Biochimica et Biophysica Acta, 767 (1984) 80–86
Elsevier

BBA 41589

COMPARISON OF ATP-INDUCED AND DCMU-INDUCED INCREASES OF CHLOROPHYLL FLUORESCENCE

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(Received February 28th, 1984)
(Revised manuscript received May 29th, 1984)

Key words: ATPase; Chlorophyll fluorescence; Photosystem II; Electron transport; Dichlorophenyldimethylurea; (Spinach chloroplast)

A comparative study of the ATP-induced and the DCMU-induced increases of dark chlorophyll fluorescence after activation of the latent ATPase gave the following results: (1) The ATP-induced fluorescence rise exceeds the DCMU-induced rise by an amount equivalent to the rapid component of the biphasic ATP-induced change. There is complementarity between the slow component and any preceding DCMU-induced fluorescence rise. (2) Up to 10^{-4} M DCMU (3-(3′,4′-dichlorophenyl)-1,1′-dimethylurea)), with the slow component being completely suppressed, the rapid ATP-induced phase is unaffected. It becomes eliminated, though, with an I_{50} of about $3 \cdot 10^{-4}$ M. (3) No binary oscillations in dependence of the number of preilluminating flashes are observed for the rapid ATP-induced fluorescence increase. Under identical conditions such oscillations are found upon DCMU-addition. (4) The amplitude of the rapid ATP-induced fluorescence rise is unaffected by closure of Photosystem II reaction centers in presence of DCMU and NH_2OH by a single saturating flash (removal of about 50% of total quenching). With further flashes and gradual complete removal of quenching, the rapid ATP-induced change is eliminated with a two-step dependency. It is concluded that the rapid phase of the ATP-induced increase in fluorescence reflects reverse electron flow at non-B-type reaction centers, while the slow phase is linked to reverse electron flow at B type centers. On the basis of these results a model is proposed for heterogeneous interactions between the ATPase and B-type and non-B-type electron-transport chains. 'Direct coupling' appears to be possible between CF_0-CF_1 and those electron-transport chains which are located in the stroma-exposed margin region of the grana stacks (PS II_β units with non-B-type properties).

Introduction

Important insights into the mechanism of electron transfer at the PS-II acceptor side has been gained by studies of the DCMU-induced increase of dark fluorescence yield, $(\Delta F)_{DCMU}$. [1–3]. Velthuys and Amesz [1] observed binary oscillations of $(\Delta F)_{DCMU}$ depending on the number of preilluminating flashes and interpreted these oscillations as expression of a two-electron gating mechanism between Q (one-electron carrier) and the pool of plastoquinone (two-electron carrier). Secondary quinone acceptor, called R [1] or B [4], was postulated to store one electron until upon a second charge separation at the same center the accumulated two electrons are released into the pool. Recent fluorescence work [5] and studies of

Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1′-dimethyl urea; Q, the primary acceptor of Photosystem II and quencher of chlorophyll fluorescence; R or B, secondary acceptor of Photosystem II; PS I and PS II, Photosystem I and II; PQ, plastoquinone; F_v, variable fluorescence, i.e., difference between any dark-level fluorescence and maximal fluorescence; P 515, field-indicating pigments of the thylakoid membrane; I_{50}, concentration for half-maximal inhibition; CF, coupling factor; Cyt, cytochrome.

[^{14}C]DCMU binding [6,7] support a model by Velthuys [8] according to which DCMU-type inhibitors compete with plastoquinone for a common binding site at the PS-II acceptor complex. There is strong binding of the plastosemiquinone anion to this site. Inhibitor-binding appears to be possible only during the fraction of time while the binding site is vacant, and a stored electron dwells on the primary acceptor, or when the acceptor complex is oxidized:

$$Q \cdot PQ^- \rightleftarrows Q^- \cdot PQ \rightleftarrows Q^- + PQ \quad (1)$$

$$Q^- + I \rightleftarrows Q^- \cdot I \quad (2)$$

$$Q + I \rightleftarrows Q \cdot I \quad (3)$$

On the basis of this model, the $(\Delta F)_{DCMU}$ reflects the transformation of centers $Q \cdot PQ^-$ into the state $Q^- \cdot$(DCMU) (Eqns. 1 and 2).

In the preceding paper [9] an analysis of the ATP-induced rise in chlorophyll fluorescence, $(\Delta F)_{ATP}$, was presented. It was shown that $(\Delta F)_{ATP}$ consists of a rapid and a slow phase with vastly differing properties. Only the slow phase is dependent on external reductants and on the build-up of a ΔpH and only the slow phase can be blocked by inhibitors which are known to act between the two photosystems at the level of the Cyt *b/f* Fe-S complex [9]. In previous work with class C chloroplasts, which show only a minor rapid component of $(\Delta F)_{ATP}$, it was concluded that there is complementarity between $(\Delta F)_{DCMU}$ and $(\Delta F)_{ATP}$ [10]. This finding suggested that as a consequence of ATP-hydrolysis an electron is reversed from a bound plastosemiquinone anion on the primary PS-II acceptor Q, as was concluded for DCMU-binding.

In the present communication, comparison of $(\Delta F)_{ATP}$ and $(\Delta F)_{DCMU}$ is extended to also include the rapid component of $(\Delta F)_{ATP}$ which is pronounced in class D chloroplasts [11]. It will be shown that the rapid phase of $(\Delta F)_{ATP}$ is not complementary with $(\Delta F)_{DCMU}$ and does not show binary oscillations with preilluminating flashes. Hence, reversed electron flow plastosemiquinone on Q appears not to be involved. The properties of this phase rather suggest involvement of 'non-B' centers [12,13] or β-centers [14–18].

Materials and Methods

Isolation of intact chloroplasts from spinach leaves and preparation of class D chloroplasts, as well as conditions of activation and reaction, were the same a described in the preceding paper [9].

Chlorophyll fluorescence was monitored by an extremely weak 550 nm measuring beam (about 10^{-4} W/m^2) which by itself did not induce any fluorescence rise even in presence of DCMU and NH_2OH. The measuring system for ATP-, DCMU- and light-induced changes of fluorescence and P 515-absorbance was as described before [9,19–21].

Results

Induction of $(\Delta F)_{ATP}$ and $(\Delta F)_{DCMU}$

Fig. 1 shows the ATP- and DCMU-induced increases of chlorophyll fluorescence, recorded under identical conditions. Each sample was preilluminated for 15 s with bright white light to activate the ATPase while chloroplasts were still intact. Then chloroplasts were given 30 s hypotonic treatment and resuspended in isotonic reaction medium in the presence of $5 \cdot 10^{-5}$ M diaminodurene and $5 \cdot 10^{-3}$ M NH_2OH. The first addition of ATP or DCMU was 135 s following light activation. It is apparent that DCMU can

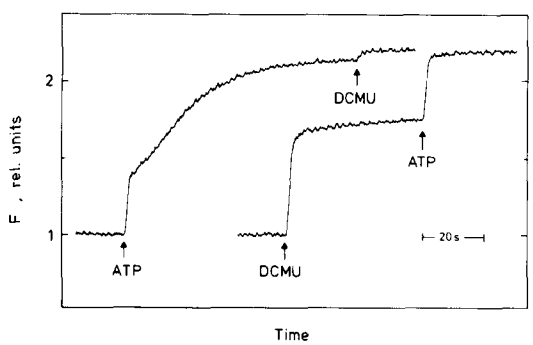

Fig. 1. Comparison of dark-fluorescence increases produced upon ATP hydrolysis and with DCMU-addition. Samples were preilluminated to activate the latent ATP-hydrolase and containing $5 \cdot 10^{-5}$ M diaminodurene and $5 \cdot 10^{-3}$ M NH_2OH. Where indicated, ATP (final concentration, $3 \cdot 10^{-4}$ M) or DCMU (final concentration, $3 \cdot 10^{-5}$ M) were injected into the rapidly stirred samples. Temperature, 10 °C. Chlorophyll concentration, 35 μg/ml. Intensity of the 550 nm measuring beam, approx. 10^{-4} W/m^2. One relative ordinate unit corresponds to the dark-fluorescence level before ATP-addition.

induce only that part of $(\Delta F)_{ATP}$ which corresponds to the slow phase. When ATP is added on top of DCMU, there is selective induction of the rapid phase. The fluorescence rise induced by DCMU of top of the ATP-induced change may be considered negligible.

In Fig. 2, $(\Delta F)_{DCMU}$ traces and the thereafter induced transients of $(\Delta F)_{ATP}$ are depicted for different concentrations of DCMU. Fig. 3 shows corresponding plots of the amplitudes of $(\Delta F)_{DCMU}$ as well as the rapid and slow phases of $(\Delta F)_{ATP}$. The slow $(\Delta F)_{ATP}$ is suppressed in parallel with the rise of $(\Delta F)_{DCMU}$, confirming the previously reported complementarity of these two signals [10]. Half-maximal effect is observed at about $2.5 \cdot 10^{-7}$ M DCMU. On the other hand, the rapid phase of $(\Delta F)_{ATP}$ is even slightly stimulated up to 10^{-4} M DCMU. However, at concentrations exceeding 10^{-4} M also the rapid phase becomes severely inhibited, with 50% suppression at about $3 \cdot 10^{-4}$ M DCMU. In the same concentration range there is also a small decrease in $(\Delta F)_{DCMU}$.

The data of Figs. 1–3 suggest that the rapid phase of $(\Delta F)_{ATP}$ is not related to the same type of reversed electron flow which is involved in $(\Delta F)_{ATP}$ (slow) and in $(\Delta F)_{DCMU}$ (see Introduction). This conclusion is further supported by the results of Fig. 4, which compares the dependency of $(\Delta F)_{DCMU}$ and $(\Delta F)_{ATP}$ (rapid) on the number of preilluminating flashes. Both samples were pre-

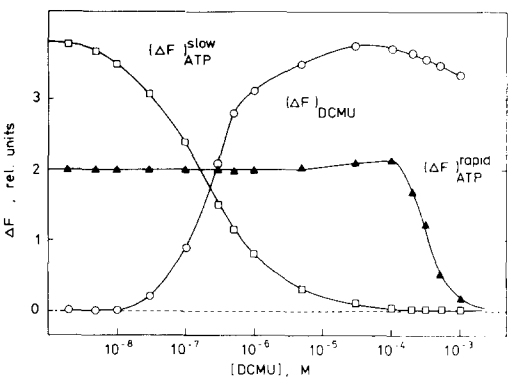

Fig. 3. Plots of the increases in dark-fluorescence induced by consecutive additions of DCMU and ATP. The data points are derived from kinetic traces, representative examples of which are displayed in Fig. 2.

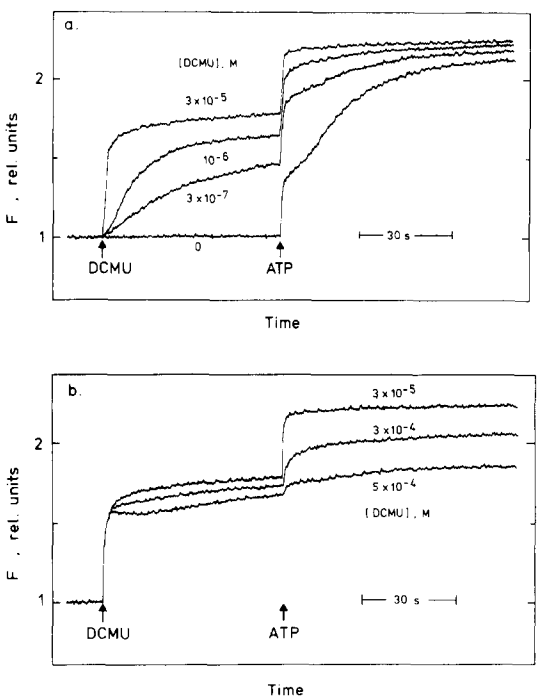

Fig. 2. Consecutive induction of $(\Delta F)_{DCMU}$ and $(\Delta F)_{ATP}$ at varying concentrations of the added DCMU. (a) Concentration range in which the slow phase of $(\Delta F)_{ATP}$ is selectively eliminated. (b) Suppression of rapid phase of $(\Delta F)_{ATP}$ at concentrations exceeding 10^{-4} M DCMU. DCMU addition was about 100 s following light activation. The amount of methanol added together with the DCMU did not exceed 0.3 vol.%.

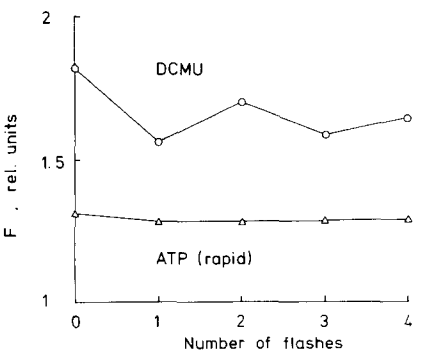

Fig. 4. Amplitudes of the DCMU- and ATP-induced fluorescence increases in dependence of the number of single turnover saturating light-flashes. Conditions as in Fig. 1, except for omission of diaminodurene from the reaction medium. Preilluminating flashes were given at 1 s intervals, with the last flash 15 s before DCMU- or ATP-injection.

illuminated in the same way, but only $(\Delta F)_{DCMU}$ shows the expected binary oscillations. The amplitudes are higher with zero or an even number of preilluminating flashes, opposite to the usual pattern in dark-adapted, preoxidized chloroplasts [1–3]. Hence, the ATPase activating preillumination results in a large population of centers with a bound plastosemiquinone anion. Obviously, there is no correspondence between this population and the rapid phase of $(\Delta F)_{ATP}$.

Elimination of the rapid $(\Delta F)_{ATP}$ upon closure of PS-II centers

In presence of DCMU and NH_2OH, reoxidation of PS-II acceptors is severely inhibited, and illumination results in an almost irreversible increase of fluorescence to a maximal level [22]. The characteristics of the flash-induced fluorescence rise in presence of DCMU and NH_2OH have led to the distinction of two different types of PS-II acceptors, Q_1 and Q_2, according to the nomenclature of Joliot and Joliot [23,24]. The quencher Q_1 can be removed by one single turnover saturating flash, while elimination of Q_2 requires several additional flashes [23,24]. Fig. 5 describes an experiment in which $(\Delta F)_{ATP}$ (rapid) as induced at different levels of fluorescence which was varied by flash preillumination in presence of DCMU and NH_2OH. As already shown in Fig. 4, $(\Delta F)_{ATP}$ (rapid) is completely unaffected by a preceding induction of $(\Delta F)_{DCMU}$. Also application of one saturating flash following DCMU-addition, which can be assumed to eliminate Q_1-quenching, does not suppress $(\Delta F)_{ATP}$ (rapid). However, with additional flashes between DCMU- and ATP-injection, there is gradual suppression of $(\Delta F)_{ATP}$ (rapid). The complete dependency of $(\Delta F)_{ATP}$ (rapid) on the state of quenching at PS-II reaction centers is depicted in Fig. 6. Interestingly, this dependency displays two phases. About 50% of F_v can be removed without any appreciable effect on $(\Delta F)_{ATP}$. Then, with the F_v-level reaching about 75% of the maximal value, $(\Delta F)_{ATP}$ is cut down by approx. a factor 2 in a first step. Complete suppression of $(\Delta F)_{ATP}$ (rapid) only occurs with removal of the last 5–10% of quenching. It should be noted, that the proportion of the two steps varied considerably with different chloroplast preparations and at different days, suggesting that this phenomenon involves a type of heterogeneity at the level of PS-II acceptors which is rather variable. Fig. 7 shows traces of the ATP-induced increase in P515 absorbance under conditions of maximal (absence of DCMU; no flash-preillumination) and minimal fluorescence quenching (presence of DCMU + NH_2OH, flash-preillumination). There is no difference in the ATP-induced $\Delta A_{525} - \Delta A_{535}$ with PS-II centers being either closed or all open. Hence, complete closure

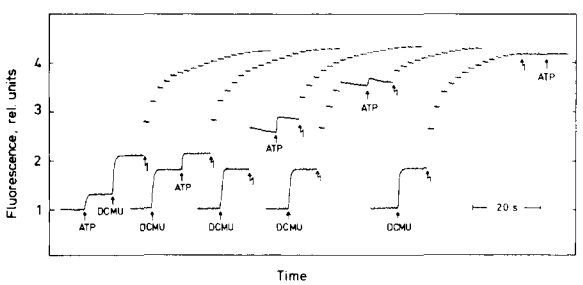

Fig. 5. Induction of $(\Delta F)_{ATP}$ at different fluorescence levels caused by controlled closure of PS-II reaction centers. Centers are blocked by application of single turnover saturating flashes in presence of NH_2OH and DCMU. Flash frequency, about 0.5 Hz. In all experiments ATP was added 135 s after termination of light-activation (final concentration, $3 \cdot 10^{-4}$ M). NH_2OH (final concentration, $5 \cdot 10^{-3}$ M) was added 60 s following activation. DCMU-concentration, $3 \cdot 10^{-5}$ M. For reasons of comparison with corresponding P 515 changes (see Fig. 7) these experiments were carried out with dual-wavelength excitation 525 nm/535 nm (2500 Hz alternating) at an integrated light intensity of about $3 \cdot 10^{-4}$ W/m^2.

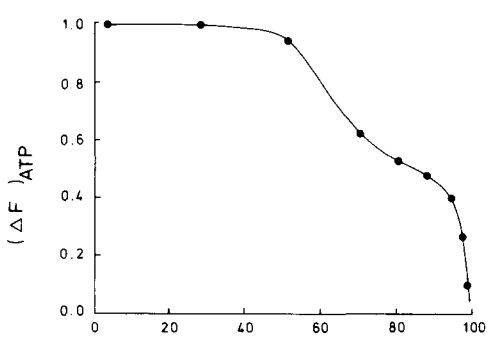

Fig. 6. Suppression of the rapid phase of $(\Delta F)_{ATP}$ with elimination of variable fluorescence upon closure of PS II reaction centers. The data points are derived from kinetic traces of the type presented in Fig. 5.

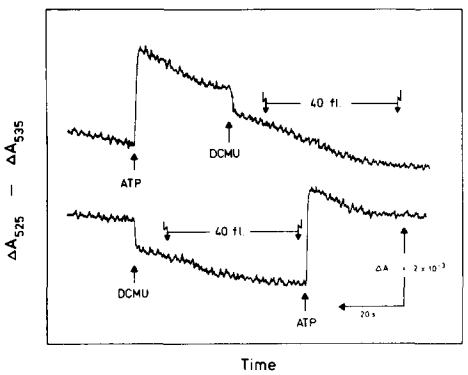

Fig. 7, ATP-induced increase in P 515 absorbance under conditions of minimal and of maximal closure of PS II reaction centers. P 515 absorbance is measured dual-wavelength at 525 versus 535 nm to minimize non-specific absorbance changes and stirring noise. The experiments were carried out in the same cuvette and under the same conditions as those of Figs. 5 and 6.

of non-B type (or Q_2-type) PS-II centers does not affect the ATP-hydrolase and the ATP-induced build-up of a membrane potential. Furthermore, these data exclude the possibility that $(\Delta F)_{ATP}$ (rapid) involves a type of fluorescence quenching (e.g., affected by a membrane potential or other forms of membrane energization) which is not related to the closure of PS-II acceptors.

Discussion

The presented comparative study of the ATP-induced and DCMU-induced dark-increase of chlorophyll fluorescence gives strong support to the conclusions of the preceding paper [9], by suggesting the involvement of two different types of fluorescence quenchers in the rapid and slow phases of the ATP-induced fluorescence increase. As demonstrated in Figs. 1–3, only the slow phase is complementary with a preceding $(\Delta F)_{DCMU}$. The rapid phase lacks the decisive property of the B-type acceptor complex (displayed upon DCMU-injection), namely the dependence of reverse electron flow on the redox state of B (see Fig. 4). This finding gives rise to the suggestion that only the slow phase of the ATP-induced fluorescence increase reflects reverse electron flow in 'normal', i.e., B-type electron-transport chains. According to Van Gorkom and Thielen [25], β-centers [14–18] are not connected to the plastoquinone pool via a secondary acceptor Q_B. Lavergne [12,13] distinguishes B-type and non-B-type PS-II centers on the basis of their capacity to show dark-induction of $(\Delta F)_{DCMU}$. Hence, it appears that the rapid component of $(\Delta F)_{ATP}$ is involving β-centers and/or non-B-type centers. Possibly, β-centers represent a subpopulation of non-B-type centers. The inhibition of the rapid component of $(\Delta F)_{ATP}$ at very high DCMU-concentration (see Figs. 2 and 3) also argues in favor of β-centers being involved. Brearley and Horton [26] recently suggested the I_{50} for β-center inhibition by DCMU to be $3.65 \cdot 10^{-4}$ M. On the other hand, the data of Figs. 5–7, are interpreted most readily in terms of Joliot's PS-II acceptors Q_1 and Q_2 [23,24]. Reduction of Q_1 by a saturating flash in the presence of DCMU and NH_2OH does not eliminate the rapid phase of $(\Delta F)_{ATP}$. Hence, following Joliot's definition, this phase should involve removal of Q_2-quenching. However, a possible reverse electron flow from Cyt *b* (563) to Q_2 is questioned again by the finding [27] that half-maximal inhibition of electron-flow between Q_2 and Cyt *b* (563) is at $3 \cdot 10^{-5}$ M DCMU. More insight into the precise nature of PS-II acceptors involved in the rapid phase of $(\Delta F)_{ATP}$ may be expected from redox titration experiments in presence of lipophilic mediators. The information available so far [9] suggests the involvement of high-potential electron carriers in a highly hydrophobic surrounding (see Fig. 9 of preceding paper [9]. In this context it may be important to note that the redox potential of Q_β is +120 mV [28] and, hence, considerable higher than that of the high and low potential forms of Q, 0 mV (Q_H) and −250 mV (Q_L), respectively [28,29]. Furthermore, following preillumination in presence of DCMU and NH_2OH, Q_β is reoxidised by ferricyanide much less readily than Q_α (Melis, A. and Schreiber, U., unpublished data). The latter observation suggests, that the vicinity of Q_β is more hydrophobic than that of Q_α.

With the present state of information the following working hypothesis may be proposed which links the observation of two distinctly different types of reverse coupling reactions with structural and functional heterogeneities at the level of PS-II centers. This working hypothesis, which in princi-

ple has been envisaged several years ago [30], is illustrated in the scheme of Fig. 8. The model suggests that the coupling mechanism between the ATPase and electron transport is heterogeneous, depending on whether protolytic electron-transfer steps take place in oppressed membrane regions or in stroma-exposed regions of the thylakoids. Contrary to the main part of PS-II centers which are located in the partition region of the grana, a population of about 20% of PS II (presumably identical to β-centers) is located at the margins [18,31] in direct vicinity of the coupling factors [32,33]. It appears feasible that such a close contact between coupling factors and certain electron-transport chains may allow a more direct mechanism of coupling, not requiring energy transduction via a bulk-phase proton motive force. The properties of the rapid phase of $(\Delta F)_{ATP}$ appear to be in agreement as well with the involvement of β-centers as with particularly close interaction between these centers and the ATPase. There are reports of dynamic changes in the relative ratio of α/β centers [34,35] with the amount of β-centers increasing upon illumination. Therefore, in strong light the type of coupling reflected by $(\Delta F)_{ATP}$ (in virtual darkness) may further increase in importance and account for observations of high-rates of ATP-synthesis at lowered levels of bulk protonmotive force [36–38]. One way of testing the validity of this hypothesis is the use of comparative measurements of reverse coupling reactions and flash-induced ATP synthesis (in presence of valinomycin) in chloroplasts with varying grana content. Such investigations are in progress.

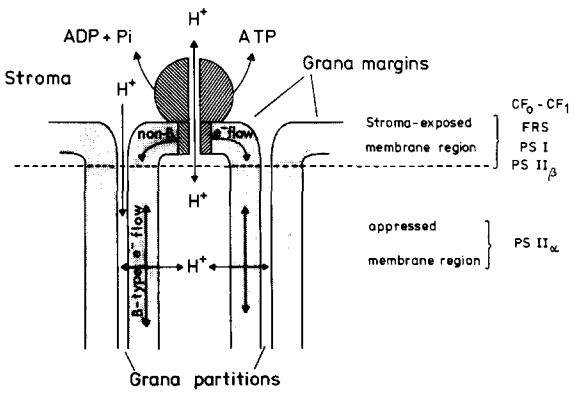

Fig. 8. Model for heterogeneous interactions of the reversible ATPase with B type and non-B-type electron flow. It is proposed that 'direct coupling' is possible between CF_0–CF_1 and those electron-transport chains which are located in close vicinity of the ATPase in the stroma-exposed margin region of the grana stacks. The properties of the rapid phase of reverse coupling reactions suggest the involvement of non-B-type electron-transport chains or PS II_β units in this proposed 'direct coupling'. On the other hand, the properties of the slow phase of reverse coupling are indicative of the involvement of B-type electron transport chains or PS II_α units, and a decisive role of a transmembrane proton gradient for transduction of the proton motive force from the ATPase into the oppressed membrane region and vice versa.

Acknowledgements

Thanks are due to Annette Weber and Ulrich Schliwa for excellent technical assistance. Keith Rienits, Secundino del Valle-Tascon, Dick Dilley and Ulrich Heber are thanked for stimulating discussions. Support by the Deutsche Forschungsgemeinschaft is gratefully acknowledged.

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