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Two distinct states of the thylakoid *bf* complex

Sebastian Heimann, Christof Klughammer, Ulrich Schreiber*

Julius-von-Sachs-Institut für Biowissenschaften, Lehrstuhl Botanik I, Universität Würzburg, Julius-von-Sachs Platz 2, D-97082 Würzburg, Germany

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Abstract Under normal physiological conditions the state of the cyt *bf* complex is characterized by rapid reoxidation kinetics of cyt *b*-563 following flash-illumination. It is known that these kinetics are dramatically slowed down under oxidizing conditions. Here we show that this slow-down of cyt *b*-563 oxidation is the consequence of a relatively slow (half-time of several minutes) transformation of the cyt *bf* complex into a distinctly different state (termed state-s). Reversal to the normal state requires strong reductive treatment or light-induced electron transport. The results are in line with a recent model of functional cyt *bf* dimers [Cramer et al., Annu. Rev. Plant Physiol. Plant Mol. Biol. 47 (1996), 477–508], if it is assumed that state-s reflects the monomeric state of the *bf* complex.

Key words: Cytochrome *b*-563 reduction; Cytochrome *bf* complex; Q-cycle; Photosynthesis

1. Introduction

The cyt *bf* complex of thylakoid membranes plays a central role in photosynthetic electron flow between the two photosystems (PS I and PS II) and associated proton translocation reactions [1–3]. In addition, there is increasing evidence that the cyt *bf* complex is also involved in redox control of regulated light distribution between PS II and PS I [4,5]. However, there are still open questions concerning details of electron transport reactions at the cyt *bf* complex, particularly with respect to steps involving the low potential chain. It is not finally settled whether a modified Q-, SQ- or b-cycle mechanism applies and whether such cycle is obligatory or facultative [1–3]. Furthermore, it remains to be clarified to what extent structural and functional dimers of the cyt *bf* complex play a role in vivo [6,7]. Progress in this field of research depends on reliable measurements and interpretation of light-induced redox changes of cyt *f* and cyt *b*-563. In the past, there has been some controversy concerning the interpretation of extremely slow cyt *b*-563 reoxidation kinetics under oxidizing conditions which appeared to be finally settled by the elaborate Q-cycle model of Kramer and Crofts [8].

Here we show that the slow cyt *b*-563 reoxidation kinetics under oxidizing conditions are not caused by the momentary redox conditions. Rather they are the result of a fundamental change in the state of the cyt *bf* complex which takes place under oxidizing conditions and requires several minutes for completion.

*Corresponding author. Fax: (49) (931) 888-6158.
E-mail: schreibe@botanik.uni-wuerzburg.de

Abbreviations: chl, chlorophyll; cyt, cytochrome; DAD, diaminodurene; LHC, light harvesting complex; PQ, plastoquinone; Q_N, plastoquinone reducing site of the cyt *bf* complex; Q_P, plastoquinol oxidizing site of the cyt *bf* complex

2. Materials and methods

Intact spinach chloroplasts were freshly prepared as previously described [9] except that no additional ascorbate was added during isolation. Thylakoids were obtained directly before each experiment by rupturing intact chloroplasts in 10-fold diluted standard suspension buffer to which 10 mM $MgCl_2$ was added. Isoosmotic conditions were re-established after 30 s by addition of double-strength suspension buffer. Absorbance changes were recorded with an improved version of a laboratory-built LED array spectrophotometer [10]. This computer-controlled kinetic spectrophotometer measures absorbance changes in the 500–590 nm wavelength range simultaneously at 16 different discrete wavelengths (ca. 2 nm half-band widths). The time-resolved difference spectra were deconvoluted on the basis of model difference spectra for C550, cyt *f*, cyt *b*-559 and cyt *b*-563. Possible contributions of plastocyanin and P700, which are relatively flat in the given wavelength range, were taken account of by including a second order polynomial function in the deconvolution procedure. All experiments were carried out at 20°C with 40 μg chl/ml in the presence of 400 μM methylviologen, 0.5 μM nigericin, 0.5 μM nonactin and, if no ferricyanide was present, 1000 U/ml catalase. The intensity of actinic red light ($\lambda > 630$ nm) was 4400 $\mu E/m^2s$ and of far-red light ($\lambda > 720$ nm) 70 $\mu E/m^2s$ in all experiments. White saturating flashes ($t_{1/2} = 7$ μs) were applied with a stroboscope flash lamp (Polytec, Waldbronn, Germany). Time resolution was 6.5 ms in flash experiments and 65 ms in continuous light. Duroquinol was prepared as described in [11].

3. Results and discussion

3.1. Flash-induced kinetics

It has been known for some time that under oxidizing conditions, when the plastoquinone (PQ) pool is completely oxidized, cyt *b*-563 reoxidation following flash-illumination is much slower than at medium potential [12,13]. Kramer and Crofts [8] have proposed a modified Q-cycle model which in principle can explain the paradoxical behavior of this catalytic step. In this model, reoxidation of cyt *b*-563 decisively depends on the availability of free PQH_2 and reduced plastocyanin. In Fig. 1 data are presented which on the one hand confirm the observations made by the above authors, but on the other hand give additional, new information which makes previous results appear in a new light. Fig. 1A shows the slow cyt *b*-563 reoxidation kinetics following flash-illumination of spinach thylakoids under oxidizing conditions. Thylakoids were incubated with 200 μM ferricyanide for 5 min and preilluminated with far-red light. Under these conditions, not only PQ but also cyt *f* is completely oxidized, as indicated by the lack of flash-induced cyt *f* oxidation. Plastocyanin should also be nearly fully oxidized, considering the very similar redox potential of cyt *f* and plastocyanin [14]. Fig. 1B is presented to demonstrate the commonly observed, much more rapid cyt *b*-563 reoxidation kinetics under medium potential conditions, paralleled by rapid kinetics of flash-induced cyt *f* oxidation and re-reduction. New information is presented in Fig. 1C. This figure shows the flash-induced kinetics in an experiment identical to that of Fig. 1A, except that there

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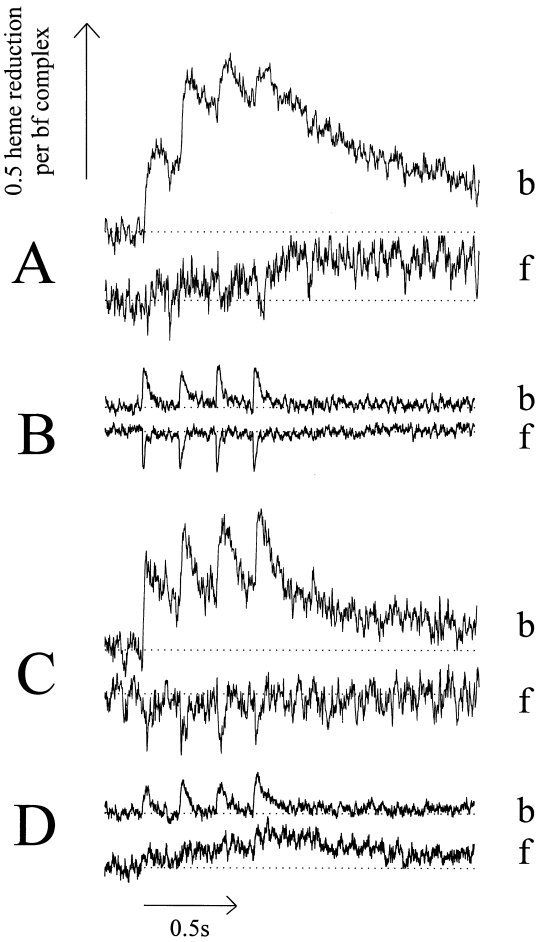

Fig. 1. Flash-induced redox changes of cyt *b*-563 (upper traces b) and cyt *f* (lower traces f). Fresh thylakoids were illuminated by four saturating flashes applied at 200 ms intervals. A: Following 5 min incubation with 200 μM ferricyanide and far-red preillumination during the first minute. Average of 20 measurements, each done with fresh samples. B: Following 1 min incubation with 5 mM ascorbate and 250 μM duroquinol. Average of 200 measurements done on four samples at 0.2 Hz. C: As A, but with 5 mM ascorbate and 250 μM duroquinol added 1 min before measurement. D: With continuous far-red background illumination, started 30 s before averaging. Average of 200 measurements done on four samples at 0.2 Hz. Note that averaging was increased in B and D as compared to A and C for the sake of sufficient signal-to-noise ratio while time resolution was kept the same (6.5 ms).

was a change from oxidizing to medium potential conditions 1 min before start of flash-illumination. Surprisingly, cyt *b*-563 reoxidation kinetics are still rather slow, although the presence of 5 mM ascorbate and 250 μM duroquinol should have ensured an excess of free PQH_2 as well as reduction of plastocyanin. Actually, the cyt *f* traces confirm that medium potential conditions really were established. On the other hand, Fig. 1D presents the example of an experiment where rapid cyt *b*-563 reoxidation kinetics were observed under conditions when the presence of free PQH_2 appears unlikely, as continuous far-red light was applied in the presence of methylviologen/O_2, an effective electron acceptor system (see also Section 3.3).

3.2. Cyt b-563 reduction in saturating continuous light

The rate of cyt *b*-563 oxidation is also expressed in the extent of cyt *b*-563 reduction in saturating continuous light. When strongly oxidizing conditions are established by ferricyanide incubation, a transiently high level of cyt *b*-563 reduction upon onset of saturating light is observed (Fig. 2A). Interestingly, however, the observed decline in reduction level is relatively slow ($t_{1/2} \sim 6$ s). Actually, it is much slower than the expected accumulation of PQH_2. This assessment is confirmed by the data in Fig. 2B–D. In the experiment of Fig. 2B, 5 mM ascorbate and 1 mM duroquinol were added 5 min before the measurement, a treatment which should ensure thorough reduction of the PQ pool [15]. The thus established conditions, however, did not prevent a rather high initial reduction level of cyt *b*-563.

Instead of ferricyanide, far-red light can also be used to oxidize the intersystem electron transport chain. The experiments of Fig. 2C,D were carried out under almost identical conditions, except for a 1 min far-red preillumination in the case of Fig. 2C 10 min before the measurement. Most importantly, in both cases the redox state of the intersystem chain was made equal by 30 s far-red illumination immediately before onset of saturating light. The equal extent of cyt *f* oxi-

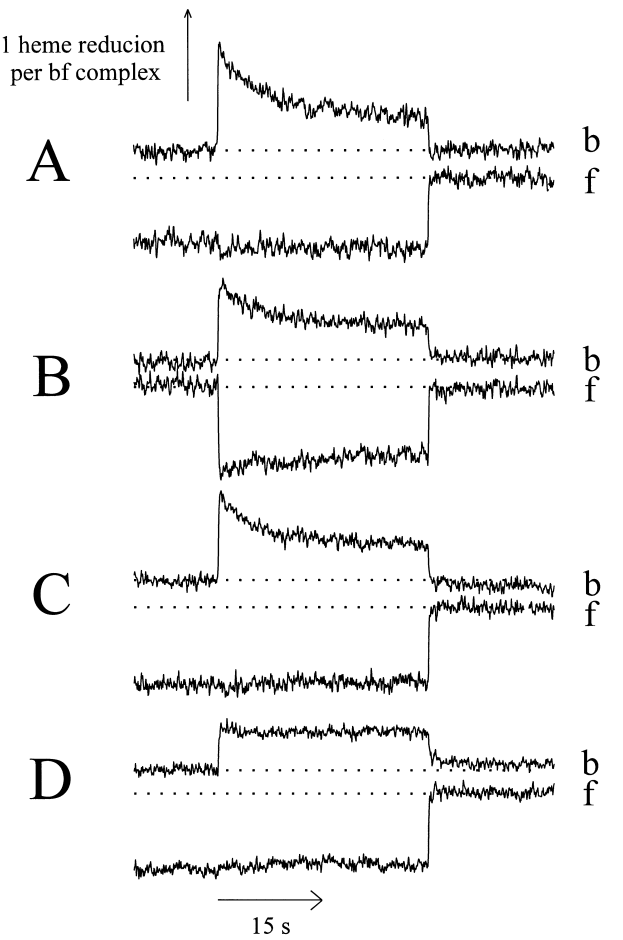

Fig. 2. Redox changes of cyt *b*-563 (upper traces b) and cyt *f* (lower traces f) upon illumination with saturating light. The depicted traces are the average of four experiments using fresh samples. A: Following 10 min dark incubation with 2.5 mM ferri-/ferrocyanide each and 1 μM DAD. B: As A, but with addition of 5 mM ascorbate and 1 mM duroquinol 5 min before onset of illumination. C: 60 s far-red preillumination followed by 10 min dark time and another 30 s far-red illumination immediately preceding onset of actinic illumination. D: As C, but without the first far-red preillumination.

dation during preillumination is confirmed by the same amplitudes of cyt *f* re-reduction upon light-off. However, there are dramatic differences in the extent of light-induced cyt *b*-563 reduction. As it appears, the far-red preillumination 10 min before illumination has induced a particular state which is characterized by slow cyt *b*-563 oxidation. Obviously, the same state cannot be induced by far-red illumination shortly before actinic illumination. For this particular state of the cyt *bf* complex, which to our knowledge has not so far been described in the literature, we propose the term 'state-s', with the 's' standing for slow.

A higher amplitude of cyt *b*-563 reduction after far-red as compared to red illumination was already reported by Böhme [16]. While a detailed discussion of that work would be beyond the scope of the present communication, it may be noted that we were not able to reproduce Böhme's observation that following red illumination a substantial fraction of cyt *b*-563 remains reduced in the dark (see Fig. 2). This discrepancy could be due to overlapping changes of cyt *b*-559, P515, P700, PC and light scattering, which may have affected Böhme's measurements with coupled chloroplasts using dual-wavelength spectroscopy. The present measurements were carried out in the presence of nigericin and nonactin using a 16-wavelength deconvolution method (see Section 2) and, hence, are much less affected by such overlapping changes.

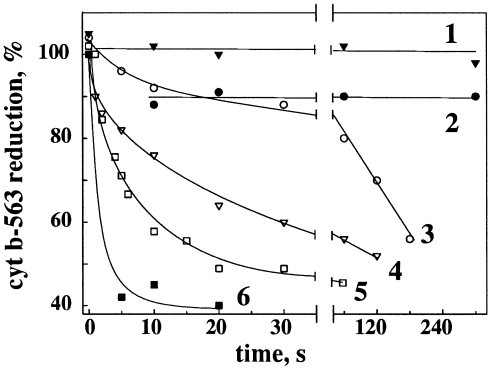

Fig. 4. Kinetics of state-s reversal by illumination and reducing conditions. The amplitude of initial cyt *b*-563 reduction (100% corresponding to one heme per *bf* complex) was measured as described in Figs. 2 and 3. The varied parameter is the time under reducing conditions after preceding establishment of state-s. Curve 1, incubation with 5 mM ascorbate, added after 10 min. incubation with ferri-/ferrocyanide (as described for Fig. 2A). Curve 2, incubation with 5 mM ascorbate and 1 mM duroquinol, added after 10 min ferri-/ferrocyanide. Curves 3–5, illumination with 40, 200 and 4400 μE/m^{2}s red light, respectively, following state-s formation induced by far-red preillumination (as described for Fig. 2C). Curve 6, incubation with 5 mM ascorbate plus 1 mM dithionite, added after 10 min ferri-/ferrocyanide. Dithionite action was stopped by adding 4 mM ferricyanide. 10 s later 4 mM ascorbate was added and 1 min thereafter the measurement was carried out.

3.3. Relatively slow formation of state-s

The results presented in Figs. 1 and 2 indicate that formation of state-s requires oxidizing conditions. In addition, as suggested by Fig. 2C,D, in the case of far-red preillumination a certain dark time following oxidation of intersystem electron carriers appears to be important. This aspect was further investigated by the experiments of Fig. 3. State-s was assessed via the amplitude of initial cyt *b*-563 reduction upon onset of saturating illumination (see examples in Fig. 2). Curve 1 in Fig. 3 shows the time course of state-s formation following 1 min far-red preillumination. Again it is observed that immediately after far-red illumination almost no state-s is formed. Under the given conditions, a half-maximal change is found after ca. 2 min and maximal transformation is observed after 5 min. Once formed, state-s is stable for prolonged periods of time in darkness, even under medium redox potential conditions (not shown). When continuous far-red light was applied for longer time periods preceding actinic illumination, there was also state-s formation (see curve 3). However, as is apparent from a comparison of curves 1 and 3, a dark period following short far-red preillumination is much more effective than continuous far-red.

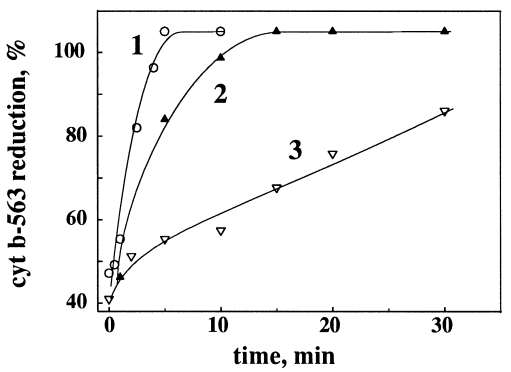

Fig. 3. Kinetics of state-s formation under oxidizing conditions. The amplitude of initial cyt *b*-563 reduction (100% corresponding to one heme per *bf* complex) upon onset of continuous saturating red light is plotted versus time of preceding oxidizing treatment. Measurement of light-induced redox changes as described for Fig. 2. Curve 1, darkness following a 1 min far-red preillumination. Curve 2, after incubation with 2.5 mM ferri-/ferrocyanide each plus 1 μM DAD. Curve 3, after continuous far-red preillumination.

Curve 2 in Fig. 3 shows the time course of state-s formation upon incubation under oxidizing conditions at a relatively high ferri-/ferrocyanide concentration (2.5 mM) and in the presence of a low concentration of an effective redox mediator (1 μM DAD). Under these conditions the induced formation of state-s is rather slow, requiring approximately 3 min for a half-maximal change. This confirms the findings in Figs. 1A,C and 2A,B which already suggested that the ambient redox conditions alone are not the decisive parameter for expression of state-s.

3.4. Reversal of state-s

As already suggested by data in Figs. 1 and 2, not only formation of state-s but also its reversal is a relatively slow process. Actually, it appears that once formed, state-s is remarkably stable under medium redox potential conditions (see Figs. 1C and 2B). Treatments which cause a reversal to the normal state are actinic illumination and incubation with strong reducing agents like dithionite. These aspects are further illustrated by the experiments in Fig. 4. Curves 1 and 2 confirm that there is reversal of state-s neither by 5 mM ascorbate nor by additional 1 mM duroquinol, even if these redox agents are applied over several minutes. Duroquinol generally somewhat lowers the maximal amplitude of reduction. Curves 3–5 illustrate the reversal of state-s by actinic light, with the rate of reversal increasing with light intensity.

The most rapid and complete reversal can be induced by a brief reductive treatment with dithionite (curve 6). In order to assess the amplitude of light-induced cyt *b*-563 reduction, in this experiment the added dithionite was first oxidized by an excess of ferricyanide and then medium potential conditions established by adding ascorbate. When the last step (addition of ascorbate) was omitted, after 10 min ferricyanide incubation state-s again was observed (data not shown).

3.5. Possible identity of state-s

The main result of the present study is the finding that the well-known slow reoxidation kinetics of cyt *b*-563 under oxidizing conditions are not due to the ambient redox conditions as such, but are the consequence of a relatively slow state change of the cyt *bf* complex induced by oxidizing conditions. The question arises as to the identity of the resulting state which we have termed state-s. While it would be premature to suggest a definite answer to this question, there are some aspects which in this context should be considered.

(1) Recently, in crystallographic structure determinations of the mitochondrial cyt bc_1 complex the Rieske subunit has been found in two different positions ca. 16 Å distant from each other [17–20]. One position is oriented towards cyt c_1, the other towards cyt b_L. Since the mitochondrial cyt bc_1 complex and the thylakoidal cyt *bf* complex principally have considerable similarity in function and structure, one may now imagine that state-s of the cyt *bf* complex could be due to a repositioning of the thylakoidal Rieske protein. However, state-s is characterized by an extremely slow reoxidation of cyt *b*-563, a redox reaction at the Q_N site on the stromal side of the membrane, while the Rieske protein is involved in plastoquinol oxidation at the Q_P site on the lumenal side. Also, flash-induced reduction of cyt *b*-563 is known to be scarcely influenced by oxidizing conditions [21], and therefore we deem this interpretation unlikely. Furthermore, neither position of the mitochondrial Rieske protein by itself is thought to allow Q-cycle-type electron transfer, due to the unusually large distance either between cyt c_1 and Rieske protein in one position, or cyt b_L and Rieske protein in the other position. Therefore, a recent hypothesis states that the mobile part of the Rieske protein may oscillate between these two positions in order to bridge this gap between cyt c_1 and cyt b_L, thus enforcing an obligate Q-cycle mechanism [19,20]. If this hypothesis is correct, normal activity of the cyt bc_1 complex depends on the proposed flexibility of the mitochondrial Rieske protein, and the observed different positions would not indicate the existence of two states of the cyt bc_1 complex with different kinetic properties.

(2) State 1/state 2 changes of thylakoid membrane organization [22] are known to be closely associated with properties of the cyt *bf* complex. Plastoquinol at the quinol oxidation site of reduced cyt *bf* complex appears to be crucial for kinase activation which leads to LHC II phosphorylation and state 2 formation [5]. State 2 is associated with a lateral movement of cyt *bf* complexes from granal membranes into the PS I-containing stroma lamellae [23]. In view of the fact that both state 1 and state-s are induced by far-red light, one might expect that they are identical. However, the currently available evidence does not support this suggestion. Dark-adapted spinach chloroplasts under medium redox conditions tend to be in state 1 without any need for far-red illumination, and are characterized by rapid cyt *b*-563 reoxidation kinetics. Furthermore, we did not find any effect of NaF when state-s formation was induced by far-red illumination (data not shown), whereas NaF is known to prevent state 1 formation by inhibiting dephosphorylation [24].

(3) There are indications that the cyt *bf* complex may be organized in the thylakoid membrane as a structural and functional dimer whereas the monomeric form is inactive [6]. Cramer et al. [7] speculate that the dimer might be needed to provide sufficient surface area, to allow the docking of extrinsic proteins. It may be allowed to further speculate that state-s could correspond to the monomeric state of the cyt *bf* complex. If in the dimer two high potential cyt *b*-563 cooperated in a concerted reduction of PQ at the Q_N site, then monomer formation would affect cyt *b*-563 reoxidation. Within the framework of a cyt *bf* dimer/monomer model, the monomers could have the function of the 'transport form' in lateral movements of cyt *bf* complexes during state transitions.

Formation of state-s requires oxidation of a redox component within the cyt *bf* complex, the identity of which is currently unknown. Our attempts to characterize this component by redox titration have so far been unsatisfactory due to an extreme hysteresis. While on the one hand quite high potentials were required to initiate state-s formation by oxidation, on the other hand a strong reducing agent like dithionite was required for reversal. Medium potential conditions affected neither the normal state (characterized by rapid cyt *b*-563 oxidation) nor state-s. Further work will be required to clarify the identity of state-s as well as of the redox sensor which initiates its formation.

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