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A RAPID VECTORIAL BACK REACTION AT THE REACTION CENTERS OF PHOTOSYSTEM II IN TRIS-WASHED CHLOROPLASTS INDUCED BY REPETITIVE FLASH EXCITATION

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Summary

In Tris-washed chloroplasts, completely lacking the oxygen-evolving capacity, absorption changes in the range of 420—560 nm induced by repetitive flash excitation have been measured in the presence and absence of electron donors. It was found:

(1) At 520 nm flash-induced absorption changes are observed, which predominantly decay via a 100—200-μs exponential kinetics corresponding to that of the back reaction between the primary electron donor and acceptor of Photosystem II (Haveman, J. and Mathis, P. (1976) Biochim. Biophys. Acta 440, 346—355; Renger, G. and Wolff, Ch. (1976) Biochim. Biophys. Acta 423, 610—614). In the presence of hydroquinone/ascorbate as donor couple the amplitude is nearly doubled and the decay becomes significantly slowed down.

(2) The difference spectrum of the absorption changes obtained in the presence of hydroquinone/ascorbate, which are sensitive to ionophores, is nearly identical with that of normal chloroplasts in the range of 460—560 nm (Emrich, H.M., Junge, W. and Witt, H.T. (1969) Z. Naturforsch. 24b, 1144—1146). In the absence of hydroquinone/ascorbate the difference spectrum of the absorption changes, characterized by a 100—200-μs decay kinetics, differs in the range of 460—500 nm and by a hump in the range of 530—560 nm. The hump is shown to be attributable to the socalled C550 absorption change, which reflects the turnover of the primary acceptor of Photosystem II (van Gorkom, H.J. (1976) Thesis, Leiden), while the deviations in the range of 460—500 nm are understandable as to be due to the overlapping absorption changes of

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Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; BPh, bacteriopheophytin.

chlorophyll a_{II}^{+}. The problems arising with the latter explanation are discussed.

(3) The electron transfer due to the rapid turnover at Photosystem II, which can be induced by flash groups with a short dark time between the flashes, is not able to energize the ATPase and to drive photophosphorylation.

On the basis of the present results it is inferred, that in Tris-washed chloroplasts under repetitive flash excitation a rapid transmembrane vectorial electron shuttle takes place between the primary acceptor (X320) and donor (Chl a_{II}) of Photosystem II, which is not able to energize the photophosphorylation. Furthermore, the data are shown to confirm the localization of X320 and Chl a_{II} within the thylakoid membrane at the outer and inner side, respectively.

Introduction

The primary photochemical electron transfers at the reaction centers of System I and II were found to generate an electric potential gradient across the thylakoid membrane (for reviews see Ref. 1—3). Measurements with macroscopic electrodes indicated, that gradients parallel to the membrane plane are very rapidly equilibrated [4]. Subsequent dark electron transfer reactions of the 'primary charges' coupled with protonations and deprotonations lead to the transformation of the original electric gradient into an electrochemical proton gradient [1—3]. Normally, the decay of the transmembrane potential gradient occurs competitively via the specific proton channel of the ATP synthetase, giving rise to ATP formation, and by nonspecific ion movements. However, under certain circumstances another pathway for the decay of a transmembrane electric potential gradient can arise: the decay via an electrogenic vectorial dark electron transfer within the membrane, which is of opposite direction to that of the primary charge transfer processes at the reaction centers, provided that this reaction is fast in comparison with the ionic movements across the ATP synthetase and the nonspecific iontransport channels, respectively. In normal chloroplasts the electric field decay via electrogenic dark reactions does not seem to play a significant role.

In mistreated chloroplasts however, where the linear electron transport is interrupted without blockage of the function of the reaction centers, an electrogenic intramembrane decay is anticipated to become the main route for the dissipation of electric potential gradients, if a vectorial charge recombination is established, which is much faster than the passive ion fluxes through the thylakoid membrane.

The linear electron transport of System II can be blocked either by 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU)-type inhibitors interrupting the electron flux from the reduced primary electron acceptor, $X320^{-}$, into the plastoquinone pool [5,6], or by destruction of the water-splitting enzyme system Y by treatments, such as Tris washing [7] or incubation with NH_2OH [8] and chaotropic agents [9]. In both cases the photochemistry of the reaction centers remains unimpaired and cyclic electron pathways arise. The cycling around System II in DCMU-type blocked chloroplasts is much slower than the ionic flux through the membrane [10,11] and therefore the decay of the light-

induced electric potential was found to be determined by the membrane permeability [11]. On the other hand, in Tris-washed chloroplasts a fast charge recombination between the reduced primary electron acceptor, $X320^-$ and the photooxidized chlorophyll *a* complex (Chl a_{II}^+) was shown to take place under repetitive flash excitation conditions, characterized by a half-life of the order of 100—200 μs at room temperature [12—14]. As this electron transfer involves $X320^-$, which is known to be located at the outer side of the thylakoid membrane [15,16], and Chl a_{II}^+, which is inferred to be intercalated near to the inner surface of the thylakoid membrane [17,18], the above-mentioned charge recombination is a vectorial electron transport opposite to that of the light-induced primary charge separation. Accordingly, under repetitive flash excitation, in Tris-washed chloroplasts a transmembrane electric potential gradient should be generated within 20 ns [19] and subsequently collapse in the dark with a half-life of 100—200 μs, coinciding in its kinetics with the electrogenic charge recombination between $X320^-$ and Chl a_{II}^+. This reaction is anticipated to be purely dissipative with respect to phosphorylation, because the field strength is too weak and the decay is too fast to allow an energization of the ATP synthetase [20]. On the other hand, in the presence of powerful electron donors the charge recombination is expected to be substituted by a linear electron flow leading to an electrochemical potential gradient sufficient for phosphorylation, provided that the thylakoid membrane is not seriously damaged by Tris treatment.

The present investigation confirms the formation and the fast dissipative collapse of an electric potential gradient via the electrogenic charge recombination at System II in Tris-washed chloroplasts. Furthermore, it is shown, that this reaction occurs across the thylakoid membrane. Accordingly, the donors participating in the above-mentioned reactions are inferred to be located near the inner side of the membrane.

Materials and Methods

Class II chloroplasts were prepared from market spinach according to the method of Winget et al. [21], except that 10 mM ascorbate was present in the grinding medium. Tris-washed chloroplasts were obtained from isolated chloroplasts by incubation with 0.8 M Tris-HCl, pH 8.0, by the procedure of Yamashita and Butler [22], as described in Ref. 23.

The standard reaction mixture contained: chloroplasts (5 μM chlorophyll), 10 mM KCl, 2 mM $MgCl_2$, 20 mM buffer (in the range $4.5 \leqslant pH \leqslant 7.0$ morpholinoethanesulfonate/NaOH, in $7.0 \leqslant pH \leqslant 8.5$ *N*-Tris(hydroxymethyl)-methylglycine(Tricine)/NaOH) and 100 μM benzylviologen or 670 μM $K_3Fe(CN)_6$ as electron acceptor. Other additions as indicated in the legends.

The absorption changes were recorded by a repetitive flash photometer similar to that described in Ref. 24. 32—1024 signals were averaged in a Fabri-Tek, model 1062. Excitation with 20-μs flashes, which passed through a cut-off filter Schott RG 610; optical pathlength as indicated in the legends, optical bandwidth, 5 nm.

The spectra of Figs. 4 and 5 as well as the absorption changes at 334 nm are not corrected for the flattening effect, at 520 nm this effect is negligibly small [25].

Results

The decay kinetics of the absorption change at 334 nm, which are inferred to reflect mainly the reoxidation of the reduced 'primary' electron acceptor of System II, was shown to be characterized by a 100—200 μs half-life in Tris-washed chloroplasts [12]. This reaction probably indicates the direct back reaction between photooxidized Chl a_{II}^{+} and $X320^{-}$ [12,25]. Beyond the μsecond kinetics there exists a much slower component in the range of a few 100 ms, which was assumed to be due to the cyclic reaction between the secondary electron acceptor R and the donor side of System II [25,26]. The relative contribution of both kinetics to the overall reaction is strongly dependent on the repetition frequency of the exciting flashies [25,26]. The results depicted in Fig. 1 (left side) confirm these data. Furthermore, at a constant frequency the relative contribution is dependent on the pH of the chloroplast suspension (Fig. 1, right side). This might reflect the participation of a System II component with a p*K* value around 6.5, which protonated form is responsible for the slow cyclic electron flow. At the present stage of knowledge no unambigious conclusion can be drawn about the nature of this substance and its localization within System II. For the investigation of the electric field decay by a fast intramembrane electrogenic reaction only the rapid 100—200-μs kinetics is of interest, because the slower cyclic reaction cannot effectively compete with the transmembrane ion transport. The absorption change at 520 nm arising in Tris-washed chloroplasts at a repetition rate of 40 Hz is shown in Fig. 2 (top). The absolute initial amplitude is about 40—50% of that observed in normal chloroplasts. A semilogarithmic plot at the bottom of Fig. 2 indicates, that the decay occurs predominantly (approx. 90% of the total amplitude) via a kinetics characterized by a half-life of 135 μs. There appear slight variations for different preparations but the half-life times of the fast kinetics are generally in the range of 100—200 μs. These values correspond with that observed for the recovery kinetics of X320 [12,25] and Chl a_{II} [13,27,28], respectively. Accordingly, the observed 100—200 μs decay at 520 nm is

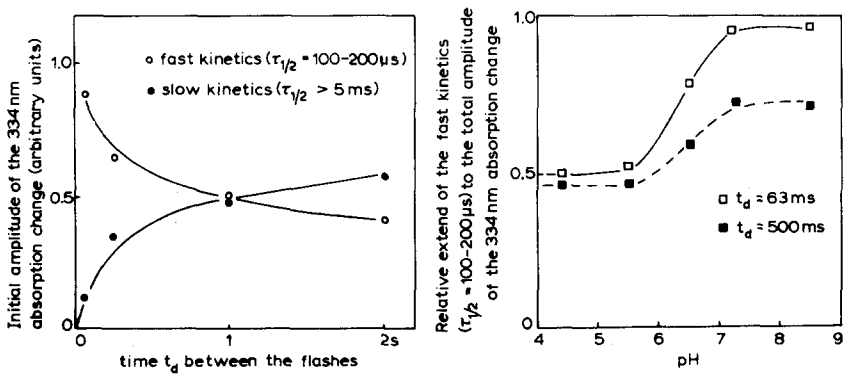

Fig. 1. Initial amplitude of the absorption changes at 334 nm as a function of time t_d between the flashes (left side) and of the pH of the suspension (right side) in Tris-washed chloroplasts. Chlorophyll concentration: 100 μM, 1 mM $K_3Fe(CN)_6$. Optical pathlength: 1.4 mm. Other conditions as described in Materials and Methods.

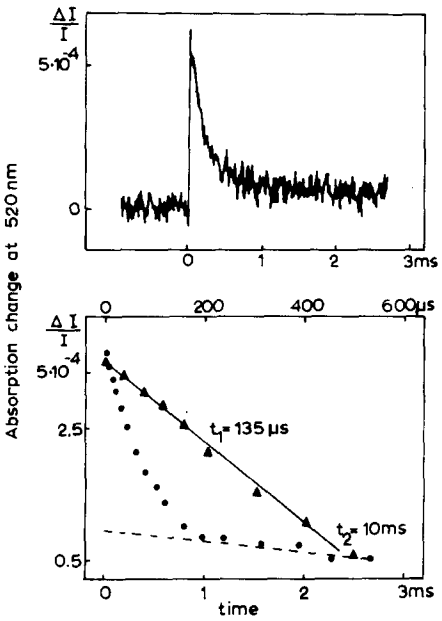

Fig. 2. Absorption change at 520 nm as a function of time. Chlorophyll concentration: 5 µM, 670 µM $K_3Fe(CN)_6$, 20 mM Tricine/NaOH, pH 7.0; optical pathlength: 20 mm. Other conditions as described in Materials and Methods. Bottom: semilogarithmic plot of the signal on the top. The 135-µs kinetics is obtained after subtration of the small contribution of the slow kinetics (greater than 10 ms) from the total signal.

assumed to reflect the electrogenic back reaction between Chl a_{II}^+ and $X320^-$. If this interpretation is correct, then the addition of an efficient exogeneous System II electron donor should cause two effects: (a) The initial amplitude of the 520 nm absorption change is expected to become nearly doubled due to the additional participation of System I, and (b) the decay kinetics of the 520

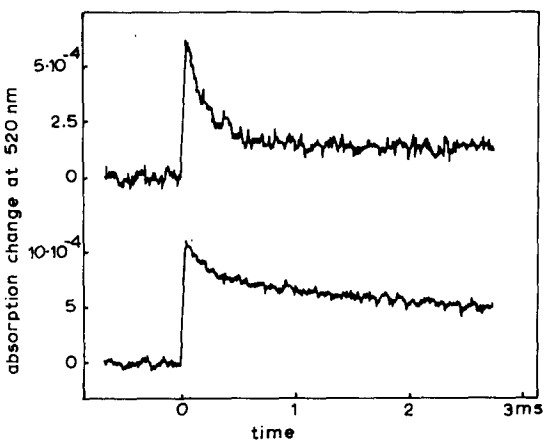

Fig. 3. Absorption change at 520 nm as a function of time. Chlorophyll concentration: 5 µM, 100 µM benzylviologen, 20 mM Tricine/NaOH, pH 7.0; optical pathlength: 20 mm. Upper curve: without donor; lower curve: 67 µM hydroquinone, 3.3 mM ascorbate. Other conditions as described in Materials and Methods.

nm absorption is anticipated to be slowed down, because the fast electrogenic back reaction should be 'switched off' by the reduction of the donor side of System II. Accordingly, the electric field decay should occur via the passive ion flux across the thylakoid membrane. As is shown in Fig. 3 both effects were found to arise in the presence of hydroquinone and ascorbate, which are known to act as efficient System II electron donor couple [22]. There appears a remaining small part of the 100—200 μs decay kinetics which is probably due to inefficiencies of the donor couple. In general the results are consistent with the above-mentioned ideas. Furthermore, the redox couple hydroquinone/ascorbate is confirmed to act as System II electron donor because the 520 nm absorption change observed in Tris-washed chloroplast in the presence of hydroquinone/ascorbate is strongly suppressed by 1.3 μM DCMU (data not shown). The electrochromic nature of these absorption changes is shown by the sensitivity to 0.1 μM valinomycin (not shown). If the 100—200-μs kinetics is really caused by the electrogenic back reaction between $X320^-$ and Chl a_{II}^+, then the difference spectrum of this reaction should contain only the difference spectra of the electrochromic effect and of the redox reactions $X320^-$/X320 and Chl a_{II}^+/Chl a_{II}, respectively. On the other hand, in the presence of hydroquinone/ascorbate the predominant decay kinetics of the electric field is expected to be much slower than the recovery of Chl a_{II} and X320, which were found to occur in the μsecond range [12,28]. Accordingly, the slower (greater than 1 ms) components of the absorption changes in Tris-washed chloroplasts in

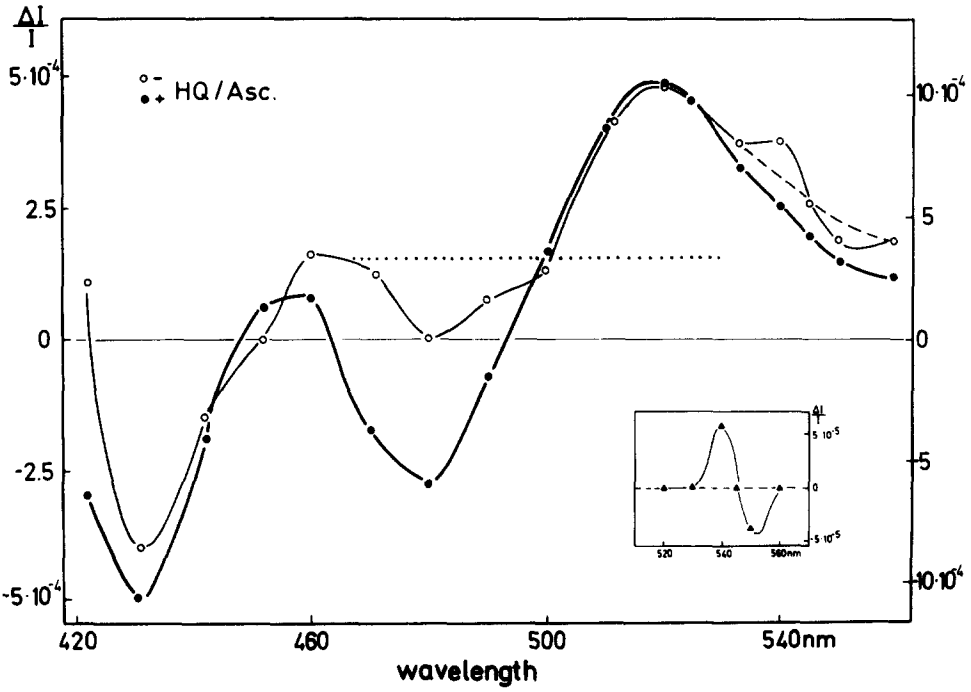

Fig. 4. Flash-induced absorption changes as a function of wavelength. Chlorophyll concentration: 5 μM, 100 μM benzylviologen, 20 mM Tricine/NaOH, pH 7.0; optical pathlength: 20 mm. ○, 100—200 μs decay component in the absence of donors (see Fig. 2). ●, addition of 67 μM hydroquinone (HQ), 3.3 mM ascorbate (asc). Insert: absorption changes in the absence of donors in the range of 530—560 nm, separated on the dashed baseline (for details see text).

the presence of hydroquinone/ascorbate reflect mainly the difference spectrum of the electrochromic effect. The data of Fig. 4 in the range of 450—550 nm (closed circles) closely resembles those reported for the electrochromic difference spectrum in normal chloroplasts [29]. Therefore, these results indicate that Tris washing does not perturb the nature of the field-indicating pigments and/or pigment complexes. Accordingly, if one admits that the electric field generated at the reaction centers of System II is delocalized over the area of a whole electron transport chain within 0.1 μs [4], then the wavelength dependency of the electrochromic contribution to the overall difference spectrum of the fast electrogenic back reaction occurring in the absence of electron donors should be identical to that observed in the presence of hydroquinone/ascorbate. The open circles in Fig. 4 show the difference spectrum of the 100-200-μs kinetics. For the sake of a simple comparison a suitable scale was used (see different units on the ordinates of Fig. 4). The results obtained show: (a) In the range of 500—530 nm the difference spectrum of the 100—200-μs kinetics in Tris-washed chloroplasts is practically identical to that of the 'normal' electrochromic effect; (b) a remarkable deviation is observed in the range of 450—500 nm, and (c) a characteristic hump appears in the range of 530—560 nm. The deviation in the range of 530—560 nm is understandable as to be caused by the C550 absorption change reflecting the turnover (probably via a local electrochromic pheophytin bandshift) of the primary System II acceptor [14]. As in the presence of hydroquinone/ascorbate in Tris-washed chloroplasts $X320^-$ becomes reoxidized with nearly the same kinetics as in normal chloroplasts [12], the C550 absorption changes are expected to have a half-life of the order of 500 μs. Accordingly, in the range of 530—560 nm the difference between the normalized initial amplitudes of the absorption changes obtained in Tris-washed chloroplasts in the absence of electron donors and the normalized amplitudes of the slower kinetics (greater than 1 ms) measured in Tris-washed chloroplasts in the presence of hydroquinone/ascorbate should result in the difference spectrum of C550. If one furthermore admits, that there exists a small deviation in the reference line, as is indicated by the dashed line in Fig. 4, then the difference spectrum given in the insert is obtained. This spectrum closely corresponds with that reported by van Gorkom [14] for the C550 absorption changes. In principle, the C550 contribution to the difference spectrum should also be observed in normal chloroplasts. However, as the decay of the electrochromic absorption changes is comparatively slow, they are often measured only with a msecond time resolution, so that the absorption changes of C550 escape the detection, because of the rapid decay. Furthermore, the relative contribution of the rather small C550 absorption changes to the overall difference spectrum becomes approximately halfed due to the contribution of System I to the electrochromic effect, which does not involve C550. An additional complication in normal chloroplasts arises by the overlapping with the absorption changes caused by cytochrome *f*, whose oxidation kinetics might interfere [30,31] with the decay of C550 determined by the reoxidation of reduced $X320^-$.

The deviation in the range of 450—500 nm, which quantitatively varies for different preparations and excitation conditions, is not well understood. In the simplest way it could be explained by overlapping positive absorption changes

due to Chl a_{II}^{+} and X320^{-} which should counterbalance the negative electrochromic absorption changes in this wavelength region. In contrast to the situation in the presence of hydroquinone/ascorbate, in the absence of electron donors the contributions due to the turnover of Chl a_{II} and X320, respectively, cannot be separated kinetically from the electrochromic absorption changes.

The contribution due to the X320 turnover is negligibly in the above-mentioned wavelength region [14]. On the other hand, in the same range the difference spectrum of Chl a_{II}^{+} versus Chl a_{II} is characterized by small positive absorption changes [32]. According to in vitro measurements of Borg et al. [33] the difference spectrum for the formation of π-cation radical of Chl *a* in CH_2Cl_2 is positive and nearly constant in the wavelength region of 460—530 nm with an approximate difference extinction coefficient $\Delta\epsilon$(Chl a^{+}/Chl *a*) of about 3000—4000 $M^{-1} \cdot cm^{-1}$. If one takes this value as to be valid also for Chl a_{II} in chloroplasts and additionally to be constant in a rough approximation, then the $\Delta I/I$ contribution in the above-mentioned wavelength range is estimated to be about $(1.4—1.8) \cdot 10^{-4}$ on the basis of 500 chlorophylls/Chl a_{II}. This would lead to a nearly constant shift of the electrochromic difference spectrum in the above-mentioned range. Accordingly the real baseline from 460 to 530 nm of the electrochromic effect in Tris-washed chloroplasts in the absence of electron donors should roughly be given by the dotted line in Fig. 4. By the application of this crude procedure the 'corrected' electrochromic difference spectrum due to the fast electrogenic back reaction in Tris-washed chloroplasts in the absence of electron donors (open circles) fairly corresponds with the electrochromic difference spectrum obtained in the presence of hydroquinone/ascorbate (closed circles) as is shown in Fig. 5 for the range of 450—530 nm. Accordingly, the differences of the normalized spectra in the absence and presence of hydroquinone/ascorbate, respectively, observed in the range of 450—500 nm can be explained as to be caused predominantly

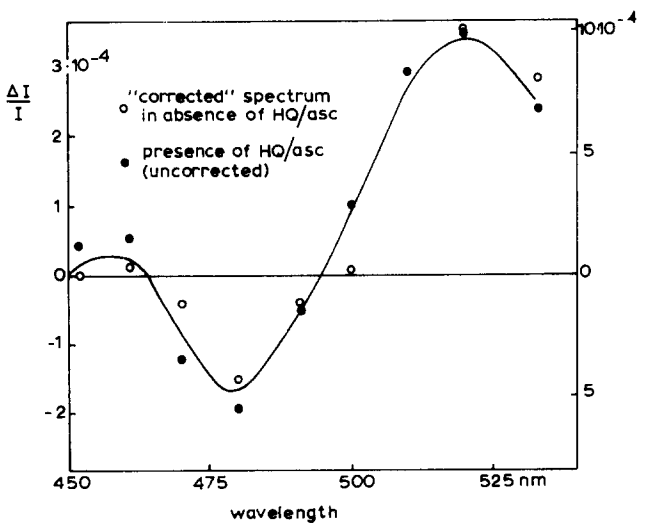

Fig. 5. Flash-induced absorption changes as a function of wavelength. Experimental conditions as in Fig. 4. ○, 100—200 μs component in the absence of donors, corrected for the contribution due to Chl a_{II}^{+} (for details see text). ●, same values as in Fig. 4.

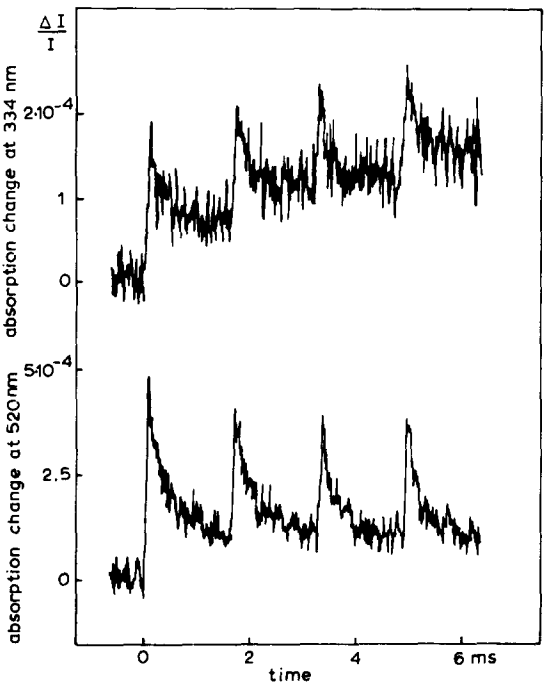

Fig. 6. Absorption changes at 334 nm and 520 nm as a function of time in Tris-washed chloroplasts in the absence of electron donors. Chlorophyll concentration: 90 μM, 1 mM $K_3Fe(CN)_6$, 20 mM Tricine/NaOH, pH 7.0; optical pathlength: 1.4 mm. Excitation: repetitive flash groups, repetition rate 2 Hz, time between the flashes of a group 1.6 ms. Other conditions as described in Materials and Methods.

by the contribution due to the turnover of Chl a_{II} (see also Discussion).

The negative absorption changes in Tris-washed chloroplasts peaking around 430 nm reflect the turnover of Chl a_{II} in the absence and the turnover of Chl a_I and Chl a_{II} in the presence of external electron donors, respectively (the small positive absorption changes due to the X320 turnovers, see Ref. 14, can be neglected).

If the 100—200-μs kinetics of the absorption changes observed in Tris-washed chloroplasts under repetitive flash excitation conditions reflect the electrogenic back reaction between $X320^-$ and Chl a_{II}^+, the fast turnover should take place also at a very high repetition rate. The absorption changes at 334 nm and 520 nm obtained by excitation with repetitive flash groups at a time of 1.6 ms between the flashes of a group are depicted in Fig. 6. The data indicate that the rapid back reaction really occurs at a high flash frequency. This result is in contrast with recent findings of Pulles [25]. The results are very reproducible and are obtained for different preparations and experimental conditions. Therefore, there is no explanation for the failing of Pulles [25] to observe the absorption changes at 3 ms dark time between the flashes of a group.

The present results indicate, that under high rate repetitive excitation the light-induced electric field in Tris-washed chloroplasts in the absence of exogenous electron donors is predominantly dissipated via the intramembrane

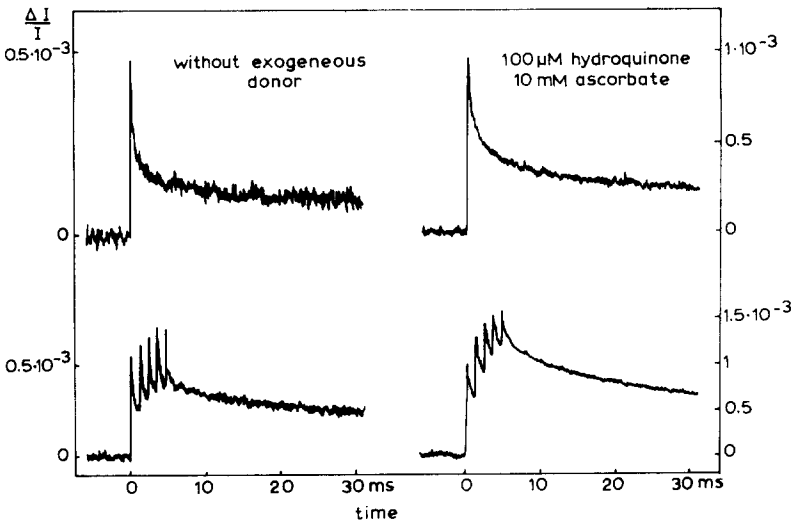

Fig. 7. Absorption changes at 520 nm as a function of time in Tris-washed chloroplasts in the presence of ADP and phosphate and in the absence and presence of hydroquinone/ascorbate as donor couple. Chlorophyll concentration: 100 µM, 100 µM benzylviologen, 10 mM KCl, 2 mM $MgCl_2$, 5 mM K_2HPO_4, 30 mM ADP, 20 mM Tricine/NaOH, pH 8.0. Excitation: repetitive flashes (2 Hz) or flash groups (2 Hz), each containing five flashes at a dark time of 1.6 ms. Other conditions as described in Materials and Methods.

electrogenic back reactions (between $X320^-$ and Chl a_{II}) and that the total extent of the electric potential gradient cannot be enhanced by excitation with repetitive flash groups. Furthermore, as the back reaction is not coupled with a proton transport (Renger, G., unpublished results) and the electrogenic field decay is very rapid, Tris-washed chloroplasts in the absence of an electron donor are expected to be inactive for photophosphorylation. On the contrary, in the presence of the donor couple hydroquinone/ascorbate the electric field decay is slowed down, due to 'switching off' the back reaction and in addition a proton gradient should be established across the thylakoid membrane (at least by excitation with flash groups) provided that the membrane permeability is not drastically enhanced by the Tris-washing procedure. The results depicted in Fig. 7 show that in the presence of hydroquinone/ascorbate an electric field is generated, which extent is approximately the same as in normal chloroplasts. Furthermore, a large part of the light-induced field decays slow enough to allow a photophosphorylation. Preliminary experiments (data not shown) indicate that in the presence of hydroquinone/ascorbate Tris-washed chloroplasts synthesize ATP under flash group excitation. This is in correspondence with former results obtained under continuous illumination (for review see Ref. 34).

Discussion

The difference spectrum obtained in Tris-washed chloroplasts under repetitive flash excitation conditions in the absence of electron donors can consistently be explained as to be caused solely by the redox reactions of Chl a_{II} and X320 (C550) and a concomitant electrochromic effect. There is no need to

assume the participation of another component in the reaction sequence characterized by a rapid flash-induced rise time and a subsequent dark recovery with a half-life of 100—200 μs. Of course, it could be possible, that there exists an intermediate electron carrier, which supports the photochemical electron transfer from Chl a_{II} to X320 in a similar way as bacteriopheophytin (BPh) within the reaction centers of bacteria [35]. This bacteriopheophytin reaction was found to be accomplished in less than 1 ns, while the back reaction between BPh^- and P^+ takes place in about 15 ns [36] provided that the dark electron transfer from BPh^- to the ubiquinone-Fe complex is functionally blocked.

An analogous reaction in Photosystem II would escape the detection due to the limited time resolution of the equipment used in this study and of the even much higher time resolution achieved for the investigation of the rise time of the 520 nm absorption change in normal chloroplasts [19]. Therefore, the present data cannot provide information about the mechanistic details of the realization of the electron transfer from the excited state of Chl a_{II} (Chl* a_{II}) to the quinoid acceptor component X320. For an unambigious decision about the participation of intermediary electron transfer steps in the reaction X320 · Chl* $a_{II} \rightarrow X320^- \cdot$ Chl a_{II}^+ more refined kinetic and spectroscopic studies are required. Very recent experiments show, that Chl a_{II} can react with another acceptor component under condition of X320 being functionally blocked in its reduced state [37]. These results are shown to be explainable within the framework of a model [38] assuming a redox carrier X_a to act as intermediate between Chl a_{II} and X320 in an analogous way as I(BPh) functions within the reaction centers of bacteria. However, the possible existence of a component X_a with very fast turnover kinetics does not influence the main conclusions drawn from the present results. Accordingly, as the simplest model to explain the data it is inferred, that the light-induced electron transfer from Chl a_{II} to X320 leads to the formation of a transmembrane electric field, which predominantly decays via the eletrogenic back reaction between Chl a_{II}^+ and $X320^-$. If the 100—200-μs kinetics can exclusively be ascribed to the back reaction, then the amplitude of the electrochromic absorption changes caused by the electrogenic field decay should provide further information about the structural arrangement of Chl a_{II} and X320 in the thylakoid membrane. Despite of the limitations of accuracy to account for the contribution of the Chl a_{II} turnover, the 'corrected' difference spectrum of Fig. 5 indicates that the amplitude of the electrochromic absorption changes caused by the electrogenic back reactions amounts approx. 35—40%. As in the presence of hydroquinone/ascorbate both photoreactions contributes with nearly the same degree to the total electric field (and hence to the amplitude of the electrochromic effect), the present results lead to the conclusion that the electric potential gradient due to the rapid turnover of Photosystem II, which depends on the electrogenic back reaction between Chl a_{II}^+ and $X320^-$, is nearly the same as in normal chloroplasts. This 'corrected' amplitude of the 100—200-μs decay kinetics at 520 nm in Tris-washed chloroplasts is 70—80% of that observed for the 520 nm electrochromic absorption changes due to Photosystem II in DCMU-blocked chloroplasts [11]. However, this comparison is meaningful only if the field generated by the primary charge separation is delocalized rapidly in comparison

to the kinetics of the electrogenic back reaction. Investigations of Zickler and Renger (Zickler, A. and Renger, G., unpublished) of the electric potential difference flash induced at macroscopic electrodes [4] and its relaxation kinetics indicate that in Tris-washed chloroplasts charge delocalization at the membrane surfaces takes place as fast as in normal chloroplasts. Accordingly, as the membrane capacity was found to be practically invariant to Tris treatment (this is inferred to be valid on the basis of the experiments in the presence of hydroquinone/ascorbate and their comparison with normal chloroplasts), it is concluded that the electron transfer between the components Chl a_{II} and X320 (either photoinduced forward direction or electrogenic back reaction between Chl a_{II}^{+} and $X320^{-}$) involves nearly the total span of the impermeable core of the thylakoid membrane. That means: Chl a_{II} is located near to the inner side of the thylakoid membrane in accordance with former conclusions [17,18]. In a very recent study of Conjeaud et al. [39], which came to my knowledge after the completion of the present investigations, quite similar conclusions have been drawn. The interpretation of the difference spectrum of Fig. 4 for Tris-washed chloroplasts as to contain a non-negligible contribution of the Chl a_{II} reaction imposes an important question about the rise kinetics of the field-indicating absorption changes at 520 and 480 nm, respectively, in normal chloroplasts. If one supposes that the difference spectrum of Chl a_I [40,41] in the range of 460—540 nm closely resembles that of Chl a_{II} [32], then the flash-induced rise kinetics of the absorption changes at 480 nm and 520 nm should significantly differ. At 520 nm the absorption changes of the electrochromic effect and of Chl a_I and Chl a_{II} photooxidation have the same sign. Accordingly, the rise time at 520 nm should coincide with the processes leading to a stabilized charge separation, and the contribution of the recovery reactions of Chl a_I and Chl a_{II} give rise to a rapid decay kinetics. As the reduction kinetics of Chl a_{II}^{+} is characterized by half-lifes in the μsecond and submicrosecond range [42,43] and also the reduction of Chl a_I involves μsecond kinetics, the fast decay at 520 nm due to the above-mentioned recovery kinetics should be observed mainly in the short microsecond range and therefore would be overlapped by the triplet valve reactions of the carotenoids [44]. On the other hand, at 480 nm the positive absorption changes due to photooxidation of Chl a_I and Chl a_{II} overlaps with the negative absorption change of the electrochromic effect. Hence, the observed rise time should be biphasic: a fast phase coinciding with the electric field formation and slower μsecond components determined by the recovery of Chl a_I and Chl a_{II}. At 480-nm absorption changes caused by the carotenoid triplet-valve reaction can be neglected [44]. Despite slow rise kinetics at 480 nm have really been measured [45] they do not provide an experimental confirmation, because they were shown to be caused predominantly by double-hit effects at the reaction centers [46]. On the other hand, the relative contribution of the μsecond rise at 480 nm was found to be higher than at 515 nm, which would be in line with the hypothesis proposed here. However, further experiments are required for an unambigious interpretation. The results described in the present study indicate, that in Tris-washed chloroplasts in the absence of artificial electron donors under repetitive flash excitation a rapid turnover of Photosystem II takes place, which involves Chl a_{II} and the primary acceptor of the stable charge separation, X320. This

process is a transmembrane electron transfer between both components in the forward and back reaction, which cannot be used for energization of the ATP-ase. The above-mentioned reaction was found to cause singlet exciton formation, as is shown by delayed fluorescence [47,48], but its quantum yield has been estimated to be very low [49]. Therefore, the back reaction appears to be a nearly pure dissipative process. The functional role under physiological conditions, which might come into play in cases, where the water-splitting enzyme System Y is inoperative (e.g. at the early stages of chloroplast development) still remains an open question.

Furthermore, the results provide additional evidence for the localization of the components of the donor side of System II, especially Chl a_{II}, near to the inner phase of the thylakoids, in agreement with very recent findings of Conjeaud et al. [39].

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