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Volume 153, number 2 FEBS LETTERS March 1983

Cytochrome b_6 from isolated cytochrome b_6f complexes

Evidence for two spectral forms with different midpoint potentials

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Received 26 January 1983

Cytochrome b_6 from spinach chloroplasts (either within the purified cytochrome b_6f complex, or in its isolated form) exhibits two spectral species, which correspond to two midpoint potentials. This can be demonstrated by low temperature difference spectroscopy at fixed redox potentials. The high potential form of cytochrome b_6 has a split α-peak at 557.5 and 561.5 nm, the low potential form has a symmetrical α-peak at 560.5 nm. Similar results were obtained with cytochrome b_6 in the isolated cytochrome b_6f complex from the cyanobacterium *Anabaena variabilis*.

Cytochrome b_6 *Cytochrome* b_6f *complex* *Chloroplast electron transport* *Cyanobacteria* *Low temperature difference spectroscopy* *Redox potential*

1. INTRODUCTION

Isolated cytochrome b_6f/bc_1 complexes from spinach chloroplasts, cyanobacteria, mitochondria and photosynthetic bacteria exhibit a universal redox center composition comprising two hemes b, one heme c_1 or f, the high potential Rieske FeS center and possibly bound quinone [1]. The double amount of heme b compared to heme c is explained by a cytochrome b hetero- or homodimer [1]. Cytochrome b in the isolated mitochondrial and bacterial complex is potentiometrically and spectroscopically heterogeneous [2–4]; however, the corresponding cytochrome b_6 in the spinach complex reveals only one α-peak at 563 nm during a reductive titration, although redox heterogeneity was found [5]. With low temperature spectroscopy only an asymmetrical, but not a split α-peak at 561 nm was observed for the fully reduced cytochrome b_6 [6]. Asymmetry was also demonstrated for cytochrome b_6 within a cytochrome-enriched fraction from chloroplasts obtained by digitonin treatment [7].

Here we show that cytochrome b_6, either within the cytochrome b_6f complex or in its isolated form, can be separated in two distinct spectral forms as derived from low temperature spectroscopy at fixed redox potentials. Also for cytochrome b_6 of the cyanobacterium *Anabaena variabilis* a similar redox heterogeneity and two spectral species were observed.

Abbreviations: MOPS, 3-(*N*-morpholino)propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris, Tris(hydroxymethyl) aminomethane

2. MATERIALS AND METHODS

Cytochrome b_6f complexes from spinach chloroplasts and *A. variabilis* were isolated as in [5,8]. Cytochrome b_6 was purified from the spinach complex as in [5] using Triton X-100/urea for the extraction followed by hydroxyapatite chromatography. Redox titration was as in [9]; experimental details and redox mediators are specified in [5]. Low temperature spectra over liquid nitrogen at fixed redox potentials were measured in an Aminco DW 2 UV/VIS spectrophotometer equipped with low temperature accessory using the 2 mm cuvettes. Isolated spinach

Published by Elsevier Biomedical Press

00145793/83/0000–0000/$3.00 © Federation of European Biochemical Societies

cytochrome b_6f complex was suspended to 10 μM cytochrome b_6, isolated cytochrome b_6 to 2.5 μM and isolated cytochrome b_6f complex from *A. variabilis* to 3 μM in 20 mM MES/20% glycerol (pH 6.0); the following redox mediators were added to 8 μM: 2 hydroxy-1,4-naphthoquinone, 1,4-naphthoquinone, 2,3,5,6-tetramethyl-*p*-benzoquinone, anthraquinone-2-sulfonate, antraquinone-2,6-disulfonate.

The reductive titration was performed by small additions of a concentrated dithionite solution. After stabilization of the ambient redox potential, the suspension was transferred from the titration cuvette, using a nitrogen-flushed syringe, to the low temperature cuvette. The sample was quickly frozen in liquid nitrogen. When redox difference spectra were taken against fully oxidized cytochrome b_6 (ascorbate was added to reduce cytochrome *f*), the reference cuvette contained the oxidized cytochrome b_6. However, when spectra were recorded against fully reduced cytochrome b_6, excess of dithionite was added to the sample cuvette and the reference cuvette contained the samples at fixed redox potentials.

Protein was determined as in [10] or [11]. [Cytochrome b_6] was determined as in [12]. An M_r of 23 500 for cytochrome b_6 was taken [5] for calculating the mol heme *b*/mol protein ratio.

3. RESULTS AND DISCUSSION

In [5] we demonstrated redox heterogeneity of cytochrome b_6 within the isolated cytochrome b_6f complex from spinach chloroplasts. It was suggested that cytochrome b_6 is at least composed of two components exhibiting pH-dependent midpoint potentials. However spectroscopically, cytochrome b_6 appeared as a single species during a reductive titration. There was no indication for two spectral forms as it is known for the corresponding cytochromes *b* from mitochondria and photosynthetic bacteria [1–4]. However, it was shown that cytochrome b_6 within the isolated cytochrome b_6f complex has an asymmetrical α-peak at 561 nm at low temperature [6]. Fig. 1(●) shows a redox titration of cytochrome b_6 within the intact cytochrome b_6f complex at pH 5.6, similar to that in [5]. With a computer-best-fit program, on the basis of linear regression, and the assumption that the low potential form of cytochrome b_6 is still fully oxidized when the high potential form is fully reduced, we estimated 62% cytochrome b_6 low potential with a Nernst slope $n = 1.01$ and $E_{m5.6}$ −146 mV and 38% cytochrome b_6 high potential with $n = 1.03$ and $E_{m5.6}$ −3 mV. Redox heterogeneity is also observed when spectra were recorded at low temperature at fixed redox potentials (fig. 1(○)). The corresponding low temperature difference spectra of the points A–C and F, with the reference cuvette poised at 120 mV, are shown in fig. 2. At an ambient redox potential of + 42 mV in the sample cuvette (fig. 2A) [conditions where selectively low potential cytochrome *b*-559 should be reduced

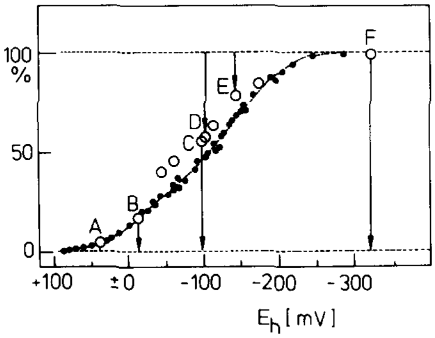
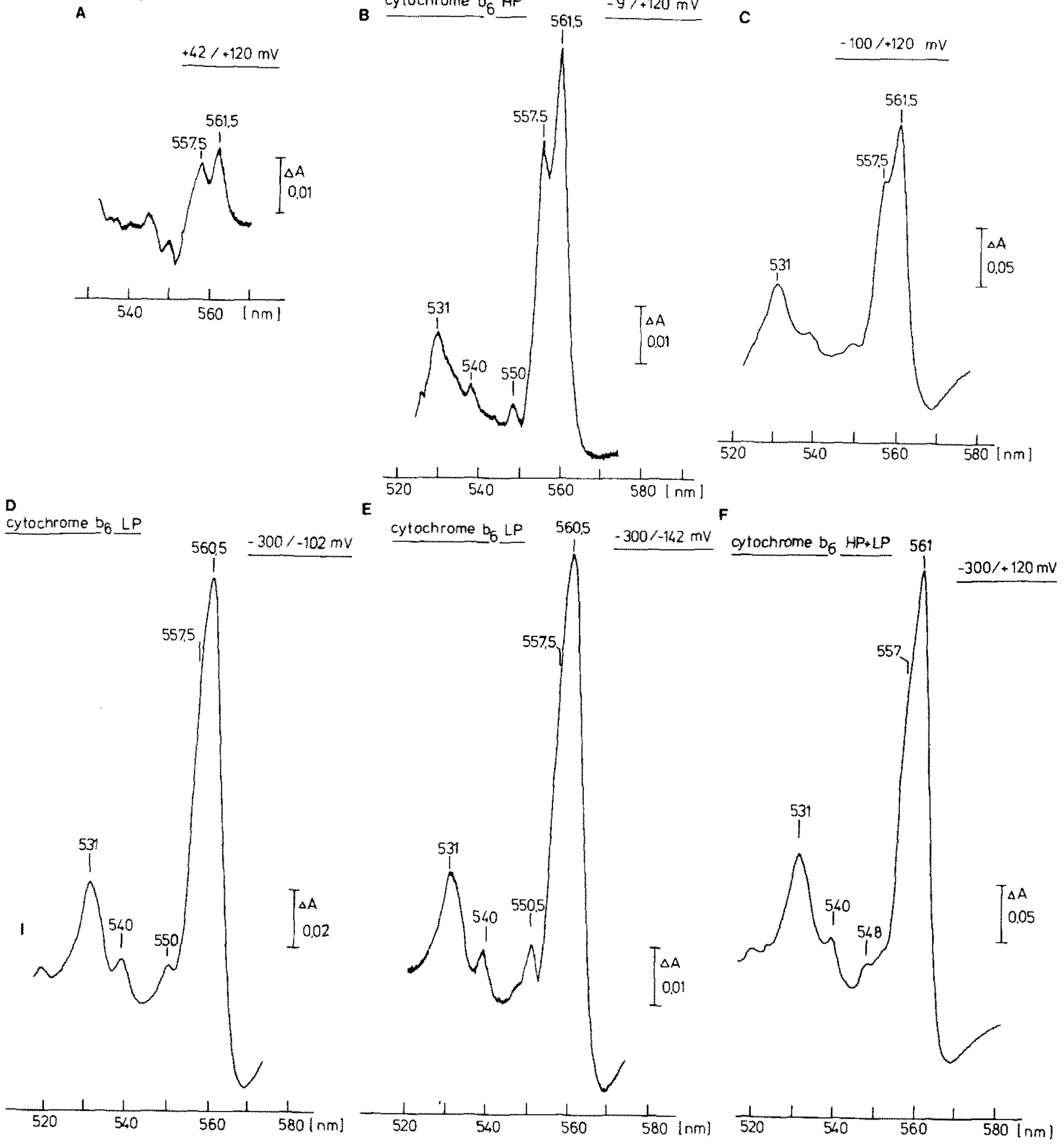


Fig. 1. Redox titrations of cytochrome b_6 within the isolated cytochrome b_6f complex from spinach chloroplasts. Redox titration was performed as in [5]: (●) reductive titration performed in 30 mM Tris–succinate (pH 5.6) using the redox mediators in [5]; (○) titration as in section 2 taking low temperature spectra to estimate the degree of cytochrome b_6 reduction; (→) low temperature spectra records.

Fig. 2. Low temperature difference spectra of cytochrome b_6 within the cytochrome b_6f complex from spinach chloroplasts poised at different redox potentials. During a reductive redox titration (section 2) samples were taken at fixed redox potentials and were quickly frozen in liquid nitrogen before low temperature difference spectra were recorded. Difference spectra of A, B, C and F were measured at the indicated fixed redox potential against fully oxidized cytochrome b_6 (reference cuvette contained ascorbate ambient redox potential of 120 mV); difference spectra D and E, however, were recorded against fully reduced cytochrome b_6 (dithionite in the sample cuvette, ambient redox potential −300 mV). HP and LP stand for high and low potential.

→

Fig. 2.


A
+42 / +120 mV
557,5
561,5
ΔA
0,01
540
560
[nm]
B
cytochrome b_6 HP
-9 / +120 mV
561,5
557,5
531
540
550
ΔA
0,01
520
540
560
580 [nm]
C
-100 / +120 mV
561,5
557,5
531
ΔA
0,05
520
540
560
580 [nm]
D
cytochrome b_6 LP
-300 / -102 mV
560,5
557,5
531
540
550
ΔA
0,02
520
540
560
580 [nm]
E
cytochrome b_6 LP
-300 / -142 mV
560,5
557,5
531
540
550,5
ΔA
0,01
520
540
560
580 [nm]
F
cytochrome b_6 HP+LP
561
-300 / +120 mV
557
531
540
548
ΔA
0,05
520
540
560
580 [nm]

($E_{m7.0} + 85$ mV [13])] only very little of total cytochrome *b* is reduced. There is a peak at 557 nm, which could indicate the presence of low potential cytochrome *b*-559, but it is not the dominant peak. Low potential cytochrome *b*-559 could not be detected in the isolated cytochrome b_6f complex before [5,6]. The occurrence of the low potential cytochrome *b*-559 in other cytochrome b_6f particles was demonstrated by redox titration or by low temperature spectroscopy [13,14]. By progressively lowering the ambient redox potential in the sample cuvette down to about −100 mV, the spectra exhibit a split peak at 557.5 and 561.5 nm (fig. 2A–C). The splitting is lost but asymmetry is still obvious when cytochrome b_6 is fully reduced (fig. 2F). When complementary low temperature difference spectra were recorded in the potential region between −100 and −200 mV using fully reduced cytochrome b_6 as reference (fig. 2D, E), a symmetric α-peak at 560.5 nm was found. These findings demonstrate that cytochrome b_6 of the isolated cytochrome b_6f complex can be separated into two distinct spectral forms by low temperature difference spectroscopy at fixed redox potentials. Cytochrome *b* in isolated cytochrome bc_1 complexes from mitochondria [2] and the photosynthetic bacterium *Rhodopseudomonas sphaeroides* [3] is spectroscopically heterogeneous, but in these cases, the low potential form has the split-, and the high potential form has the symmetric α-peak. Interestingly, the different cytochromes *b* with split α-peaks (i.e., the high potential forms in the cytochrome b_6f complexes and the low potential forms in the cytochrome bc_1 complexes) have close midpoint potentials ([2,3,5]; see [1]).

We also looked for the spectral and redox properties of cytochrome b_6 isolated from the cytochrome b_6f complex which in SDS–polyacrylamide gel electrophoresis migrates as a single polypeptide with app. M_r 23 500 [5]. This polypeptide is identical with subunit II of the cytochrome b_6f complex [5,6]. Isolated b_6 contained 41 nmol heme *b*/mg protein (protein determined as in [10]) which corresponds to 0.97 mol heme *b*/mol M_r 23 500 protein, or contained 53 nmol heme *b*/mg protein (protein determined as in [11]) which corresponds to 1.25 mol heme *b*/mol M_r 23 500 protein. Since *b*-type cytochromes exhibit abnormally fast migration on SDS–polyacrylamide gel electrophoresis [1], the M_r of 23 000 as well as the calculated molar ratios of heme and protein might be underestimated. More than one heme group/polypeptide chain suggests a two-headed heme protein which has also been proposed for mitochondrial cytochrome *b* [15]. During the purification of spinach cytochrome b_6 using Triton X-100/urea as extraction mixture [5] redox heterogeneity is partially lost (fig. 3). Especially at alkaline pH the titration curve corresponds rather to a slope $n = 1$ (fig. 3(■)) with a midpoint potential of −86 mV. However, at pH 5.8 (fig. 3(●)) redox heterogeneity is still observed. This implies that the midpoint potential of the high potential form of isolated cytochrome b_6 is more pH-dependent compared to the low potential form. Also the high potential form of cytochrome b_6 within the intact cytochrome b_6f complex exhibits a more pronounced pH-dependence compared to the low potential form [5], but there, even at pH 8.3, redox heterogeneity is clearly observed. Therefore the two midpoint potentials of cyto-

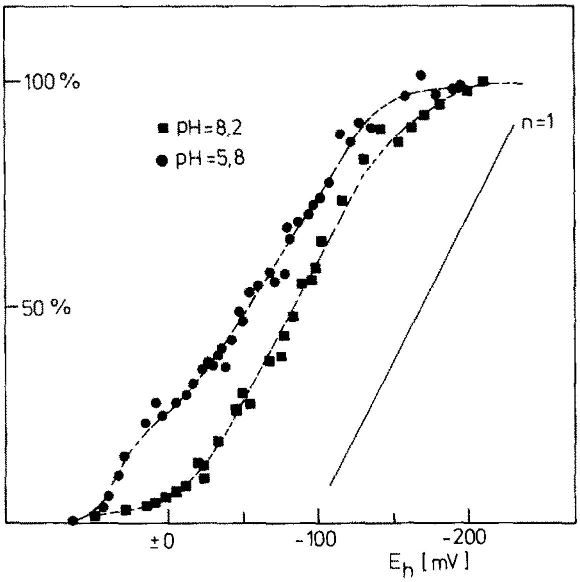


Fig. 3. Redox titration of isolated cytochrome b_6 from spinach chloroplasts at pH 5.6 and 8.2. The redox titration was performed in [5] using the same redox mediators. The buffer was 30 mM MES/MOPS/Tris, the pH was adjusted by addition of HCl or NaOH; 100% reduced cytochrome b_6 corresponds to 3.5 μM cytochrome b_6. A theoretical Nernst curve for $n = 1$ drawn through the inflexion point of the titration curve, is also given.

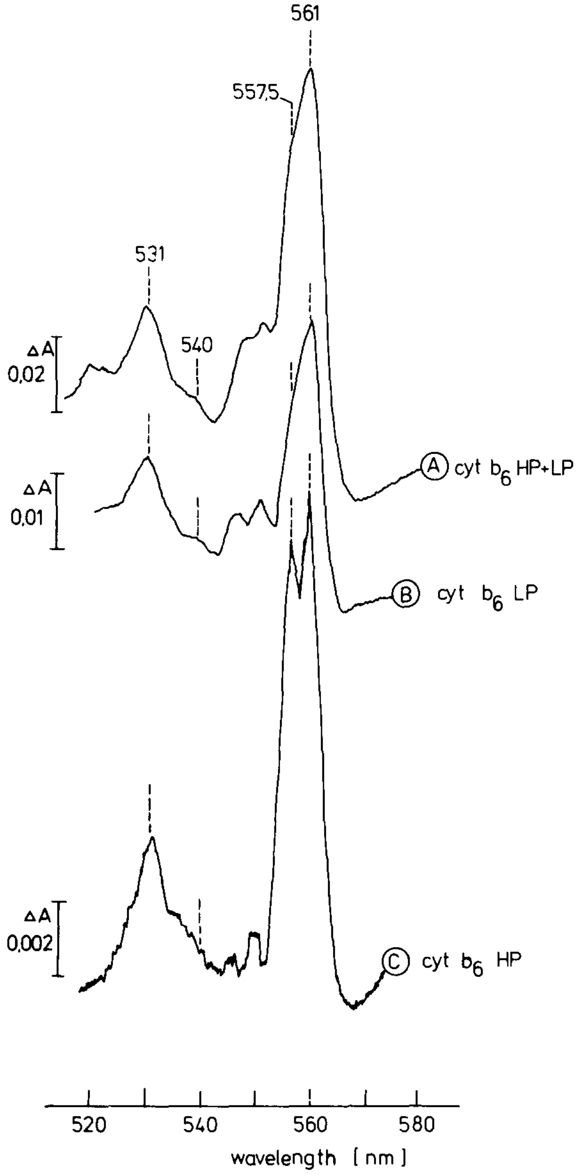


Fig. 4. Low temperature difference spectra of isolated cytochrome b_6 from spinach chloroplasts poised at different redox potentials: experimental conditions as in section 2 and fig. 2; low temperature difference spectra were taken at −300/+130 mV for A, at −300/−130 mV at B and −70 mV/+130 mV at C.

chrome b_6 seem to come closer during isolation. Also cytochromes b from mitochondria [16] or photosynthetic bacteria [3] show the tendency to lose redox heterogeneity during purification.

When isolated cytochrome b_6 is analyzed at acidic pH by low temperature difference spectroscopy at fixed redox potentials, again two spectral forms, a high potential cytochrome b_6 with a split α-peak at 561 nm and 557.5 nm and a low potential form with a single α-peak at 561 nm are found (fig. 4). Both spectral forms have the maximal peak at 561 nm which is different to cytochrome b_6 within the cytochrome b_6f complex (see fig. 2). In addition, the low potential form of isolated cytochrome b_6 exhibits a modified difference spectrum compared to the intact cytochrome b_6 with additional absorptions at 551 and 548 nm (cf. fig. 2D and fig. 4B). However, both high potential forms are very similar (fig. 2B and fig. 4C) suggesting that they are more protected against damage during isolation.

Cytochrome b_6 in the isolated cytochrome b_6f complex from the cyanobacterium *Anabaena variabilis* also shows redox- and spectral heterogeneity (fig. 5,6). The midpoint potentials of the two forms are not so clearly separated (cf. fig. 1 and 5); however, as in the case of spinach cytochrome b_6, $E_{m(1)6.0}$ + 24 mV and $E_{m(2)6.0}$ −80 mV can be derived from the redox titration in fig. 5(●). The two midpoint potentials of cytochrome b_6 from *Anabaena variabilis* are more positive than the redox potentials of spinach cytochrome b_6. Low temperature difference spec-

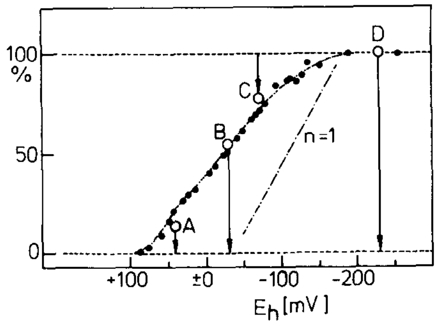


Fig. 5. Redox titrations of cytochrome b_6 within the isolated cytochrome b_6f complex from *A. variabilis*. The redox titration was done as in [5] using the following redox mediators (at 15 μM): 2-hydrox-1,4-naphthoquinone; 2,3,5,6-tetramethyl-*p*-benzoquinone; 1,4-naphthoquinone; 1,2-naphthoquinone; anthraquinone-2-sulfonate; anthroquinone-2,6-disulfonate. (●) Reductive titration in 20 mM MES (pH 6.0); 100% reduced b_6 corresponds to 3 μM cytochrome b_6; (○) A–D come from a titration as in section 2.

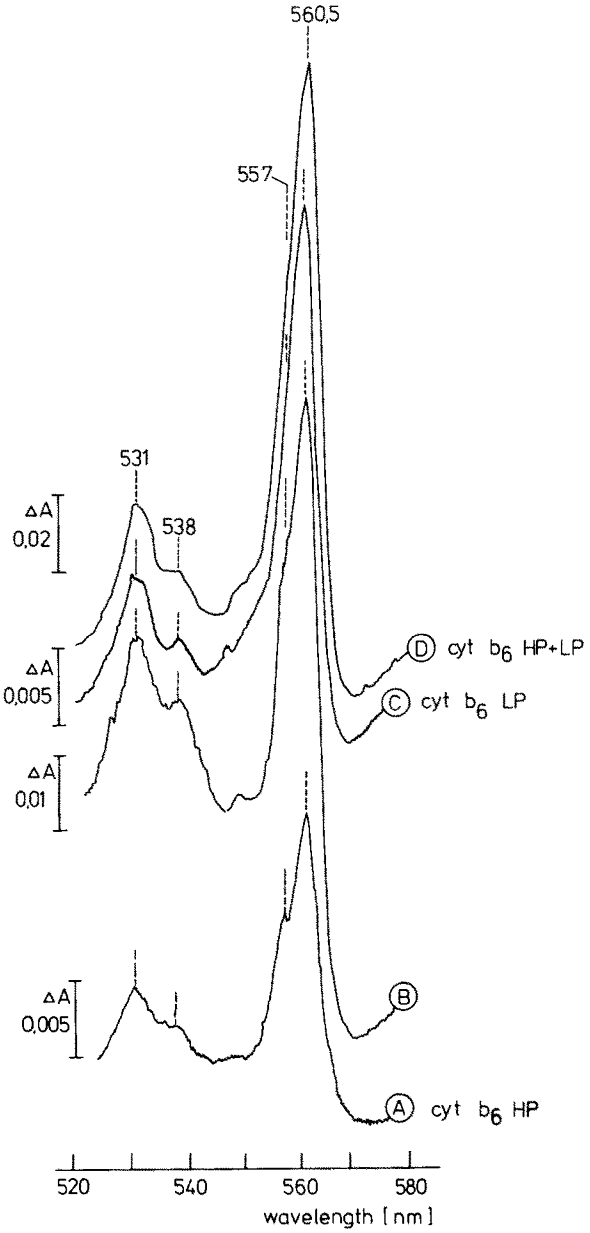


Fig. 6. Low temperature difference spectra of cytochrome b_6 within the cytochrome b_6f complex from *A. variabilis* at fixed redox potentials. Low temperature difference spectra at poised redox potentials were obtained as in section 2 and fig. 2. Difference spectra were recorded at: (A) + 40/ + 190 mV; (B) – 30/ + 190 mV; (C) – 250/ – 70 mV; (D) – 230/ + 190 mV. Cyt, HP and LP: cytochrome, high potential and low potential, respectively.

troscopy at fixed redox potentials (fig. 5(○)) showed that the high potential form has a split α-peak at 560.5 nm and at 557 nm, the low potential form a single α-peak at 560 nm.

We would like to propose (see [1]) that, according to protonmotive Q-cycle [17] or a *b*-cycle [18], the two different forms of cytochrome b_6 in the cytochrome b_6f complexes of chloroplasts and cyanobacteria function between two states of plastoquinone. In this scheme, low potential cytochrome b_6 is reduced via an unstable semiquinone created during oxidation of plastoquinol in one state, and high potential cytochrome b_6 is oxidized by quinone or stabilized semiquinone in the other state.

ACKNOWLEDGEMENT

The preparation of cytochrome b_6f complex from *Anabaena variabilis* by Miss S. Pfeffer is gratefully acknowledged. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 43 C 2).

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