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THE CONTROL OF THE REDUCTION KINETICS IN THE DARK OF PHOTO-OXIDIZED CHLOROPHYLL a_{II}^+ BY THE INNER THYLAKOID PROTON CONCENTRATION

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SUMMARY

The 690 nm absorption change reflecting the turnover of the reaction center chlorophyll of System II, chlorophyll a_{II}, (see Döring, G., Renger, G., Vater, J. and Witt, H. T. (1969) Z. Naturforsch. 31c, 712–721 and Gläser, M., Wolff, Ch., Buchwald, H. E. and Witt, H. T. (1974) FEBS. Lett. 42, 81–85) has been investigated as a function of pH of the chloroplast suspension. It was found:

(1) In the range $6.0 \leqslant pH \leqslant 8.0$ the amplitude of the measured chlorophyll a_{II} absorption change, ΔA_0 (chlorophyll-a_{II}), markedly declines with increasing pH, whereas the average oxygen yield per flash remains nearly unaffected.

(2) The dependency on actinic flash intensity of the amplitudes ΔA_0 (chlorophyll-a_{II}) at pH 6.0 closely resembles that for the amplitudes of the chlorophyll a_I absorption change. On the contrary, at pH 7.5 a higher flash intensity is required for half saturation of ΔA_0 (chlorophyll-a_{II}), whereas the corresponding curve for ΔA_0 (chlorophyll-a_I) is practically invariant to pH.

(3) Addition of ionophores at concentrations sufficiently high for a complete collaps of proton gradients across the thylakoid membrane shifts the pH-dependence of ΔA_0 (chlorophyll-a_{II}) by more than 2 units towards the acidic range.

On the basis of a hypothesis presented very recently (Gläser, M., Wolff, Ch. and Renger, G. (1976) Z. Naturforsch. 31c, 712–721) it is assumed, that the reduction in the dark of photooxidized chlorophyll a_{II}^+ is regulated by the proton concentration in the inner thylakoid space. At lower pH the reaction proceeds predominantly via the well known 35 μs kinetics, whereas at higher pH a very rapid kinetics ($\leqslant 1$ μs), too fast to be resolvable by our measuring device, additionally contributes to a significant degree to the overall reduction of chlorophyll a_{II}^+. Furthermore, on the basis of Lavorels model (Lavorel, J. (1975) in Bioenergetics of Photosysthesis (Govindjee, ed.), pp. 223–317, Academic Press, New York) the close interrelationship between ΔA_0 (chlorophyll-a_{II}) and delayed fluorescence intensity in the μs range is shown to be interpretable mainly by kinetical rather than by energetical effects.

INTRODUCTION

The photosynthetic cleavage of water accomplished by System II via electron abstraction leads to the evolution of free molecular oxygen and the concomitant extrusion of protons to the inner phase of the thylakoids. The rate limiting step of the overall electron transport from water to $NADP^+$ (see Fig. 1) has been found to be the reoxidation of the reduced plastoquinone [1], a process which is accompanied by proton release into the inner thylakoid space. The reaction rate, symbolized by the constant $k_{PQ}([H^+])$ in Fig. 1, was shown to increase as the inner proton concentration, $[H^+]_{in}$, decreases [2]. Accordingly, the reactions leading to water oxidation could be anticipated to depend similarly on the proton concentration in the environment of the water-splitting enzyme system Y. Double flash group experiments led to the conclusion, that the rate limiting step between system Y and the PQ pool has a half life time of about 600 μs [3]. This time was shown to be nearly independent of the pH of chloroplast suspensions in the range of 6 to 8.5 [4]. Later, at pH 5 for double flash groups and at higher pH values by excitation with repetitive groups containing more than 3 flashes a pH-dependence of the oxidizing side of System II was found [5]. The water oxidation is caused by the positive charges produced via photoreactions at the primary electron donor of System II, which was identified as a special chlorophyll *a*, designated as chlorophyll a_{II} [6, 7]. The electron transfer reactions leading to chlorophyll a_{II}^+ reduction by water, symbolized in Fig. 1 by an overall rate constant k_{Chl}, could be the candidates for a possible proton effect. The reduction of chlorophyll a_{II}^+ occurs via different kinetics. Experimentally two phases were detected with half-life times of 35 μs [8] and 200 μs [6]. Very recently, indirect evidence was given for the existence of even much faster reduction kinetics of chlorophyll a_{II}^+, which were estimated to take place with $\tau_{\frac{1}{2}} \leqslant 1$ μs [9]. In the light of the above mentioned remarks it seems to be worthwhile to investigate the effect of pH on the recovery kinetics of chlorophyll a_{II}.

Recent experiments led to the conclusion that the functional connection between chlorophyll a_{II} and the water-splitting enzyme system Y becomes disturbed at

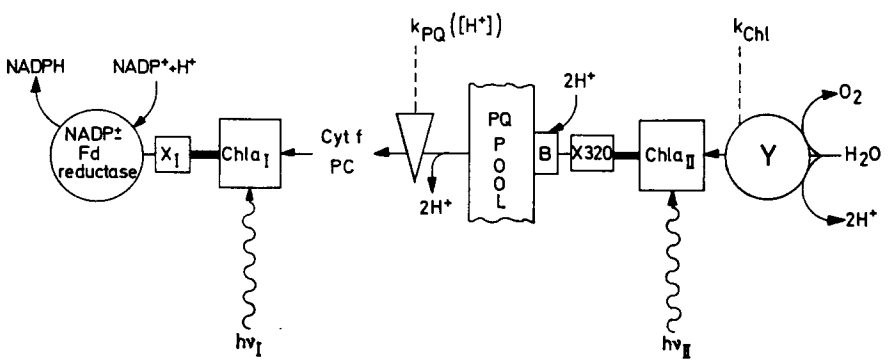

Fig. 1. Scheme of the linear electron transport chain. Abbreviations: B = intermediary carrier (special plastoquinone) Chl-a_I = chlorophyll a_I, Chl-a_{II} = chlorophyll a_{II}, Cyt *f* = cytochrome *f*, Fd = ferredoxin, PC = plastocyanine, PQ = plastoquinone, X 320 = primary electron acceptor of Photosystem II (special plastoquinone), X_I = primary electron acceptor of Photosystem I, Y = water-splitting enzyme system, $k_{PQ}[H^+]$ and k_{Chl} = rate constants (see text).

pH values below 5 of the chloroplast suspensions [10, 11]. On the other hand, at alkaline pH values the water-splitting enzyme system Y itself degrades [4, 12]. Therefore, the present study is mainly restricted to that pH range, where System II as a whole appears to be not seriously modified, i.e. $5 \leqslant pH < 8.5$.

The data presented in this study indicate that the reduction kinetics of chlorophyll a_{II}^+ are strictly dependent on the pH value of the chloroplast suspensions. It is inferred, that the protonation of a specific group, located near the inner side of the thylakoid membrane, with a pK value near 5 regulates the reduction kinetics of photooxidized chlorophyll a_{II}^+.

MATERIALS AND METHODS

The chloroplasts were prepared from market spinach according to the method of Winget et al. [13], except that 10 mM ascorbate was present in the grinding medium. For storage in liquid nitrogen 5 % dimethylsulfoxide was added. The oxygen evolution activity of the stored chloroplasts after thawing was nearly the same as that of freshly prepared chloroplasts.

The reaction mixture for the measurements of the absorption changes contained: chloroplasts (5 μM chlorophyll), 2 mM $MgCl_2$, 10 mM KCl, 20 mM morpholinoethanesulfonate/NaOH in the range $4.5 \leqslant pH \leqslant 7.0$ and N tris-(hydroxymethyl)methylglycine/NaOH in the range $7.0 \leqslant pH \leqslant 9.0$ as buffers, 100 μM benzylviologen or 250 μM $K_3[Fe(CN)_6]$ as electron acceptors. The assay conditions for the measurements of the oxygen evolution are the same, except that a 10-fold higher chlorophyll-concentration has been used and only $K_3[Fe(CN)_6]$ served as electron acceptor.

Absorption changes were recorded by a repetitive flash photometer similar to that described in ref. 14. The details of the measurements are described in ref. 9.

Oxygen evolution was detected by a repetitive amperometric method as is described in ref. 15.

RESULTS

If one presupposes that there exists a 1 : 1 functional and structural connection between chlorophyll a_{II} and the watersplitting enzyme system Y (for other models, e.g. that reported in ref. 16, see Discussion), then the number of intact System II electron transport chains is given by the amount of the average oxygen yield per short flash, M_{O_2}, (for details see ref. 17). The dependence of M_{O_2} on pH of the chloroplast suspension is given in Fig. 2. It is seen that within the range of $5.5 \leqslant pH \leqslant 8$ M_{O_2} is nearly constant. On the contrary, in the same pH range the amplitudes of the "detected" absorption changes due to chlorophyll a_{II} turnover, ΔA_0(Chl-a_{II}), measured at 690 nm, drastically decrease with increasing pH. Furthermore, Fig. 2 shows, that the "detected" kinetics of chlorophyll a_{II} recovery with half life times of 35 μs and 200 μs, respectively, depend in a different manner on the pH of the suspension. Especially at low pH values there occurs from pH 5.0 towards 4.5 a steep increase of the amplitude of the 200 μs kinetics, whereas the 35 μs kinetics remain nearly unaffected. In the same range the average oxygen yield per flash, M_{O_2}, markedly declines, indicating damage of the oxygen evolving apparatus. The effect of acidic treatment on the recovery

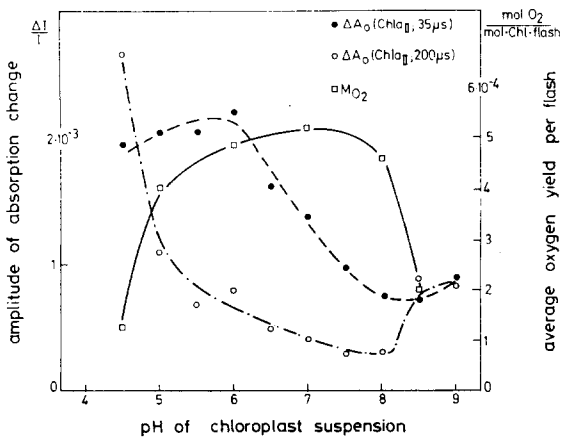

Fig. 2. Amplitude of absorption change of chlorophyll a_{II} (Chl-a_{II}) and of the average oxygen yield per flash as a function of pH of the chloroplast suspension. Experimental conditions as described in Materials and Methods.

kinetics of chlorophyll a_{II} has been thoroughly investigated by other groups [10, 11]. The present results confirm the conclusions of refs. 10 and 11 and will not be discussed extensively in this paper.

The far most important fact depicted in Fig. 2 is the discrepancy between the dependencies on pH in the range from 6 to 8 of the "detected" amplitudes ΔA_0(Chl-a_{II}) and of M_{O_2}, respectively. As remarkable pH-dependent bandshifts in the red of the difference spectrum for the chlorophyll a_{II}^+ formation can be excluded (at pH 6.0 and 7.5 the peaks were found to be centered around 685±1 nm and 686±1 nm, respectively), the above mentioned discrepancy can be simply explained by two different models: (a) if the quantum yield of oxygen evolution is assumed to be nearly constant, then the "detected" amplitudes ΔA_0(Chl-a_{II}) are not a direct measure of the number of positive charges produced per flash. (b) If on the other hand ΔA_0(Chl-a_{II}) would reflect linearly the number of chlorophyll a_{II}^+ generated per flash, then at least one dissipative pathway strongly dependent on pH, has to be assumed to exist for the reactions of the positive charges of chlorophyll a_{II}^+ so that at pH 6.0 more than 50 % of the charges should be wasted. As the latter model is in contradiction with quantum yield measurements, only the former one seems to be appropriate for the interpretation of the data of Fig. 2. Very recently, we came to the conclusion [9], that in normal chloroplasts there exists a very rapid chlorophyll a_{II} recovery kinetics, too fast to be resolvable by our measuring device*. If this supposition is correct, then the results of Fig. 2 are easily understandable by the assumption that the contribution of the fast "undetected" kinetics of the total absorption change at 690 nm is strongly dependent on pH, which becomes more pronounced at higher pH values.

The occurrence of an "undetected" very rapid reduction of chlorophyll a_{II}^+ and the assumption of its transformation into a "detected" kinetics by a second type of light reaction occurring with low quantum yield under multiphotonic excitation of the centers were claimed to be responsible for the divergence in the behaviour of the

* Accordingly, these kinetics have been referred to as "undetected."

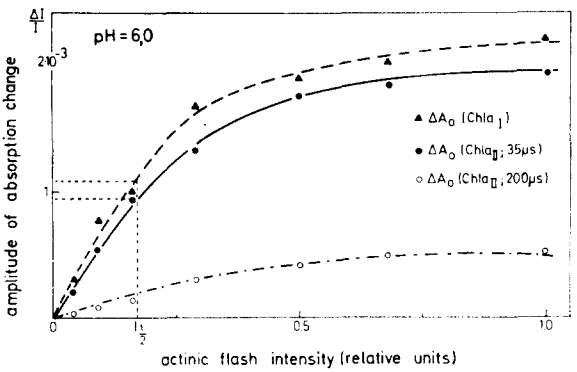

Fig. 3. Amplitude of absorption changes of chlorophylls a_I and a_{II} (Chl-a_I and Chl-a_{II}), respectively, as a function of actinic flash intensity at pH 6.0 of the chloroplast suspension. Experimental conditions as described in Materials and Methods.

saturation by flash light intensity of the measured amplitudes of the absorption changes of chlorophylls a_I and a_{II}, respectively, at pH 7.2 in normal chloroplasts [9]. Therefore, a varying amplitude ratio of "undetected" and "detected" absorption changes at 690 nm should give rise to saturation curves, which are dependent on the pH of the chloroplast suspension. On the other hand, the corresponding saturation behaviour of chlorophyll a_I is expected to remain invariant to pH in the above mentioned range. The dependency on the actinic flash intensity of the amplitudes at 690 nm, which are due to chlorophyll a_{II} turnover, and at 703 nm reflecting the reactions of chlorophyll a_I, measured at pH 6.0 and 7.5, respectively is depicted in Figs. 3 and 4. It is seen that the absolute extent at saturating intensity as well as the actinic flash intensity required for half saturation of the amplitude of chlorophyll a_I absorption changes, ΔA_0(Chl-a_I), are practically the same at pH 6.0 and 7.5. A more complex pattern is observed for the amplitudes ΔA_0(Chl-a_{II}). At pH 6.0 (see Fig. 3) the absolute amplitude of the 35 μs-kinetics ΔA_0(Chl-a_{II}, 35 μs) at saturating intensity closely

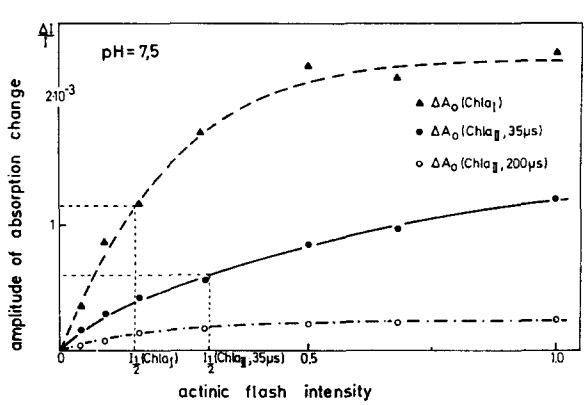

Fig. 4. Amplitude of absorption changes of chlorophylls a_I and a_{II} (Chl-a_I and Chl-a_{II}), respectively, as a function of actinic flash intensity at pH 7.5 of the chloroplast suspension. Experimental conditions as described in Materials and Methods.

resembles that of ΔA_0(Chl-a_I). Half saturation of ΔA_0(Chl-a_{II}, 35 μs) is attained at exactly the same actinic flash intensity ($I_{\frac{1}{2}} = 0.17$ on an arbitrary scale) as for ΔA_0 Chl-a_I). The amplitude of the 200 μs kinetics is comparatively small with a similar saturation behaviour as the other absorption changes. On the contrary, at pH 7.5 (see Fig. 4) the absolute amplitude of ΔA_0(Chl-a_{II}, 35 μs) reaches only about 50 % of the extent observed for ΔA_0(Chl-a_I), whereas a higher actinic flash intensity is necessary for half saturation of ΔA_0(Chl-a_{II} 35 μs). Analogous to pH 6 the amplitude of the 200 μs-kinetics is rather small with light saturation resembling that of ΔA_0(Chl-a_I).

The results of Figs. 3 and 4 are consistent with the assumption that at pH 6.0 the dark reduction of photooxidized chlorophyll a_{II}^+ occurs predominantly via an electron transfer reaction with a half life time of 35 μs, whereas at pH 7.5 additionally a very rapid ($\tau_{\frac{1}{2}} \leqslant 1$ μs) reduction takes place, thus giving rise to a significant "undetected" absorption change. The distinct saturation curve might be caused by two effects, the multiphotonic transformation process and/or different ΔpH values attained at different actinic flash intensities. However, as the curve is rather flat, a small scatter of the data will cause large changes of $I_{\frac{1}{2}}$ so that no definite conclusion can be drawn about the extent and the nature of the $I_{\frac{1}{2}}$-increase. On the basis of the data of Figs. 2–4, we conclude, that under physiological conditions the rate of the electron transfer reactions on the donor side of System II is strongly dependent on the pH of the suspension medium of the thylakoids. However, as different lines of evidence point to a localization of chlorophyll a_{II} as well as of the water-splitting enzyme system Y near the inner phase of the thylakoids [7, 18, 19], the outer pH is expected to exert only an indirect effect. It was shown that in the range of 7.0 $\leqslant$ pH $\leqslant$

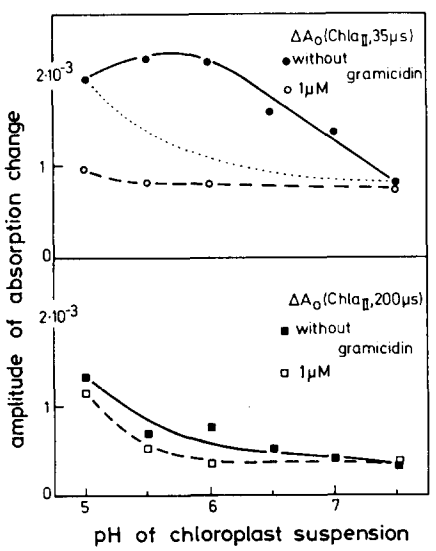

Fig. 5. Amplitudes of the 35 μs and 200 μs kinetics of the chlorophyll a_{II}-absorption change as a function of pH of the chloroplast suspension in the absence and in the presence of gramicidin. Gramicidin-concentration as indicated in the figure, other experimental conditions as described in Materials and Methods. The dotted line describes the dependence at gramicidin concentrations insufficient for a complete collapse of proton gradients.

9.0 under periodical single turnover flash excitation conditions a proton gradient of 2.3–2.7 pH units is established across the thylakoid membrane at repetitive rates of 4 Hz [20]. Therefore, if one assumes that the redox component (or components) responsible for the very fast chlorophyll a_{II}^+ reduction is accessible to protons from the inner side of the thylakoids rather than from the outside, then in the presence of uncouplers the increase of the 35 μs kinetics of ΔA_0(Chl-a_{II}) should be shifted by about 2.5 units towards the acidic range as the pH-value of the chloroplast suspension decreases. In order to avoid any interference with effects due to the charge accumulation state of the water-splitting enzyme system Y, ADRY-type uncouplers like FCCP (see ref. 21) are not applicable. Therefore the ionophoric uncoupler gramicidin was used, which is known to be unable to react as ADRY-reagent [22]. In Fig. 5 the amplitudes of the 35 μs- and of the 200 μs-kinetics of the absorption change at 690 nm as a function of the external pH are given for chloroplasts in the absence and presence of gramicidin. At concentrations sufficiently high for complete uncoupling there occurs a drastic shift, so that even at pH 5.0 the contribution of the 35 μs kinetics remains comparatively low. At lower concentrations a moderate effect is observed (dotted line). A much less pronounced dependency is found for the 200 μs kinetics. The data of Fig. 5 show that the regulation of the reduction kinetics of photooxidized chlorophyll a_{II}^+ is obviously dependent on the inner thylakoid proton concentration, $[H^+]_{in}$. A dependency on $[H^+]_{in}$ of the reduction kinetics has been reported some years ago also for chlorophyll a_I. However there seem to exist significant mechanistic differences of the steerage by $[H^+]_{in}$ of the reduction kinetics of chlorophylls a_I and a_{II}, respectively, as will be outlined in Discussion.

DISCUSSION

The experimental findings depicted in Figs. 2–5 favour the assumption, that the fast "undetected" kinetics ($\leqslant$ 1 μs) of the overall reduction of chlorophyll a_{II}^+ is

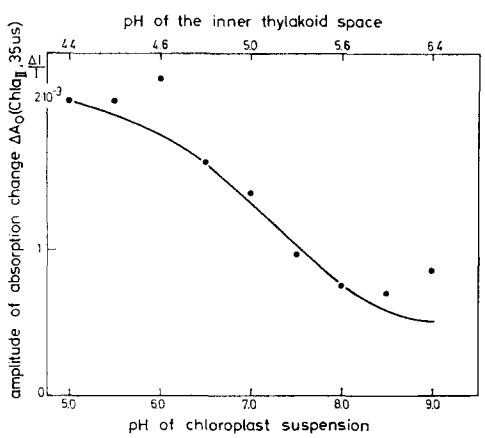

Fig. 6. Amplitude of the 35 μs kinetics of the chlorophyll a_{II}-absorption change as a function of pH of the chloroplast suspension. Top abscissae: pH of the inner thylakoid space estimated as is described in the text. The curve gives the theoretical values obtained from Eqn. 1 by numerical fitting of the data of Fig. 2.

governed by the proton concentration mainly of the inner phase of the thylakoids rather than of the outside. Therefore, for the analysis a calibration of the outer (chloroplast suspension) pH into units of internal pH is required for single turnover actinic flashes with a repetition rate of 4 Hz. As the number of flashes given to each sample is very large (1024–2048), induction phenomena can be neglected. Exact data for the steady state pH gradient under our excitation conditions are not available for the whole pH range. Gräber and Witt [20] found by application of 9-aminoacridine as fluorescing indicator, that a pH gradient of 2.3–2.7 units is established across the thylakoid membrane for $7.0 \leqslant \text{pH} \leqslant 9.0$ of the chloroplast suspension. Similar values were obtained under continuous illumination of saturating intensity by Siggel [23]. The experiments extended up to pH 6.0 show a remarkable decline of ΔpH towards the acidic range. By extrapolation of the data down to pH 5.0 and taking into account the kinetical limitations of the electron transport rate at lower pH (see ref. 4), we made a rough estimation of the inner thylakoid pH, which is given on the top of Fig. 6. Additionally we assume, that for intact thylakoids there exists an upper limit for the internal proton concentration of the order of 40–50 μM, in reasonable agreement with data found under high intensity continuous illumination [24]. For the sake of simplicity we assume, that in the range $5.0 \leqslant \text{pH} \leqslant 6.5$ of the chloroplast suspension the steady state ΔpH linearly rises from 0.6 to 1.75, whereas from pH 6.5 up to 9.0 the increment of the ΔpH-increase gradually declines, so that ΔpH varies from 1.75 up to 2.6.

It must be clearly emphasized that our calibration gives only a rough approximation, but the exact absolute values are not of crucial importance for the interpretation of the experimental results. Taking into account the above mentioned calibration, the data of fig. 2 can be simply described by an equation of the form:

$$\Delta A_0(\text{Chl-}a_{\text{II}},\ 35\ \mu\text{s}) = \frac{a + [\text{H}^+]_{\text{in}}}{b + [\text{H}^+]_{\text{in}}} \cdot c \qquad (1)$$

The best fit was obtained with $a = 2.5 \cdot 10^{-6}$ M, $b = 1.5 \cdot 10^{-5}$ M and $c = 2.6 \cdot 10^{-3}$.

For the interpretation of Eqn. 1 generally two different types of mechanism can be proposed for the regulation of the chlorophyll $a_{\text{II}}{}^+$ reduction by $[\text{H}^+]_{\text{in}}$, if one accepts the hypothesis, that a special electron carrier D_1 in its reduced form is indispensible for the occurrence of the $\leqslant 1$ μs kinetics (see ref. 9) : (a) Allosteric regulation by a protonizable group R_a, which controls either the functional connection between D_1 and chlorophyll $a_{\text{II}}{}^+$ or the redox equilibrium between D_1 and the charge accumulation state of the water-splitting enzyme system Y. R_a itself does not participate in the electron transfer reactions. In the simplest case (R_a binds only one H^+; ΔA_0(Chl-a_{II}) is proportional to the concentrations of the forms $R_a \cdot H^+$ and R_a, but with different coefficients) of this type of regulation the parameter b represents directly the dissociation constant, K_D, of the group R_a, i.e.

$$b = K_D = \frac{[\text{H}^+]\,[\text{R}_a]}{[\text{R}_a \cdot \text{H}^+]} \qquad (2)$$

(b) Direct regulation via the protonation of the intermediary species of the water-oxidation (see refs. 17, 25) and of D_1 as well as of their redox equilibrium. In this case the mathematical analysis becomes more complicated and Eqn. 1 is obtained only

under special assumptions (Renger, G., in preparation). However, also in this model protonizable groups with a dissociation constant of the order of 10^{-5} M could be of high importance.

Hence, irrespective of the mechanistic details of the regulation, which still remain to be clarified, the common feature of both models is the proton binding site for the control of the extent of the very fast ($\leqslant 1$ μs) reduction kinetics of chlorophyll a_{II}^+. This value is estimated to be of the order of p$K \approx 5$. It is interesting to note that a slightly more acidic group (p$K \approx 4.7$) was postulated to influence the rate of the electron flux from System II into the plastoquinone pool [23], but it must be emphasized that a completely different mechanism (regulation by $[H^+]_{in}$ of the number of functional active Systems II) is claimed to be operative. Nevertheless, the data so far known favour the existence of protonizable groups with pK values of the order of 4.7–5.2, which exert a regulatory role on the electron transport of System II.

Some years ago it was shown, that the reduction kinetics of chlorophyll a_I^+ is also dependent on $[H^+]_{in}$ [2]. The control by internal pH of the chlorophyll-cation radical reactions in the dark of both photosystems provide a further argument for the localization of chlorophylls a_I and a_{II} near the inner side of the thylakoid membrane.

However, it must be stressed that the regulation by $[H^+]_{in}$ of the dark reductions of chlorophylls a_I^+ and a_{II}^+, respectively, is probably based on completely different mechanism. The half life time of the reduction kinetics of chlorophyll a_I^+ continuously increases by more than one order of magnitude as the pH of the chloroplast suspension falls down from 7 to 4.5. On the contrary, according to the present results the dark reduction of chlorophyll a_{II}^+ is regulated mainly by the $[H^+]_{in}$-dependent contribution of different electron transfer reactions, with $\tau_{\frac{1}{2}}(1) \leqslant 1$ μs, $\tau_{\frac{1}{2}}(2) \approx 35$ μs and $\tau_{\frac{1}{2}}(3) \approx 200$ μs, to the overall process, whereas the half life time at least for the 35 μs kinetics seem to be influenced only slightly by pH (unpublished results).

The variation of the normalized amplitudes of the different reduction kinetics of chlorophyll a_{II}^+ should be reflected by the delayed fluorescence too, because according to Lavorels hypothesis [26] its intensity decay is linearly related to the decay of the state X320$^-$ · chlorophyll a_{II}^+. As the reoxidation of X320$^-$ ($\tau_{\frac{1}{2}} \approx 600$ μs, see refs. 1, 27) is comparatively slow, in the first 100–200 μs the delayed fluorescence decay is mainly determined by the decay of chlorophyll a_{II}^+. Recently, it was shown that the delayed fluorescence intensity measured 100 μs after the actinic flash has a similar dependency on the pH of the chloroplast suspension (see Fig. 5 of ref. 28) as the amplitude of the 35 μs kinetics, ΔA_0(Chl-a_{II}; 35 μs), depicted in Fig. 2. Furthermore, the addition of ionophoric uncouplers shifts the increase of delayed fluorescence intensity at 100 μs by more than 2 pH units towards the acidic range, analogous to the pH dependency of ΔA_0(Chl-a_{II}; 35 μs). The correspondence of the present data with the results obtained for delayed fluorescence [28] is illustrated in Fig. 7. It is seen that the dependency of the sum of the amplitudes $C_1 \cdot \Delta A_0$(Chl-a_{II}, 35 μs)$+C_2 \cdot \Delta A_0$ (Chl-a_{II}, 200 μs) on the pH value of the chloroplast suspension closely resembles that of the delayed fluorescence intensity at 100 μs after the actinic flash (redrawn from fig. 5 of ref. 28 and normalized to the corresponding value of ΔA_0(Chl-a_{II}) at pH 6.0 of the control chloroplasts) in the absence as well as in the presence of ionophoric uncouplers. $C_1 = 0.14$ and $C_2 = 0.71$ give the contribution of the 35 μs and 200 μs kinetics, respectively, to the total amplitude of ΔA_0(Chl-a_{II}) at 100 μs. Therefore, our

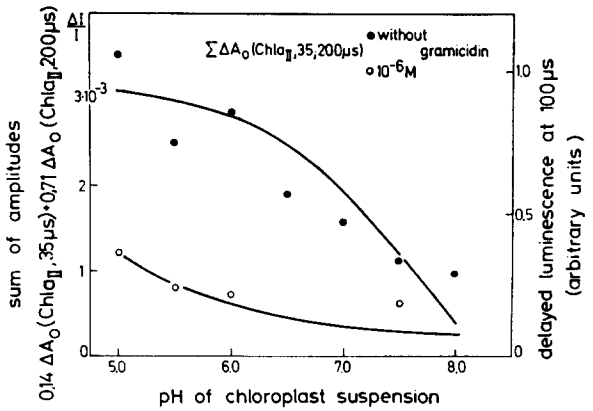

Fig. 7. Sum of the amplitudes of the 35 μs and 200 μs kinetics of the chlorophyll a_{II}-absorption change and delayed fluorescence intensity at 100 μs as a function of pH of the chloroplast suspension in the absence and in the presence of ionophoric uncouplers. The curves give the delayed fluorescence redrawn from fig. 5 of ref. 28. The normalization was made at pH 6.0 of the corresponding values of chloroplasts without uncouplers.

interpretation of the pH effect on the "detected" amplitude of the chlorophyll a_{II} absorption changes is fully consistent with the data found for delayed fluorescence.

It should be noted that a similar correlation between the amplitudes of the "detectable" absorption changes due to chlorophyll a_{II} and the delayed light intensity in the μs range has been shown to exist in ref. 9 (Fig. 6) for the oscillatory pattern in a flash train. It must be emphasized, that in both cases the parallelism between delayed fluorescence and the "detectable" chlorophyll a_{II}^+ amplitudes was explained by the same basic model, namely the existence of a very rapid ($\leqslant$ 1 μs) kinetics (not detectable by our measuring device) which extent is dependent on the charge accumulation state of the water-splitting enzyme system Y as well as on the proton concentration in the inner thylakoid space. That means, the phenomena of delayed fluorescence in the μs range are mainly explainable by the kinetic pattern of the chlorophyll a_{II}^+ reduction rather than by influences on the activation energy for charge recombination to excited singlets. As the functional integrity of the reaction center of System II was shown to remain nearly unaffected even under conditions leading to a complete destruction of the water-splitting enzyme system Y (e.g. Tris-washing, see e.g. ref. 29), the present study has been restricted to a pH range, where system Y is not seriously modified, in order to obtain information about the role of protons for the regulation of the electron flow from water to the reaction center. At pH values below 5 there occur dramatic changes, reflected by the steep increase of the 200 μs kinetics, as is shown in Fig. 2 and thoroughly discussed in refs. 10 and 11.

A last point should be mentioned. Our analysis is based on the assumption of a 1 : 1 functional and structural connection between the reaction center of Photosystem II (i.e. chlorophyll a_{II}) and the water-splitting enzyme system Y. Very recently, an interesting model was proposed, postulating a time restricted diffusive connection between chlorophyll a_{II}^+ and system Y, with chlorophyll a_{II} fixed in the membrane and Y mobile in the inner phase of the thylakoids [16]. If the cross-linkage of Y to chlorophyll a_{II}^+ is assumed to be dependent on $[H^+]_{in}$ the present data are also explainable

by this model. However, this mode of regulation by internal pH can be considered as another way of realizing the allosteric model discussed here. If our interpretation is correct, then under physiological conditions, where the inner thylakoid pH ranges between 5 to 7 [30] depending on the illumination conditions, the regulatory mechanism by $[H^+]_{in}$ of the chlorophyll $a_{II}{}^+$-reduction discussed in this paper is operative in vivo. The physiological role of the mechanism remains to be clarified.

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