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Fast electron transfer from low- to high-potential cytochrome b_6 in isolated cytochrome b_6f complex

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The two hemes of cytochrome b_6 in the cytochrome b_6f complex isolated from chloroplasts exhibit slight spectral differences in the α-band at room temperature, sufficient to deconvolute redox kinetics of cytochrome b_6 into the contributions of the two components. The deconvoluted changes observed can be explained by fast electron transfer from low- to high-potential cytochrome b_6.

Cytochrome b_6f complex; Cytochrome b; Oxidant-induced reduction; Q-cycle; Spectral deconvolution

1. INTRODUCTION

The b_6f complexes from chloroplasts and cyanobacteria catalyze a branched electron transfer from plastoquinol, measurable as oxidant-induced reduction of b_6 [1–3], which resembles the oxidation of ubiquinol by bc_1 complexes isolated from mitochondria [4] and bacteria [5]. This branched reaction is believed to be linked to proton translocation in respiratory and photosynthetic membranes, and different models for the mechanism of this energy-conserving reaction have been formulated ([6,7]; see [8] for a compendium of reviews). Among them versions of the so-called Q-cycle [9] stringently depend on a fast transmembrane electron transfer from the low- to high-potential heme b [6,8]. Indeed, EPR measurements [10,11] and more specifically the analysis of amino acid sequences [12–14] suggest that the two hemes of b or b_6 are close to opposite surfaces and perpendicular to the plane of the membranes. However, based on functional observations [15,16] and structural considerations [12,14], doubts have been raised that a fast interaction occurs between the two hemes b of b_6f, in contrast to b of bc_1 (review [17]). Therefore, we tried to deconvolute spectral changes of b_6 in isolated b_6f into the high- and low-potential components. The present results are most easily explained by fast interaction of the two hemes.

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Abbreviations: PQH_2, plastoquinol; b_h, high-potential cytochrome b; b_l, low-potential cytochrome b; f, cytochrome f; b_6f, cytochrome b_6f complex; bc_1, cytochrome bc_1 complex

2. MATERIALS AND METHODS

The b_6f was prepared using the detergent nonanoyl-*N*-methyl glucamide (MEGA-9) [18]. Plastoquinone-3 was synthesized according to Wood and Bendall [19], reduced to PQH_2-3 [20] and stored in acidic ethanolic solution. Plastocyanin was isolated from spinach leaves [21].

For all experiments described below the reaction mixture contained 50 mM NaCl, 5 mM KCl and 50 mM 2-*N*-morpholinoethanesulfonic acid (Mes)/NaOH, pH 6.7 (final concentrations in the cuvette or mixing cell). Kinetic traces were recorded by a modified Aminco-Chance DW-2 spectrophotometer supplied with an Aminco stopped-flow equipment (1 cm light path) maintained at 2°C. The spectrophotometer was used in the single-wavelength mode, as the chopper was switched off to avoid the high noise level created by the rotating mirror. The photomultiplier current was converted to voltage, amplified

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and processed in a separate amplifier unit. An output voltage signal proportional to transmission was fed into an analog-digital converter connected to a Z 80-based microcomputer system (Rechenzentrum der Universität Regensburg), where data were stored and further processed. The time resolution of the setup is limited by the dead-time of the stopped-flow apparatus, which was determined to be 4 ms.

Spectral deconvolution of components was achieved by using the procedure suggested by Rich et al. [22], which is essentially based on recording kinetics at as many different wavelengths as there are components contributing spectral changes. Data are deconvoluted according to:

$$\Delta A\mathrm{dec}^j = \epsilon_j^i \cdot \Sigma(\epsilon^{-1})_i^j \cdot \Delta A_i \quad (1)$$

where summation is performed from $i = 1$ to n (n, number of components); subscripts denote label wavelengths, superscripts designating components; $\Delta A\mathrm{dec}^j$ represents the 'pure' absorption difference due to the j-th component, where contributions of other components have been eliminated; ΔA_i denotes the measured absorption changes at the i-th wavelength; the ϵ_i^j constitute the elements of an 'extinction-coefficient matrix', where each column consists of the extinction coefficients of the respective component at certain wavelengths; the elements of the inverted matrix are denoted by $(\epsilon^{-1})_i^j$; matrix inversion was achieved by applying 'Cramers rule'. The elements of ϵ were determined from the spectra of the isolated components. For deconvolution of a system containing f, b_h, b_l and plastocyanin (PC) the following values of ϵ_i^j were used: $\epsilon^f_{554\,nm} = 17.0$; $\epsilon^f_{559\,nm} = 3.4$; $\epsilon^f_{567\,nm} = -3.8$; $\epsilon^f_{575\,nm} = -4.0$; $\epsilon^{b_h}_{554\,nm} = 1.1$; $\epsilon^{b_h}_{559\,nm} = 13.6$; $\epsilon^{b_h}_{567\,nm} = 10.6$; $\epsilon^{b_h}_{575\,nm} = -6.8$; $\epsilon^{b_l}_{554\,nm} = 1.1$; $\epsilon^{b_l}_{559\,nm} = 11.0$; $\epsilon^{b_l}_{567\,nm} = 14.4$; $\epsilon^{b_l}_{575\,nm} = -6.8$; $\epsilon^{PC}_{554\,nm} = -2.62$; $\epsilon^{PC}_{559\,nm} = -3.2$; $\epsilon^{PC}_{567\,nm} = -3.7$; $\epsilon^{PC}_{575\,nm} = -4.2$.

All coefficients are expressed in $cm^{-1} \cdot mM^{-1}$. Absorbance changes due to quinones are negligible in the analysed spectral region (550–600 nm) [23].

3. RESULTS AND DISCUSSION

Spectra of the low- and high-potential hemes in b_6 were obtained by three different methods:

(i) Ascorbate-prereduced b_6f was further reduced by dithionite. The reduction of b_6 is biphasic [24], b_h being reduced much faster than b_l. In fig.1 the spectra of the first and the last quarter of progressive reduction are shown.

(ii) The same reduction was performed in the stopped-flow apparatus and kinetics were run every 2 nm between 540 and 575 nm. They were rearranged into time-resolved spectra by means of a computer (not shown).

(iii) The b_6f was titrated anaerobically with limiting amounts of dithionite and difference spec-

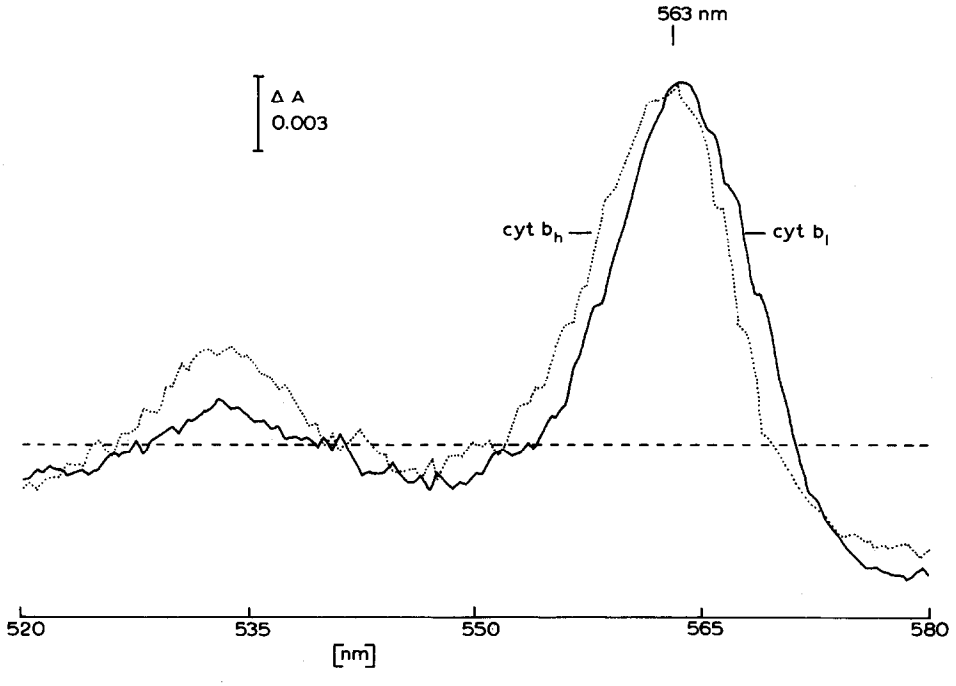

Fig.1. Spectra of low- and high-potential cytochrome b_6. Isolated b_6f complex (1 μM cyt. f) was first reduced by ascorbate and then further by 10 mM dithionite at room temperature. Spectra were recorded at 60 s intervals in a Kontron Uvikon 860 spectrophotometer. Difference spectra at 25 minus 0% (cyt. b_h) and at 100 minus 75% (cyt. b_l) reduction of total cyt. b_6 are shown.

tra were taken between different states of reduction (not shown).

All three methods gave the result shown in fig.1: The spectrum of b_h, which is reduced more rapidly, shows the peak at slightly shorter wavelength than for the spectrum of b_l, which is reduced more slowly. This has already been noted by Clark and Hind [24] during progressive reduction of b_6 by ferredoxin.

Interestingly, in *Chlorella* cells b_l absorbs at slightly shorter wavelength [25]. The spectral differences of b_h and b_l in isolated b_6f are more pronounced at cryogenic temperature [24,26], b_h showing a split α-band at 557 and 561.5 nm, while b_l has a single peak at 560.5 nm [26]. In mitochondrial [27] and bacterial [28] bc_1 the low-potential b_l shows the split α-peak, and the spectral differences vs b_h are clearly observed at room temperature in membranes.

Extinction coefficients at 559 and 567 nm for b_h and b_l of b_6 were estimated from spectra as in fig.1 assuming a ϵ_{peak} of 21 $mM^{-1} \cdot cm^{-1}$ [29] and are given in section 2. The deconvolution procedure for the kinetics at these two wavelengths, recorded as in point (ii) above, yielded traces corresponding to the rapidly reduced b_h and the slowly reduced b_l (fig.2). This result provided the means to look for the functional interaction of the two components in the b_6f complex.

Fig.3 shows the deconvoluted kinetics of b_h and b_l when fully oxidized b_6f was reduced by PQH_2-3 in a stopped-flow experiment. Since this reaction was much faster than the reduction by dithionite, the experiment was performed at 2°C. The Q_{10} value of the reaction was determined to be 3.3. The traces in fig.3 demonstrate that b_h is reduced to about 50%, whereas b_l is not reduced at all. Ascorbate completely inhibits the reduction of b_h by PQH_2 (not shown). Unlike the situation in bc_1 where ubiquinol can directly reduce b_h via reversal of the quinone reduction site [30,31], this site is irreversible in b_6f and plastoquinol can reduce b_6 only via the quinol oxidation site which requires an oxidized Rieske FeS center [2]. Since b_l and not b_h is believed to act at this quinol oxidation site in most of the mechanistic models [6,7], the lack of net reduction of b_l in fig.3 could reflect very fast donation of electrons from b_l to b_h, as demonstrated before for bc_1 in membranes of photosynthetic bacteria [28]. Recently, such a

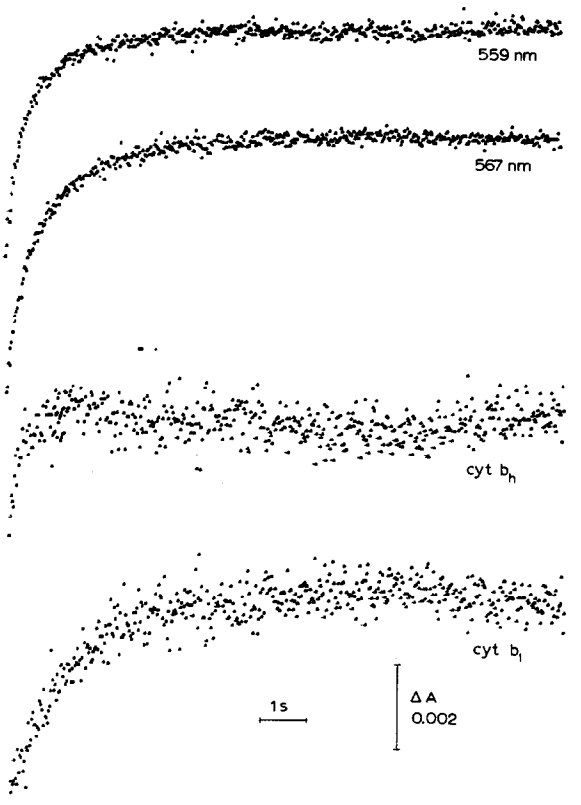

Fig.2. Deconvolution of cyt. b_6 into cyt. b_l and cyt. b_h during reduction by dithionite. Ascorbate-reduced b_6f complex (0.25 μM) was further reduced by 100 mM dithionite in the stopped-flow apparatus at room temperature. Deconvolution of the kinetics at 559 and 567 nm (upper traces) into cyt. b_h and cyt. b_l (lower traces) was achieved as described in section 2.

transfer was also found to occur in the isolated bc_1 from yeast mitochondria [31].

To test for this transfer in b_6f the deconvolution procedure was applied to stopped-flow measurements under conditions of oxidant-induced reduction of b_6 [1], in which b_h had been prereduced to increasing extents by dithionite. Fig.4 shows traces with 0% (a) and about 100% (b) of b_h reduced before mixing with oxidant. The traces in fig.4a resemble those in fig.3, i.e. no reduction of b_l is observed. In the case of prereduced b_h, however, electrons accumulate in b_l. This is most easily explained by a fast electron transfer from b_l to b_h. Alternatively, one might assume that reduction of b_h causes a conformational change of the complex which makes b_l accessible to PQH_2. It is interesting in this context that although heme-heme interac-

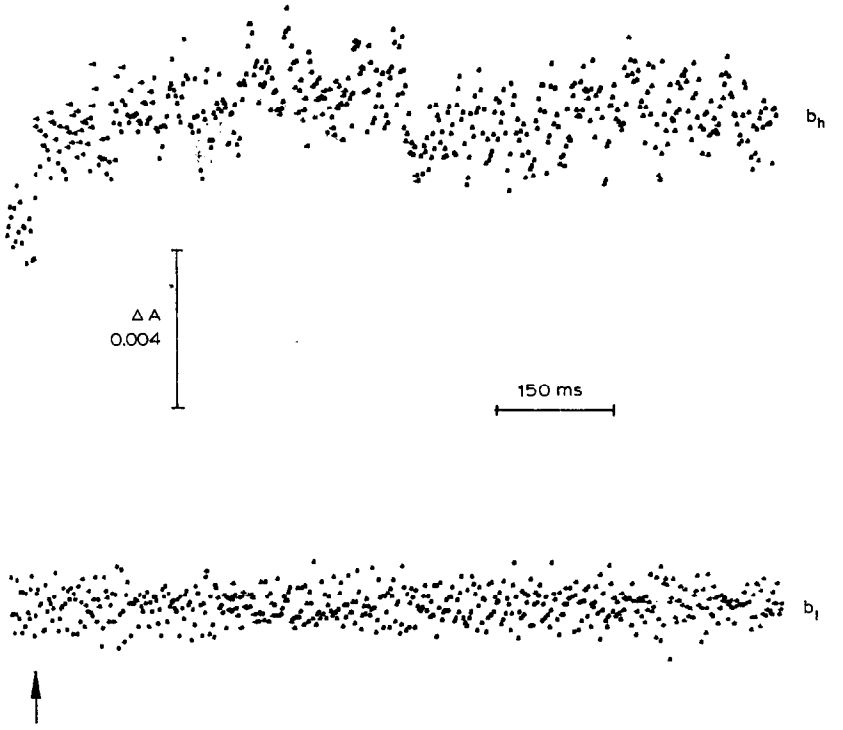

Fig.3. Reduction of cyt. b_h by PQH_2-3. Fully oxidized b_6f complex (0.5 μM cyt. *f*) was reduced by 20 μM PQH_2-3 (final concentrations) at 2°C. Traces at 554, 559 and 567 nm were recorded and deconvoluted into cyt. b_l and b_h as described in section 2.

tion in the bc_1 complex from yeast is fast during *b* reduction [31], it is inefficient during *b* oxidation [32], which indeed calls for different conformations dependent on the redox state. On the other hand, if one considers the redox potentials of PQH_2, b_h and the Rieske FeS center in isolated b_6f

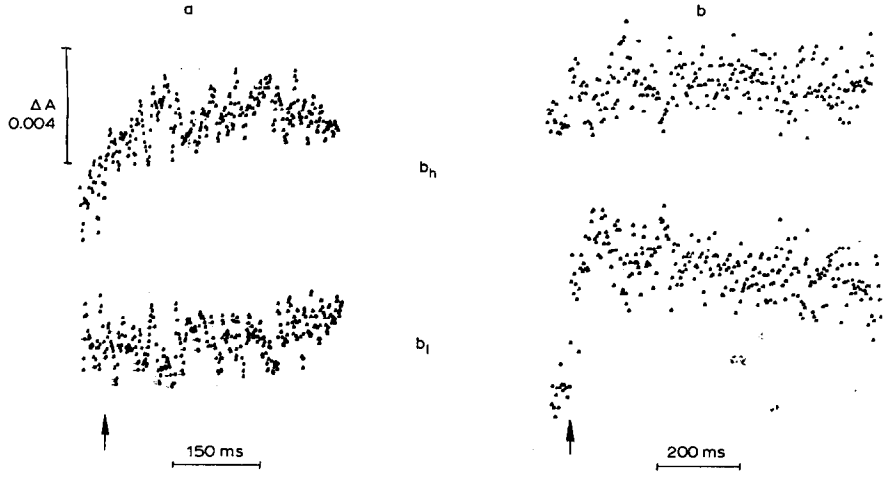

Fig.4. Oxidant-induced reduction of cyt. b_h and cyt. b_l. b_6f complex (0.5 μM cyt. *f*) preincubated with 20 μM PQH_2-3 was mixed with 4 μM oxidized plastocyanin (final concentrations) at 2°C. Traces taken at 554, 559, 567 and 575 nm were deconvoluted into cyt. b_l and b_h. (a) Both cyt. *b* components oxidized before mixing; (b) cyt. b_h prereduced by anaerobically titrating with dithionite to about 50% reduction of total cyt. b_6 (followed by taking spectra in the stopped-flow cell).

[7], a much higher degree of b_h reduction than observed is expected, if a quasi-equilibrium at the quinol oxidation site [28] is reached in the experiment of fig.3, and b_h instead of b_l accepts electrons from PQH_2. This will be elaborated elsewhere.

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