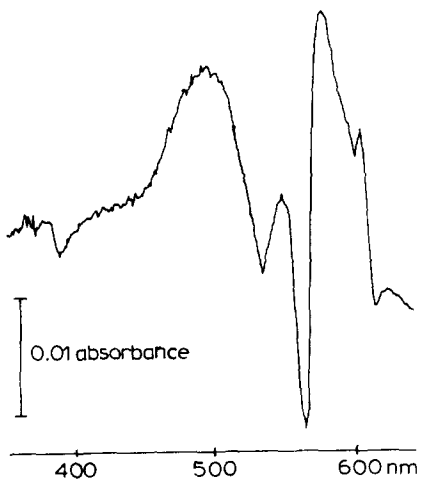

Fig. 3. Absorbance spectrum of NO-treated minus untreated cytochrome b_6f complex. The complex was 4 μM in cytochrome f. Reduction was achieved by adding a few grains of solid dithionite.

liganded [12]. In particular, the loss of the α- and β-band (563 and 534 nm) and the concomitant increase of two bands on either side of the former bands can be observed. Moreover, the redox-difference spectrum of the complex inactivated by high detergent, or of isolated cytochrome b_6 (Fig. 4) displays absorptions considered to be characteristic for oxidized high-spin cytochromes [13]: charge transfer bands at 490–500 nm ($\varepsilon = 12$ $mM^{-1} \cdot cm^{-1}$) and 610–650 nm ($\varepsilon = 3$ $mM^{-1} \cdot cm^{-1}$), seen as troughs in Fig. 4b and c, and a γ-band at 390–400 nm (Fig. 4, inset). A trough at 610–650 nm is seen only for isolated cytochrome b_6 (Fig. 4c).

The amount of cytochrome b_6 in the high-spin form can be calculated from the loss in α absorption after NO-ligation and independently from the 500 nm trough using $\varepsilon_{500} = 12$ $mM^{-1} \cdot cm^{-1}$ [13]. Maximally 44% cytochrome b_6 was found to be in the high-spin form. Therefore, we consider it quite likely that only one of the two b_6 hemes is changed to high-spin under our conditions. Accordingly, the EPR spectrum of isolated cytochrome b_6 (Fig. 1c) still shows a low-spin signal, in addition to the high one. Recent EPR experiments on the

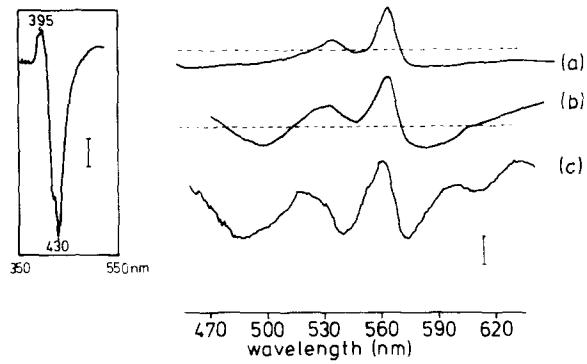

Fig. 4. Absorbance spectra of cytochrome b_6 samples. The absorption difference between dithionite and ascorbate-reduced samples is shown in (a)–(c). (a) Active complex lacking a high-spin EPR signal (1.8 μM cytochrome f). (b) Complex inactivated by exposure to 60 mM MEGA-9 showing a large high-spin EPR signal (1.8 μM cytochrome f). (c) Isolated cytochrome b_6-subunit (0.5 μM cytochrome b_6). The vertical bars indicate a ΔA of 0.05, 0.042, 0.005 and 0.004 for (a), (b), (c) and for the inset, respectively. The $\Delta A = 0$ baseline is shown as a broken line in (a) and (b). Inset: difference spectrum of ascorbate-reduced b_6f samples of (a) and (b) (inactivated–active); cytochrome b_6 is in the oxidized state.

mitochondrial bc_1 complex indicate that the change from the low- to the high-spin state proceeds via an intermediate $g_z = 3.0$ low-spin form (Ref. 14; Salerno, J.C., personal communication), which has also been observed in cytochrome *b* from yeast mitochondria after harsh treatment [15]. Several attempts to see this signal in b_6f samples at different stages of inactivation in the temperature range of 5–20 K were unsuccessful.

In an attempt to find out which heme of cytochrome b_6 is affected, we compared the reduction kinetics of active and inactivated complex. According to Clark and Hind [16], the reduction of the low potential b_6 heme is much slower than of the high-potential heme. Fig. 5 shows that by inactivation of the complex the slow reduction phase was split into at least two phases, one corresponding to the former slow phase and accounting for 20% of the low-potential b_6, and another one that is slowed down further. The fast phase remained unchanged. This indicates that about 80% of the low-potential cytochrome b_6 has been altered in the particular sample. Also the following observation supports this conclusion: as has been shown by Malkin [17], addition of the electron-transport inhibitor DBMIB to reduced, active b_6f complex results in a shift of the g_y signal of the Rieske FeS-center from $g = 1.89$ to $g = 1.94$. 'High-spin' b_6f complex, however, doesn't show this effect (Fig. 6). The fact that in the isolated Rieske subunit this shift is also not found [18] suggests that in inactive samples with high-spin cytochrome b_6 the FeS-protein is dislocated from the Q_0 site, the plastoquinol-oxidizing site which binds the inhibitor DBMIB. In active cytochrome b_6f complex this site is thought to be formed by the Rieske FeS-centre together with the low-potential heme of cytochrome b_6 [4].

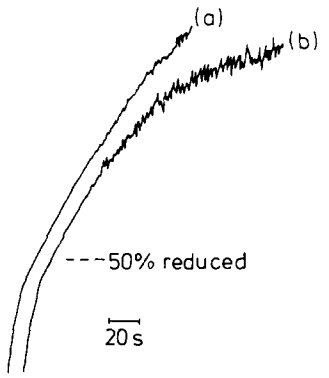

Fig. 5. Kinetics of b_6 reduction by dithionite. The kinetics were measured in a stopped-flow apparatus attached to an Aminco DW2 spectrophotometer using the wavelength combination of 563–571 nm. They are displayed in logarithmic scale (ln $[\Delta A_{tot}/\{\Delta A_{tot} - \Delta A(t)\}]$. The b_6f complex, prereduced by ascorbate and 1.8 μM in cytochrome *f*, in the 1st syringe was reduced by 5 mM dithionite from the 2nd syringe. (a) Active complex lacking a high-spin EPR signal. (b) Complex inactivated by 60 mM MEGA-9 showing a large high-spin EPR signal.

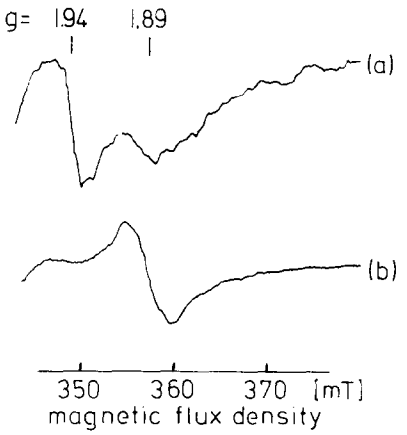

Fig. 6. High-field EPR-spectra of cytochrome b_6f complex in presence of DBMIB. The samples were 20 μM in cytochrome *f*. DBMIB was added to 100 μM. (a) Active; (b) inactivated complex. EPR-conditions: temperature, 15 K; microwave frequency, 9.45 GHz; microwave power, 20 mW; and modulation amplitude, 2.5 mT.

Discussion

The hemes of cytochrome *b* in the bc_1 or b_6f complexes can occur in four different states as indicated by the EPR-signals found so far. (1) One with two distinct low-spin signals at $g_z = 3.8$ (asymmetric) and 3.5 (broad), for low- and high-potential heme *b*, respectively; both result from strained ligations with two histidines out of plane [14]. (2) One with a broad low-spin signal with g_z around 3.6 for both hemes; this state is still strained with the histidines out of plane [14]. (3) One with a g_z signal at 3.0 reflecting a relaxed state with the histidines in plane [14,19]; it is not clear whether this signal comes from low- or high-potential heme, or from both. (4) A high-spin

state with a signal at $g = 6$ indicating loss of a histidine ligand. This might occur more readily on the low-potential heme.

The first state with two low-spin features for the two hemes *b* is well documented as the native state in mitochondrial bc_1 complexes [14,15,20], and has been observed also for the bc_1 complex from a photosynthetic bacterium [21]. The second state, lacking the asymmetric signal of the low-potential heme, is obtained by inactivating treatments [15], or by isolating cytochrome *b* from the mitochondrial [15] and bacterial [21] bc_1 complex. For the chloroplast b_6f complex no asymmetric low-spin signal for low-potential heme has been published so far. In routine measurements only the second state with resonances of the two b_6 hemes coinciding into a broad line around $g_z = 3.6$ is seen (Refs. 5–8 and 22; see also this article). Recently, we were able to detect a small, asymmetric signal at $g_z = 3.9$ in isolated cytochrome b_6f complex at T below 6 K [23], however. The state reflected by this signal is less stable for cytochrome b_6 compared to cytochrome *b* and might be largely lost already during treatment of chloroplasts for EPR measurements [5–8], or during isolation of the b_6f complex [22]. The third state with relaxed heme and a low-spin signal at $g_z = 3.0$ [14,19] has been documented for cytochrome *b* from yeast mitochondria [15], but has not been found for cytochrome b_6 so far. Finally, the high-spin state, which is readily formed in cytochrome b_6 by inactivating treatments of the b_6f complex, as demonstrated in this paper, has not been documented by EPR for cytochrome *b* of mitochondria. It has been recently described for bacterial cytochrome *b* [24], and it is also indicated for cytochrome *b* of yeast mitochondria by CO binding [15]. In summary, we conclude that the transitions from the first to the fourth state represent progressive denaturations of cytochrome *b* or b_6, and these occur more readily on cytochrome b_6 to a final high-spin state. Alternatively, the various states of cytochrome *b* or b_6 reflected by the different EPR signals might be physiological, transiently formed during turnover of the bc_1/b_6f complexes. Such transients might participate in proton translocation through the membranes via the His ligands of the hemes [25]. Therefore, it would be of interest to know how the oxidoreductase activities of the complexes are correlated with the appearance of the different EPR signals. Our experiments suggest that the high-spin state of cytochrome b_6 is inactive, but we do not know whether only the first, or also the second low-spin state represents an active form. The same is unknown for cytochrome *b* of the bc_1 complexes.

Similarly incomplete is the correlation of the different EPR features with the redox potentials of the two *b* hemes. For mitochondrial cytochrome *b* it is agreed that the asymmetric low-spin signal and the broad, symmetric low-spin signal of the first state represent low- and high-potential heme, respectively [14,15,20]. By analogy this is assumed also for bacterial cytochrome *b* [21] and chloroplast cytochrome b_6 [23]. Interestingly, the loss of the asymmetric signal during transition from the first to the second low-spin state upon isolation of cytochrome *b* from bc_1 complexes [15,21] is accompanied by a drop in redox potential [15,21,26–29]. This drop suggests a relatively higher strain in the oxidized form of the heme in the first state. In contrast, isolation of cytochrome b_6 from the b_6f complex leads to a rise of the redox potential of low potential heme, the hemes now largely titrating as a single redox component with -86 mV at pH 8 [29]. At the same time about half the heme, probably the low-potential form, changes from the low- to the high-spin state (Fig. 1c). This suggests that the reduced form of this heme is under relatively higher strain in the low-spin state compared to the high-spin state. In this context it is noteworthy that the high-spin signal of cytochrome b_6 at $g = 6$ has been reported to disappear before the broad, low-spin signal at $g = 3.6$ during stepwise reduction [22]. This low-spin signal titrates with 0 mV in chloroplast membranes [8], which matches the redox potential value of cytochrome b_6 measured for untreated membranes [30], but is in contrast to the values found for the isolated b_6f complex [2,4]. Clearly more experiments are required.

Our conclusion that the first state with the asymmetric low-spin signal is highly unstable in cytochrome b_6 gains support from a comparison of the primary structures. Hydropathy analysis of cytochrome *b* sequences suggests that the two hemes are sandwiched between membrane-span-

ning helices 2 and 5, each containing two conserved histidines in proper position, spaced by 13 residues [31,32]. Helical wheels show that ligation of the two hemes by these histidines is under strain [31], the two helices being tilted against each other [33]. The relative instability of cytochrome b_6 could be explained by the fact that helix 5 contains a 14th amino acid between the two histidines [31,34], exerting an additional torsion. This could also explain why the high-spin state with the loss of a histidine ligand is formed more easily. The fact that this transition to high spin of maximally half the hemes in cytochrome b_6 is accompanied by the loss of the shift of the g_z signal in the Rieske FeS-center by quinone analogs (Fig. 6) suggests that the site of quinol oxidation (Q_0 site) in the b_6f complex is formed by the heme which changes, probably the low-potential species, and the Rieske FeS-center.

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