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STUDIES ON WELL-COUPLED PHOTOSYSTEM-I-ENRICHED SUBCHLOROPLAST VESICLES

DISCRIMINATION OF FLASH-INDUCED FAST AND SLOW ELECTRIC POTENTIAL COMPONENTS

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This work aimed at the resolution of the multi-component electric potential changes induced by single-turnover flash illumination of Photosystem-I-enriched subchloroplast vesicles. If supplemented with ferredoxin and under carefully adjusted redox poising, these vesicles show a pronounced slow-rising and -decaying electric potential component, as monitored by endogenous and exogenous field-sensitive probes, carotenoids and oxonol VI, respectively. The fast and slow potential components can be easily discriminated without the need for computer-assisted deconvolution after selective presaturation of the slow component by preillumination or a transmembrane ΔpH, after selective suppression of the slow component by low valinomycin or uncoupler concentrations or in the absence of ferredoxin. The slow electric potential component, as compared to the fast one, is relatively sensitive to low concentrations of ionophores and uncouplers, detergent, ageing and lower temperatures (4–12°C), is associated with electrogenic proton displacements and is interpreted to respond to a field that is more located on the membrane-bulk interface. Temperature effects show transition temperatures around 20°C for both the rise and decay of the slow potential component. The results provide further evidence that the carotenoids and oxonol VI sense the same (slow) electric field, but may be differently located in the thylakoid membrane.

Introduction

Electric potential generation upon illumination of photosynthetic membranes is clearly attributed to two major distinguishable events. This is most obvious in highly intact photosynthetic systems such as whole algal cells [1,2] and carefully prepared chloroplasts, containing their envelope and stroma phase [3–6]. Kinetic discrimination of these events and/or a multi-component analysis by computer-assisted deconvolution are possible after single-turnover flash activation using the electrochromic carotenoid absorbance transient at 518 nm as the intrinsic electric potential indicator [6]. Different deconvolution methods used by several groups [3,4,7–9] depend on a number of preassumptions concerning the origin of the two electric potential components.

Joliot and Delosme [1] first observed a clear

Abbreviations: Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl) ethyl]amino}ethanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; S-13, 5-chloro-3-*t*-butyl-2′-chloro-4′-nitrosalicylanilide; Chl, chlorophyll; Qbc, plastohydroquinone-plastocyanin oxidoreductase: cytochrome *b*-*c* complex containing bound plastoquinone and Rieske (FeS) protein; PS, photosystem.

biphasic flash-induced carotenoid absorbance rise (phase *a* and *b*, respectively). Whereas the fast rise is obviously associated with charge separations in the photosynthetic reaction centres, the origin of the slower component is still a matter of debate. It has been suggested that it results from delocalization of the fast electric field [9,10] or from redox reactions in the Qbc region of the electron transfer chain [3,4,7,11,12]. Following the work of Joliot and Delosme [1], Bouges-Bocquet [4] considered phase *b* as the expression of the slow potential component. Alternatively, Crowther and Hind [3] prefered to assign the 'true' slow component to the DBMIB-sensitive part of the overall carotenoid transient. A different deconvolution into Reaction I and II by Schapendonk and Vredenberg [7,13] was based on the association of the slower component (Reaction II) with that part of the overall transient, which is abolished after a few flashes, fired in rapid succession.

Obviously, the choice of visual or computer-assisted construction of the slow-rising and decaying potential component dictates the measurement and/or identification of the kinetic profile of the fast component and vice versa. This has profound consequences for the interpretation of primary and secundary electric events during photosynthetic conversion of light energy into chemical or chemiosmotic energy.

In previous papers [12,14,15] we have described the isolation and characterization of well-coupled PS-I-enriched and PS-II-depleted subchloroplast vesicles. In these vesicles, supplemented with ferredoxin and with a carefully poised cyclic system, the slow-rising potential component is relatively well expressed as monitored by the carotenoid absorbance transient (ΔA_{518}) and by the extrinsic field-sensitive probe oxonol VI (ΔA_{590}) [12,16]. In this paper, we will present more detailed experiments aiming at the discrimination of the two major electric potential components in this more simple membrane system devoid of membrane stacking and stroma.

Materials and Methods

PS-I-enriched vesicles were isolated from market spinach as described previously [15]. The standard reaction medium contained 5 mM Tes-KOH buffer (pH 7.6)/2.5 mM KH_2PO_4/25 mM NaCl/25 mM KCl/5 mM $MgCl_2$; the chlorophyll concentration was 50 μg/ml. For optimal cyclic electron transfer [12] ferredoxin and NADPH were added to final concentrations of 5 μM and 0.5 mM, respectively, whereas the oxygen concentration was maintained at about 100 μM by periodical stirring, as controlled by a Clark oxygen electrode. Oxygen was partly depleted from the medium by pregassing with pure nitrogen gas. All experiments were carried out at 20 °C (unless stated otherwise) in a thermoelectrically controlled multipurpose cuvette (2.0 ml), the actinic light being provided from the bottom by a fiber-optic light guide [17]. When used, the concentration of oxonol VI was 0.3 μM.

A pH jump was performed by adding 20 μl 0.2 M succinic acid to the reaction mixture (final pH 5.5), followed after 20 s of equilibration by addition of 20 μl 0.4 M NaOH (final pH 7.8).

Flash-induced absorbance changes were measured with a laboratory-built fast-responding dual-wavelength spectrophotometer [8]; ΔA_{518} and ΔA_{590} were recorded against the references 545 and 603 nm, respectively. Oxonol VI shows an absorbance increase and decrease of maximal extent at 625 and 590 nm, respectively [18]; the isobestic wavelength is at 603 nm. We have measured the oxonol VI as a positive absorbance change by measuring at 603–590 nm. Saturated flash activation was provided by a General Electric FT-230 xenon flash tube (2 kV) firing flashes (5 μs at half-amplitude and tail-depressed) and passed through a filter cutting off below 695 nm (Schott, Mainz, F.R.G.). The signals of 16 or 25 flashes fired at a frequency of 0.125 Hz were averaged, except in the pH jump and preillumination experiments; in those cases two flashes with a time-interval of 4 s were averaged. On-line processing and triggering were mediated by a microprocessor-minicomputer system as described before [8].

When the reaction mixture was continuously illuminated before or during flash experiments, actinic light (light intensity 10 $mW \cdot cm^{-2}$) was provided using a bifurcated light guide, with one arm connected to the flash lamp, the other connected to a tungsten iodide light source (250 W), the light of which was passed a filter cutting off below 695 nm and 2 heat-absorbing filters (Calflex C).

Digitonin (twice recrystallized from ethanol)

was obtained from Merck, spinach ferredoxin from Sigma. S-13 was kindly donated by Dr. P.C. Hamm. Oxonol VI (bis[3-propyl-5-oxoisoxazol-4-yl]pentamethineoxonol) was synthesized and kindly provided by Prof. W.G. Hanstein (University of Bochum, F.R.G.) or synthesized according to his recipe (personal communication).

Results

When carefully prepared potentially well-coupled PS-I-vesicles are supplemented with ferredoxin under conditions for optimal redox poising with NADPH and oxygen, they show a typical biphasic flash-induced carotenoid absorbance transient with a pronounced slow-rising component, ΔA_{518} (slow) [12,16]. Fig. 1 shows this transient at different ferredoxin concentrations, together with the response of the extrinsic probe oxonol VI. In the absence of ferredoxin, only a fast-rising and -decaying electric field is observed, that is only sensed by the carotenoids (ΔA_{518} (fast), rise- and decay-halftimes of subnanosecond and 20 ms, respectively). At increasing ferredoxin concentrations (i.e., cyclic electron transfer) a slower flash-induced electric potential is generated that is sensed by both indicators (ΔA_{518} (slow) and ΔA_{590}, respectively). This is substantiated by Fig. 2, showing that the amplitude of this slow component saturates at about 5 μM ferredoxin like cyclic photophosphorylation [12] whereas the amplitude of ΔA_{518} (fast) is virtually independent on the ferredoxin concentration.

With the assumption that the kinetic profile of ΔA_{518} (fast) does not change with the ferredoxin concentration, the slow carotenoid and oxonol transients show similar kinetics at increasing ferredoxin concentration. However, the maximal oxonol amplitude is reached progressively later and does not exactly go hand in hand with that of the carotenoid transient (Fig. 1).

In order to discriminate and characterize the fast and slow electric potential components further, we have followed two approaches. First, we have created conditions under which the slow component is expected to become saturated by preenergization, i.e., by preillumination, a preestablished ΔpH or ATP hydrolysis. Second, we have studied the effects of uncouplers, ionophores,

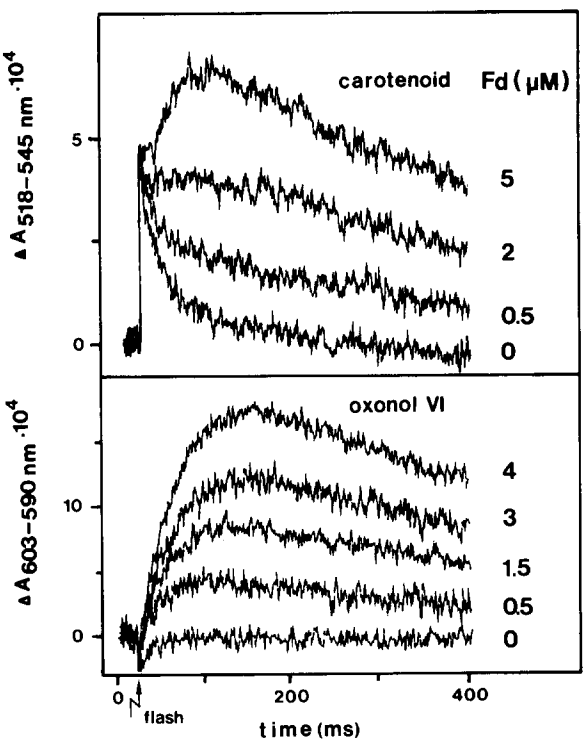

Fig. 1. Flash-induced absorbance changes of electric potential indicators in PS I vesicles at different ferredoxin concentrations. Experimental conditions as described in Materials and Methods.

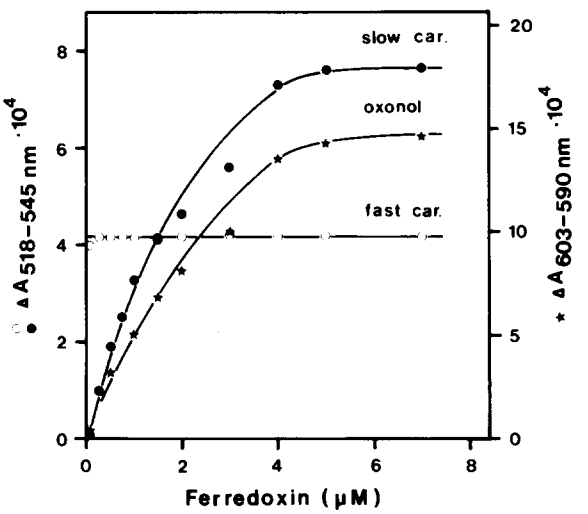

Fig. 2. Amplitudes of discriminated flash-induced absorbance changes of electric potential indicators in PS I vesicles as function of the ferredoxin concentration. Conditions as in Fig. 1. Amplitudes of the carotenoid (car.) transients were measured at 1 ms (fast carotenoid) and 80 ms (slow carotenoid) after the flash; amplitudes of the oxonol VI responses were measured at maximal extent.

detergent, ageing and temperature on the two electric potential components. Since in our reconstituted and optimized subchloroplast system the presence of the slow-rising carotenoid component is so obvious, we have not considered it useful for the purpose of this work to analyze the transients by computer-assisted kinetic deconvolution as we have previously done for broken chloroplasts [8,19].

In Fig. 3, the normalized amplitudes are given of the fast and slow carotenoid responses and the oxonol response to flashes fired before, during and after a 20 s period of continuous illumination, under optimal redox poising of the cyclic system. Whereas during the light period no flash-induced ΔA_{518} (fast) as well as ΔA_{518} (slow) and ΔA_{590} are observed, the flash responses immediately after the light periods reveal that ΔA_{518} (fast) is fully restored. However, the recovery of ΔA_{518} (slow) and the oxonol response takes about 100 s ($t_{0.5} \approx 20$ s) (see also Fig. 3B). So, at a few seconds following preillumination only ΔA_{518} (fast) is observed with a decay halftime of about 20 ms (not shown), as in the absence of ferredoxin (Fig. 1), substantiating the afore-mentioned assumption on the constancy of ΔA_{518} (fast) at different ferredoxin concentrations. The results suggest that during continuous illumination both fast and slow electric potential components become saturated and the dark relaxation of the slow component takes a much longer time than that after a single-turnover flash (about 1.5 s, result not shown). Under conditions that the slow potential is saturated, the flash photometer signal is at a higher level, but in the repetitive flash and averaging mode this would remain unnoticed due to automatic zero start resetting.

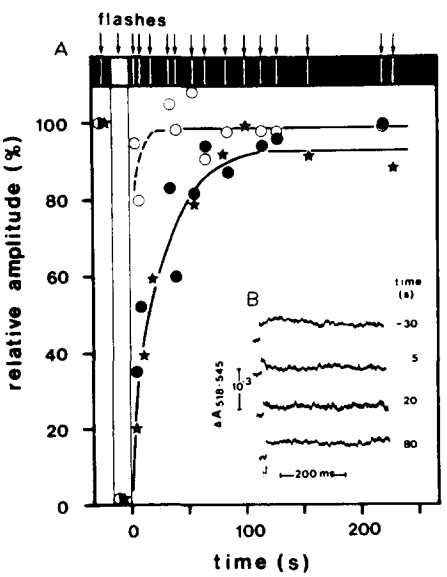

Fig. 3. (A) Effect of preillumination on the relative amplitudes of the discriminated flash-induced absorbance changes of electric potential indicators. Flashes were fired before, during and after a 20 s period of continuous illumination (as indicated) and the responses analyzed. (B) shows a representative series of carotenoid responses at different times after the preillumination period. Reaction mixtures as in Fig. 2 with 4 μM ferredoxin. Amplitudes were measured as in Fig. 2. ●, slow carotenoid; ○, fast carotenoid; ★, oxonol VI.

The same type of discrimination can be made by preacidification of the vesicle lumen with succinic acid followed by an upward pH jump, according to the classical Jagendorf and Uribe experiment [20]. Fig. 4 shows that during the relaxation of the transmembrane ΔpH (in this case with a halftime of about 50 s) the amplitudes of the ΔA_{518} (slow) and ΔA_{590} are strongly reduced and slowly regain their original levels, while ΔA_{518} (fast) remains essentially unaffected. Again, flashing during a high ΔpH only generates the fast electric potential component, the slow one already being saturated.

During ATP hydrolysis in chloroplasts, we have

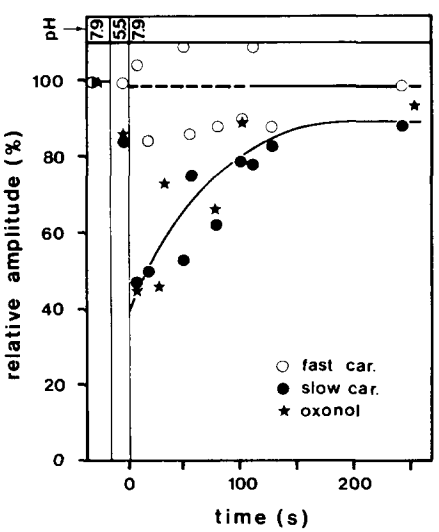

Fig. 4. Effect of an acid → base transition on the relative amplitudes of the discriminated flash-induced absorbance changes of electric potential indicators. Conditions as in Fig. 3. car., carotenoid.

shown earlier that the slow electric potential is also saturated to such a level, that additional flashing only produces ΔA_{518} (fast), ΔA_{518} (slow) and ΔA_{590} being absent [19,21]. Galmiche and Girault [22] and, more recently, Schreiber and Rienitz [23] and Peters et al. [24] have also convincingly demonstrated that the slow light-induced electric potential and the ATP hydrolysis-induced electric potential are of the same nature and in fact fully complementary. However, these experiments are difficult to perform with the ferredoxin-supplemented PS-I vesicles, since activation of the ATPase requires an artificial redox mediator (e.g., phenazine methosulfate) under which conditions the ΔA_{518} response is drastically altered (not shown).

In the following experiments we describe the effects of various chemical and physical treatments on the two electric potential components. Fig. 5, showing the effect of valinomycin in the presence of K^+ ions, illustrates that in the lower concentration range this ionophore primarily affects the magnitude of the slow potential component. A titration experiment is shown in Fig. 6A. Apparently, ΔA_{518} (slow) is somewhat more sensitive to the presence of the ionophore in the membrane than ΔA_{590}. The same results were found with the uncoupler S-13 (Fig. 6B). Membrane treatment with the mild detergent digitonin (Fig. 6C) also primarily inhibits expression of the slow potential component. In this case the oxonol response seems to be somewhat more sensitive. Treatment with the fixative glutaraldehyde (6 min, 5 mM at 4°C) completely abolishes the slow potential component but leaves the fast one unchanged [16]. Ageing of the PS-I-vesicles at 20°C (Fig. 6D) does not alter the carotenoid response for the first 30 min, but in the following few hours the amplitudes of both potential components gradually decrease, the slow component falling off more rapidly.

The ionophore nigericin presents a special case.

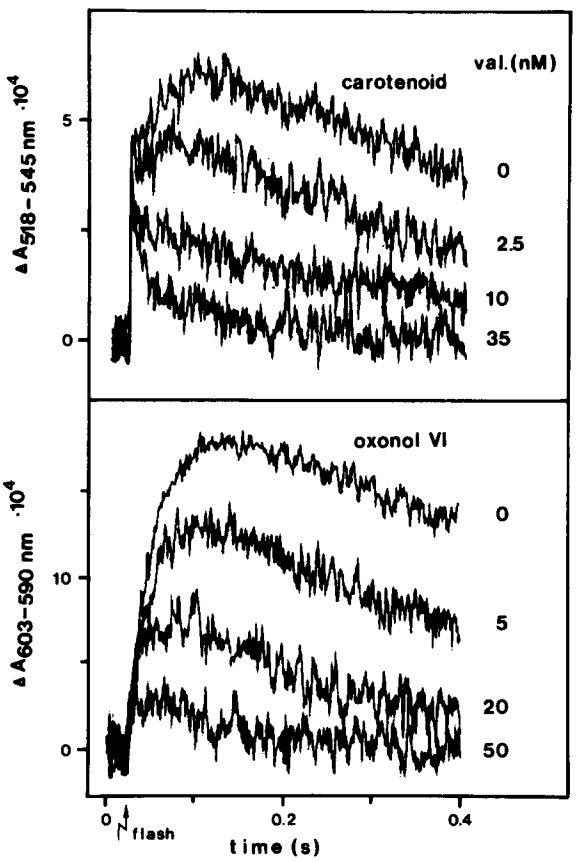

Fig. 5. Effect of valinomycin on the flash-induced transients of electric potential indicators. Conditions as in Fig. 3.

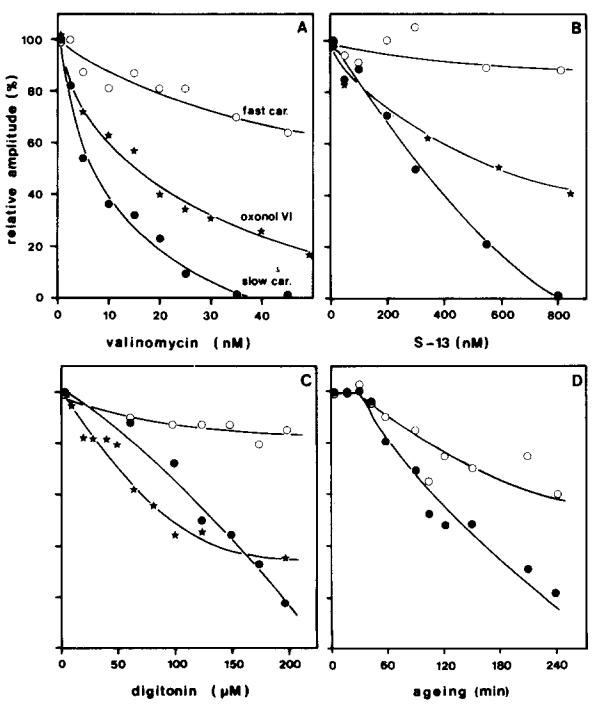

Fig. 6. Effect of valinomycin, S-13, digitonin and ageing on the relative amplitudes of discriminated flash-induced absorbance changes of electric potential indicators. Conditions as in Fig. 3.

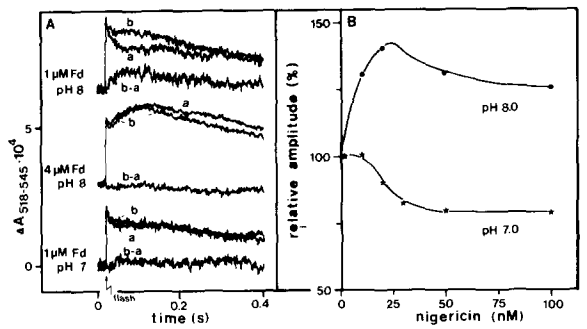

Fig. 7. (A) Effect of the nigericin on the flash-induced carotenoid transient at saturating and non-saturating ferredoxin concentrations and at pH 7.0 and 8.0; a = control, b = control with 20 nM nigericin. (B) Effect of nigericin on the relative amplitudes of the slow carotenoid transient at pH 7.0 and pH 8.0 with 1 μM ferredoxin.

Crowther and Hind [3] have observed in intact chloroplasts that nigericin increases the amplitude of the slow component of the carotenoid response.

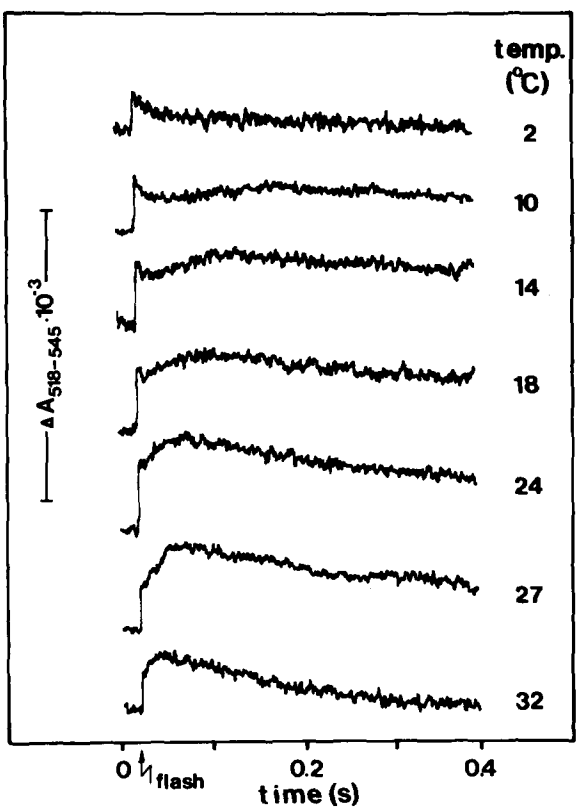

Fig. 8. Temperature dependence of the flash-induced carotenoid transient. Conditions as in Fig. 3.

Because nigericin transduces a pH gradient into an electrical gradient by H^+-K^+ exchange, this was interpreted as an indication of the involvement of protons in the generation of the slow potential component. We also observed a stimulation of ΔA_{518} (slow) by low concentrations of nigericin, as shown in Fig. 7, but only at pH 8 and under suboptimal electron transfer conditions, e.g., at a non-saturating ferredoxin concentration (1 μM). At pH 7, no significant stimulation of ΔA_{518} (slow) was observed. This suggest that under optimal conditions for cyclic electron transfer (5 μM ferredoxin) the flash-induced electric potential reaches a maximum level.

Finally, we have studied the effect of incuba-

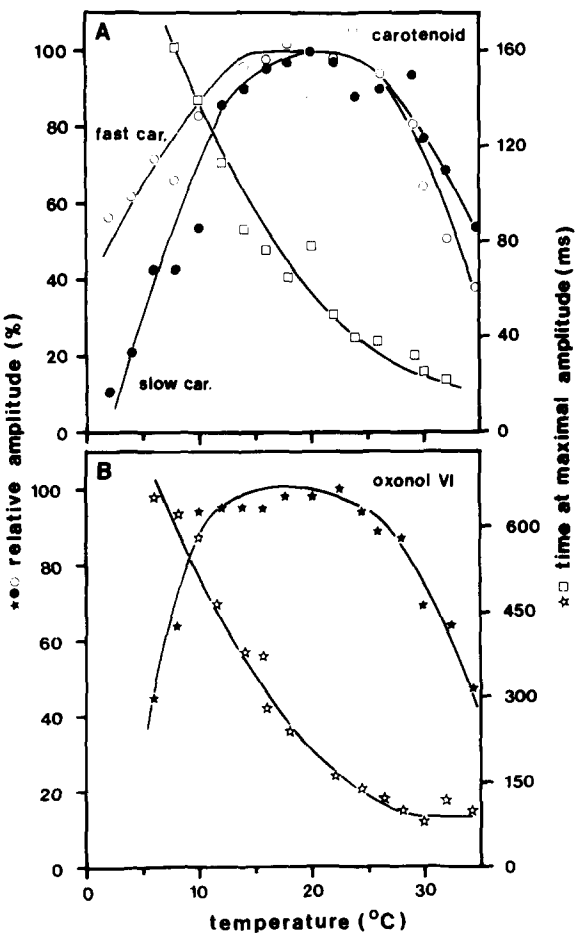

Fig. 9. Temperature dependence of the relative amplitudes and time at maximal amplitude of the discriminated flash-induced carotenoid (A) and oxonol VI (B) transients. Conditions as in Fig. 3.

tion temperature on the flash-induced carotenoid and oxonol absorbance changes. In Fig. 8, the traces of ΔA_{518} are pictured. Most obvious is a marked influence of temperature on the kinetics of the slow-rising component. The corresponding oxonol responses (not shown) resemble ΔA_{518} (slow) qualitatively, but the maximal amplitudes are reached significantly later (Fig. 9). Fig. 9 summarizes the dependencies on temperature of the amplitudes of the two carotenoid and oxonol transients as well as the times at which their maximal amplitudes are reached. The temperature optima of all electric potential indicators are rather broad. Fast and slow carotenoid response components show a somewhat different temperature sensitivity in the extreme temperature spans, especially in the lower one (4–12 °C).

The temperature dependence of the halftimes of rise and decay of ΔA_{518} (slow) and ΔA_{590} are presented in the Arrhenius plots of Fig. 10. We observe clear temperature transitions of these halftimes around 20 °C. These correspond to the transition temperatures of the decay halftimes reported for (broken) chloroplasts (Refs. 25–27, in contrast to Ref. 28). It was suggested earlier [27,29] that these discontinuities are related to the involvement of electron transfer and/or proton translocation by the Qbc redox complex.

Breaks in the Arrhenius plots at temperatures around 10 °C were observed for processes, driven by artificial cyclic electron transfer around PS I (i.e., in the presence of phenazine methosulfate or pyocyanine) [27]. Apparently, ferredoxin-mediated 'native' cyclic electric transfer in our PS-I-vesicles does not show breaks in the lower temperature span so that the occurrence of such breaks is not necessarily related to PS I or to the membrane composition of stroma lamellae [27,29,30], where the PS-I-vesicles are derived from.

Activation energies of the rise and decay of ΔA_{518} (slow) and ΔA_{590} above and below their respective transition temperature are listed in Table I. The activation energies for the rise or decay of both responses are rather different, which points to a different location of the probes in the thylakoid membrane. Remarkably, the magnitude of the changes in activation energy around the transition temperature is rather constant for both potential indicators, substantiating a common origin of the

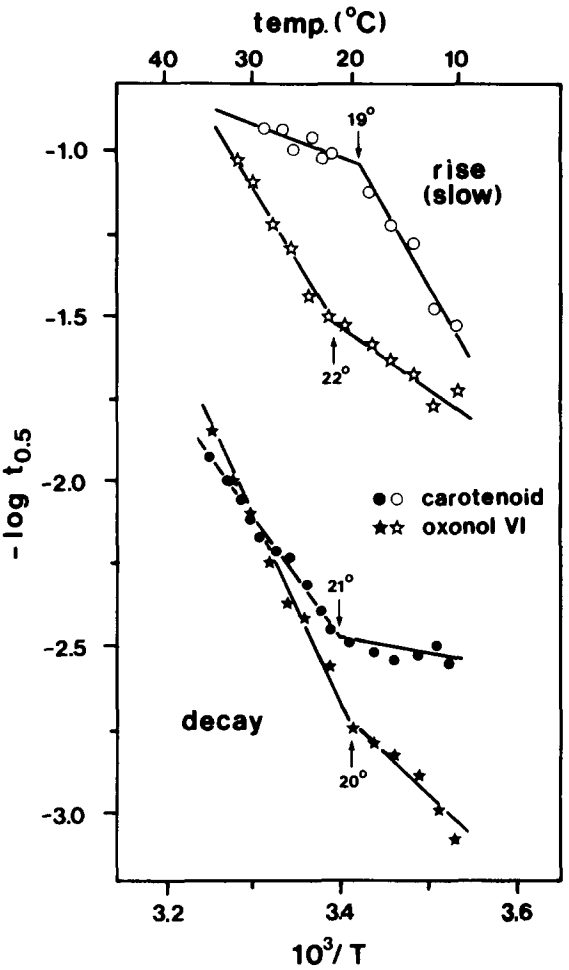

Fig. 10. Arrhenius plots of the slow rise and decay kinetics of the flash-induced transients of electric potential indicators. Conditions as in Fig. 3.

slow carotenoid and oxonol response. Whereas the change in activation energy of the rise of ΔA_{518} (slow) is in the opposite direction compared to its decay, the activation energies of the rise and decay of the oxonol transient alter in the same way (Table I).

Discussion

The experiments presented in this paper aimed at the resolution of the fast and slow components of the electric potential which became manifest in the response to single-turnover light flashes under various conditions. These conditions were created

TABLE I

ACTIVATION ENERGIES OF THE FLASH-INDUCED SLOW CAROTENOID AND OXONOL VI RESPONSES BELOW AND ABOVE THEIR RESPECTIVE TEMPERATURE TRANSITION POINTS

Activation energies (E_A) were calculated from the data in Fig. 10, according to Ref. 31. ΔE_A represents the change in E_A going from the lower to the higher temperature ranges around the transition temperatures (t).

Probe	t (°C)	E_A (kJ·mol^{-1})	ΔE_A
Carotenoid rise	< 19	79.5	(−) 61.1
	> 19	18.4	
Carotenoid decay	< 21	6.7	(+) 59.0
	> 21	65.7	
Oxonol rise	< 22	36.4	(+) 49.8
	> 22	86.2	
Oxonol decay	< 20	40.1	(+) 58.6
	> 20	98.8	

such as to allow a better discrimination of these components without the need for kinetic deconvolution. The validity of deconvolution methods heavily rests on the correctness of the necessary assumptions. In our simple model system of well-coupled PS-I-vesicles under carefully controlled conditions, the slow electric potential component (half-rise time of 10–30 ms, at room temperature) is very well expressed like in whole algae [1,2] or highly intact chloroplasts [3–5]. In the absence of cyclic electron transfer (Fig. 1) or in the presence of 35 nM valinomycin (Fig. 5), the slow-rising potential component is not generated and selectively suppressed, respectively, while the amplitude and rate of decay (halftime of 20 ms) of the fast potential component remain unchanged. Therefore, this allows a plain discrimination directly from the measured transients. Much higher valinomycin or uncoupler concentrations are required to change the kinetic profile of the fast potential component (not shown).

The two potential components show similar differences in sensitivity to detergent (Fig. 6C), glutaraldehyde [16], a short heat treatment (not shown) and ageing at 20 °C (Fig. 6D). Apparently, the slow component is much less resistant against treatments that primarily affect membrane-bulk interface processes than the fast one. So, it seems reasonable to conclude that the slow electric potential component, as compared to the fast one, responses to a field that is more located at the membrane-bulk interface. This interpretation is in harmony with findings of Schreiber and Rienitz [23], who did not observe any further acceleration of the decay of the flash-induced ΔA_{518} (fast) by valinomycin, when ΔA_{518} (slow) was presaturated by ATP hydrolysis. The different degree of exposure to the bulk aqueous phase is further substantiated by the temperature effects (Figs. 8 and 9), showing that in the 4–12 °C span ΔA_{518} (slow) and ΔA_{590} are much more decreased than ΔA_{518} (fast) on lowering the temperature.

The slow electric potential component is generated by either electron transfer (Fig. 1) most likely through the Qbc redox complex, ATP hydrolysis [19,21–24], or a preestablished ΔpH (Fig. 4) and involves electrogenic proton displacements, at least at alkaline pH, as pointed out by the increment of ΔA_{518} (slow) in the presence of nigericine at pH 8.0 (Fig. 7). These proton displacements, probably occurring at specific membrane-bound sites at the membrane-bulk interface, may result in local proton-stabilization in phases or domains adjacent to these sites. The absence of ΔA_{518} (slow) after energetization, i.e., by preillumination (Fig. 3), ΔpH (Fig. 4) or ATPase activity [19,21–24] could then be explained because these phases or domains are preloaded with protons. The reappearance of ΔA_{518} (slow) after preloading would then depend on the rate of H^+-efflux from the membrane.

The primary photophysical acts in the photosystems involve a rapid (subnanosecond) charge separation which is sensed by the carotenoids and not by extrinsic probes like oxonol VI (see, however, Ref. 32). From our experiments it is clear that oxonol VI generally responds to the slow electric potential like the ΔA_{515} (slow). There are, however, time-dependent deviations in that oxonol requires more time to reach its maximal amplitude in some cases (Figs. 1 and 9). When the carotenoid transient is measured in the presence of oxonol VI at a non-uncoupling concentration (0.3 μM) no retarding effect on ΔA_{518} is observed. Therefore, we tentatively interpret the slight differences in kinetics and sensitivity towards various treatments (Fig. 6) as due to a different localization of the

intrinsic and extrinsic potential probes in the membrane. This is further substantiated by the differences in activation energies for the rise and decay of both probe responses (Table I). During continuous or pulsed illumination of chloroplasts neither the binding of added oxonol VI nor its fluorescence lifetime do significantly change (Schuurmans, J.J. and Kraayenhof, R., unpublished data), which makes it unlikely that this probe rate-limits its own response by an electrophoretic translocation across the bulk membrane as proposed by Girault and Galmiche [32].

If one observes the flash-induced carotenoid absorbance transients in chloroplasts in the presence of a preestablished ΔpH [6] or ATP hydrolysis [1,8,23,33–35], the overall ΔA_{518} decay is apparently accelerated. This commonly has been interpreted as a consequence of a supposed higher proton efflux under these conditions [6,33,34]. Kinetic deconvolution (into two exponential components) indicates that the slow potential component is absent. We have found earlier [8,19,21] that this absence is only apparent as a trivial consequence of the repetitive transient-averaging technique that requires zero-normalization before each trigger. In fact, the slow potential component is maintained at a higher steady-state level so that, upon additional flashing, only the fast component remains to be seen. This rise of steady-state level was demonstrated by recording a series of non-averaged flash responses [19]. The same situation arises at higher flash frequencies or after double flashing [7,13] (incomplete relaxation of the slow potential component) and in the presence of DCCD or Dio-9 [21,33] (no proton transfer through the ATPase complex). The experiments with preillumination (Fig. 3) and ΔpH generation (Fig. 4) further illustrate that the fast and slow electric potential components respond differently to these pretreatments.

Acknowledgements

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