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THE REDUCTION KINETICS OF CHLOROPHYLL a_I AS AN INDICATOR FOR PROTON UPTAKE BETWEEN THE LIGHT REACTIONS IN CHLOROPLASTS

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SUMMARY

The flash-induced oxidation kinetics of the primary acceptor of light Reaction II (*X*-320) and the reduction kinetics of chlorophyll a_I (*P*-700) after far-red preillumination have been studied with high time resolution in spinach chloroplasts.

1. The kinetics of chlorophyll a_I exhibits a pronounced lag phase of 2–3 ms at the onset of reduction as would be expected for the final product of consecutive reactions. Because the oxidation of the plastoquinone pool is the rate-limiting step for the electron transport between the two light reactions, the lag indicates the maximal electron transfer time over all preceding reactions after light Reaction II.

2. The observation that the lag phase decreases with decreasing pH is evidence of an electron transfer step coupled to a proton uptake reaction.

3. Protonation of *X*-320 after reduction in the flash is excluded because a slight increase of the decay time is found at decreasing pH values.

4. The time course of plastohydroquinone formation is deduced from the first derivative of the reduction kinetics of chlorophyll a_I. This approach covers those plastohydroquinone molecules being available to the electron carriers of System I via the rate-limiting step. Direct measurements of absorbance changes would not allow to discriminate between these and functionally different plastohydroquinone molecules.

5. The derived time course of plastohydroquinone at different pH gives evidence for an additional electron transfer step with a half time of about 1 ms following the proton uptake and preceding the rate-limiting step. It is tentatively attributed to the diffusion of neutral plastohydroquinone across the hydrophobic core of the thylakoid membrane.

6. The lower limit of the rate constant for proton uptake by an electron carrier, consistent with the lag of chlorophyll a_I reduction, is estimated as $> 10^{11}\ M^{-1} \cdot s^{-1}$. The value is higher than that of the fastest diffusion controlled protonations of organic molecules in solution.

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Possible mechanisms of linear electron transport between light Reaction II and the rate-limiting oxidation of neutral plastohydroquinone are thoroughly discussed.

INTRODUCTION

In the photosynthesis of higher plants, linear electron transport is driven by two light reactions coupled in series (for a review see ref. 1). Between the light reactions there is a sequence of electron carriers beginning with the primary acceptor of light Reaction II and ending with chlorophyll a_I (P-700), the component directly oxidized by light Reaction I [2]. When all of the electron carriers are oxidized by far-red illumination [3], chlorophyll a_I^+ cannot be reduced until the electrons produced by light Reaction II pass through all of these electron carriers sequentially. In terms of kinetics, reduced chlorophyll a_I is the final product of consecutive dark reactions.

The electron transfer steps known from absorbance changes are illustrated in Fig. 1. The rate-limiting oxidation of plastoquinone ($t_{\frac{1}{2}} \approx 20$ ms) governs the rate of

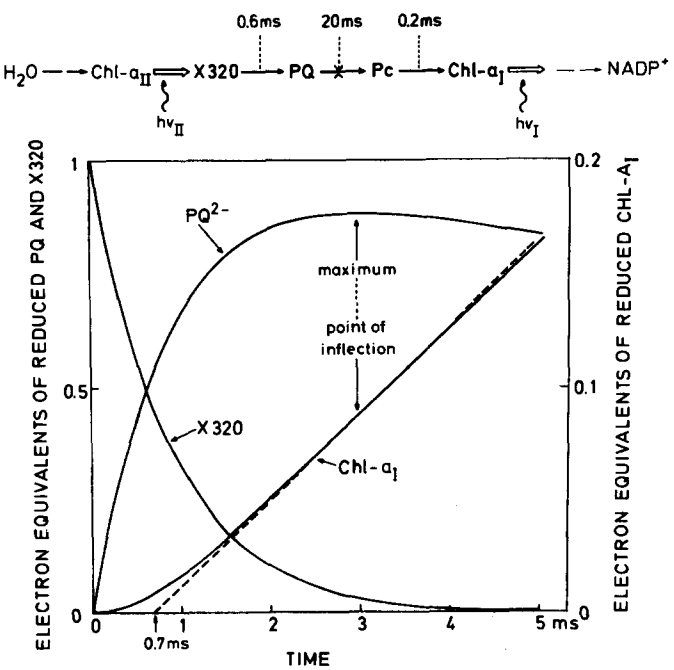


Fig. 1. Above: Simplified scheme of linear electron transport [1]. Below: Schematic illustration of the time course of X-320 oxidation [4] and the expected time course of plastoquinone and chlorophyll a_I reduction after a flash following far-red preillumination. The trace of chlorophyll a_I, plotted in the expanded scale on the right hand side, is calculated according to

$$[\text{Chl-}a_I] = 1+(k'_2 \cdot \exp(-k_x \cdot t)-k_x \cdot \exp(-k'_2 \cdot t))/(k_x-k'_2).$$

For further details see text. Abbreviations: Chl-a_I, chlorophyll a_I; Chl-a_{II}, chlorophyll a_{II}; Pc, plastocyanin; PQ, plastoquinone.

reduction of chlorophyll a_I^+ by electrons coming from light Reaction II [4, 5]. This reduction cannot be affected by the subsequent fast electron transfer from plastocyanin to chlorophyll a_I^+ ($t_{\frac{1}{2}} = 200\ \mu s$) [6, 7]. However, electron transfer from the primary acceptor of light Reaction II, called either *X*-320 [8] or Q [9], to the plastoquinone pool ($t_{\frac{1}{2}} = 0.5$–0.6 ms) [10, 11, 4] must cause a delay of the reduction of chlorophyll a_I^+ in the order of 0.6 ms resulting in a sigmoidal time course.

A good deal of evidence has been accumulated suggesting that the light induced absorbance changes of *X*-320 peaking around 320 nm [8] are those of the primary acceptor of light Reaction II [4, 12, 13]. The identity of *X*-320 with a component responsible for absorbance changes around 550 nm (*C*-550), also attributed to the primary acceptor of light Reaction II [14, 15], has recently been demonstrated [13, 16, 17]. The increase in absorbance of *X*-320 occurs within $<30\ \mu s$ and is followed by a first-order decay of $t_{\frac{1}{2}} = 0.6$ ms, the half-time for the electron transfer from water to the plastoquinone pool [4]. The involvement of a secondary acceptor of light Reaction II, called either B or R, is shown by different measurements [18–20]. Nevertheless, the expected reduction kinetics of chlorophyll a_I induced by a saturating flash after far-red preillumination can be approximated by an ordinary first-order reaction sequence of two steps with rate constants $k_x = 1150\ s^{-1}$ and $k_2' = 43\ s^{-1}$ for the rate-limiting step after one flash [4].

The time courses are shown in Fig. 1. The exponential decay of *X*-320 is an experimental result taken from Stiehl and Witt [4]. The calculated rise of the plastohydroquinone concentration (cf. ref. 1) has as yet not been experimentally resolved. The time intercept of the tangent at the point of inflection of the sigmoidal time course of chlorophyll a_I is designated in the following as the lag of the chlorophyll a_I reduction. It should be emphasized that this lag is completely different from the lag phase of the oxidation of chlorophyll a_I upon far-red illumination [21–23]. The latter depends on the intensity of the far-red light and the amount of electrons accumulated in the plastoquinone pool and in the immediate donors of chlorophyll a_I [24].

An additional reaction step not regarded in the above approach may be due to the vectorial proton translocation across the thylakoid membrane coupled to linear electron transport [25–27]. In support of a proton uptake from the outside by reduced plastoquinone, as postulated by Mitchell [28], are the high p*K* value of hydroquinones and the uptake of one proton per electron reducing the plastoquinone pool [29, 30]. However, a direct coupling of an electron transfer step with the proton uptake has not yet been demonstrated. An indication that this may be the case is seen from the lag phase of chlorophyll a_I reduction. Instead of the 0.7 ms lag phase predicted on the basis of the half time of *X*-320 oxidation, we have previously reported a longer lag phase of about 2 ms [5]. In addition, the rate of proton uptake detected with indicator dyes, which in earlier reports was in disagreement with the rate of electron transfer reactions [27, 30], has recently been measured to have a half time of 2 ms in the presence of carbonylcyanide-*p*-trifluormethoxyphenylhydrazone [31].

In this work the sigmoidal reduction kinetics of chlorophyll a_I under different experimental conditions are presented. In order to analyse the reactions causing the lag, the time courses of *X*-320 and the lag phase are studied as a function of the proton concentration. Based on the reduction kinetics of chlorophyll a_I the time course of plastohydroquinone formation, that has not yet been experimentally measured, is derived. The location as well as the magnitude of the rate constants of the proton

uptake and other reaction steps preceding the rate-limiting step between the two light reactions are also derived and discussed. A preliminary report of these results has previously been given [32].

MATERIALS AND METHODS

Envelope free spinach chloroplasts were prepared as in ref. 5. Reaction mixtures contained chloroplasts, at a concentration of 10 μM chlorophyll, 20 mM KCl, 1 mM $MgCl_2$, 10 μM benzylviologen as electron acceptor and 1 μM gramicidin D for uncoupling. The pH was adjusted at values between 5.6 and 7.0 with 20 mM 2-(*N*-morpholino)-ethanesulfonic acid/NaOH and between 7.2 and 8.4 with 20 mM *N*-tris(hydroxymethyl)methylglycine/NaOH. Reaction mixtures of a viscosity of 8 cP and 40 cP contained additionally 12 and 24 g ficoll per 100 ml, respectively. The high polymer ficoll was obtained from Pharmacia. The temperature was 20–22 °C.

Chloroplasts, suspended in a 20 × 20 mm cuvette, were illuminated repetitively with flashes of saturating light intensity at a frequency of 0.2 Hz and continuously with far-red light (720 nm, $\Delta\lambda = 15$ nm) at an intensity of 10^4 ergs · cm^{-2} · s^{-1}. The content of the sample cuvette was changed after every 64 signals. The absorbance changes of chlorophyll a_I were measured at 705 or 708 nm (band width $\Delta\lambda = 3$ nm) and those of *X*-320 at 335 nm ($\Delta\lambda = 2.5$ nm). The intensity of the monitoring light was 200 ergs · cm^{-2} · s^{-1}. The experimental technique and equipment described in ref. 5 was used. The response time of the apparatus was limited by the sweep speed of 20 μs per address of the Fabri-Tek 1072 signal averager with plug-in SD-72/IA and SW-70. To enable a high intensity of the monitoring light for an improved signal to noise ratio two of the six dynodes of a photomultiplier EMI D 214 were connected to the anode.

RESULTS

The lag of chlorophyll a_I reduction kinetics

The kinetics of chlorophyll a_I induced by a flash after far-red preillumination are shown in Fig. 2, upper curve. In continuous far-red light about 25–30 % of the total chlorophyll a_I remains reduced [3]. This fraction of chlorophyll a_I is, however, oxidized in the saturating flash. Following the flash, the reduction of chlorophyll a_I^+ has a half time of 16 ms in agreement with previous results [23, 3–5]. Fig. 2, lower curve, shows the initial kinetics at an increased resolution. A pronounced lag of the reduction of chlorophyll a_I after the flash is observed. If the far-red light is switched off 0.1 s before the flash, the lag is unchanged (see Fig. 7). Since the 2.8 ms lag is much longer than the 0.7 ms lag predicted from Fig. 1, the formation of plastohydroquinone is delayed more than corresponds to the transfer for an electron from H_2O to the plastoquinone pool [4, 11]. This cannot originate from a loss of electrons because a quantitative electron transport from H_2O to chlorophyll a_I^+ has been found under the same experimental conditions [33].

It may be due to a contribution from the proton uptake reaction. If the pH is lowered, one would expect that the proton uptake reaction is accelerated. This can be examined by measuring the lag phase as a function of the pH as shown in Fig. 3. The decrease of the lag from 2.8 ms at pH 8 to 1.6 ms at pH 6 is evidence for an

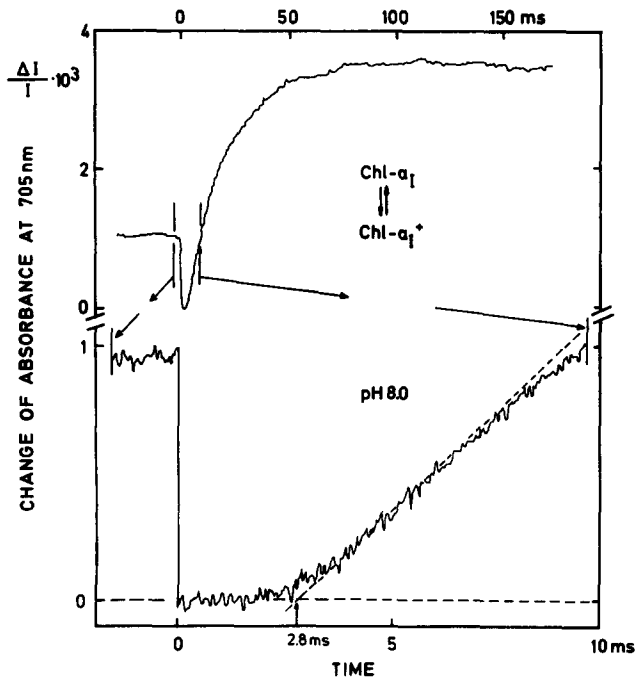


Fig. 2. Absorbance changes of chlorophyll a_I at 705 nm as a function of time induced by a flash at continuous far-red illumination at pH 8.0. Above: Average over 32 signals. Below: The marked time interval of the upper trace was recorded at a faster sweep speed of 20 μs per address. The dashed line indicates the tangent at the point of inflection. Average over 256 signals. Abbreviation: Chl-a_I, chlorophyll a_I.

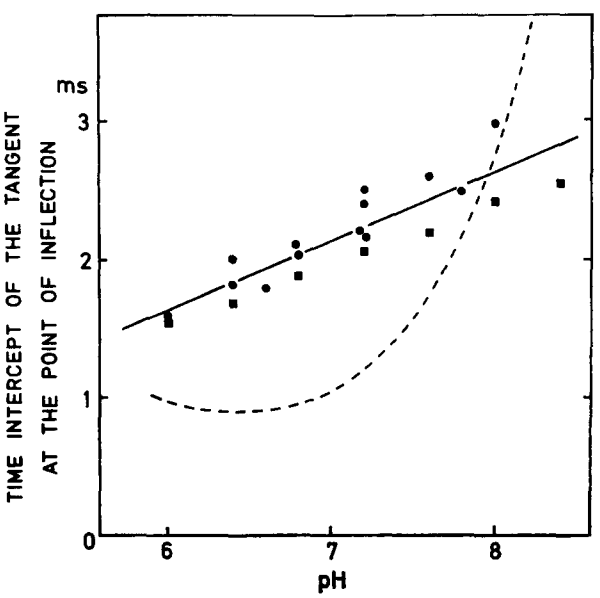


Fig. 3. The time intercept of the tangent at the point of inflection of the reduction kinetics of chlorophyll a_I at 705 nm as a function of pH. Experimental conditions as in Fig. 2, below. The dashed line results from kinetics calculated from Eqn. 1 (see text).

electron transfer step occurring before the rate-limiting step which can be accelerated by protons. At omission of the uncoupler gramicidin D the lag is not increased.

After the lag, a retardation of the rate of chlorophyll a_I reduction at decreasing pH values was observed in support of the previous results [34, 22]. The first half time increased from 16 ms at pH 8 (Fig. 2) to 23 ms at pH 6 (kinetics not shown). The decreasing slope of the reduction kinetics coupled with the concomitant decrease of the lag at decreasing pH made an estimation of the lag difficult at pH values lower than 6.

Decay kinetics of the primary acceptor of light Reaction II

The kinetics of the primary acceptor of light reaction II should help to localize the proton uptake step. The increase of absorbance of *X*-320 has been attributed to the reduction of a special plastoquinone of light Reaction II to form a plastosemiquinone anion radical [8, 12, 13, 35]. A decrease of the absorbance at 320 nm would

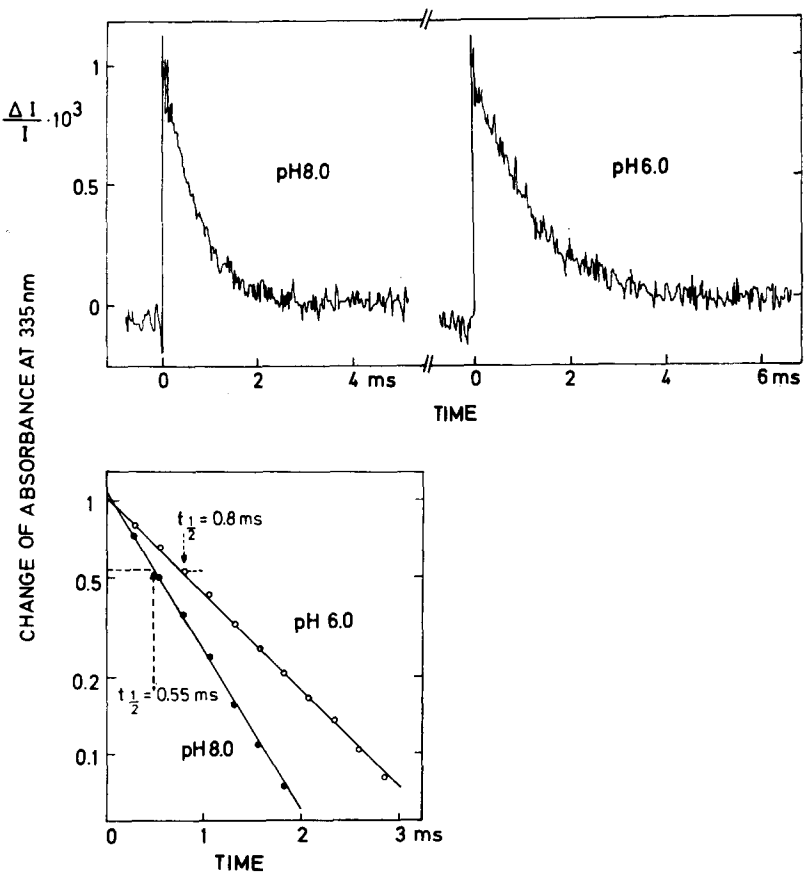


Fig. 4. Absorbance changes of *X*-320 at 335 nm as a function of time at pH 8.0 and at pH 6.0. Above with linear, below with logarithmic time scale. Both traces are the average of 1024 signals at a repetition rate of 1 Hz. The content of the sample cuvette was changed after 512 signals.

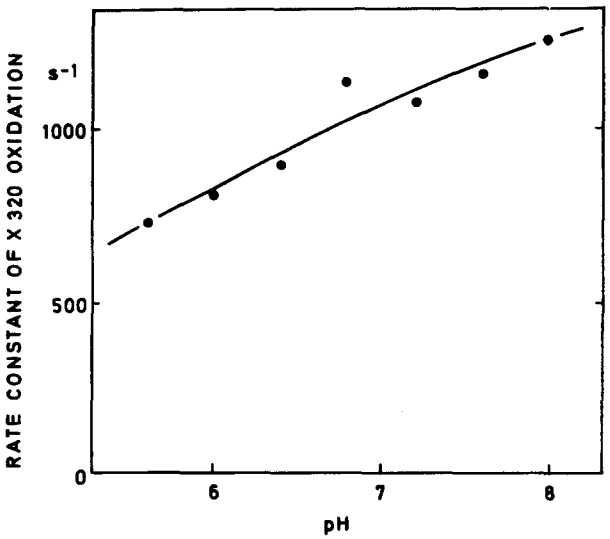


Fig. 5. Rate constant of the *X*-320 oxidation as a function of pH. Experimental conditions as in Fig. 4.

be observed if either a plastohydroquinone dianion or a neutral plastosemiquinone radical were formed from the plastosemiquinone anion radical [35]. One of these possibilities can be eliminated by the relaxation kinetics of *X*-320 at different pH values. Fig. 4 shows these kinetics at pH 8.0 and pH 6.0. The semi-logarithmic plots indicate the first-order nature of these kinetics and half times of 0.55 ms and 0.8 ms at pH 8.0 and pH 6.0, respectively. Fig. 5 shows the first-order rate constants ($k = \ln 2/t_{\frac{1}{2}}$) as a function of pH. This result was not changed by omission of gramicidin D or by increasing the repetition rate of the flashes from 0.2 Hz to 1 Hz. The slight decrease of the rate constant instead of an increase at decreasing pH provides evidence against protonation of *X*-320.

Kinetic approach to the proton uptake reaction

The proton uptake step, which may be indicated by the chlorophyll a_I reduction, lag must be located after the reoxidation of *X*-320. To better describe the reaction sequence, a proton uptake step of pseudo first-order is introduced into the basic scheme in Fig. 1. Designating the rate constant as k'_H, the sequence would then be:

$$X320 \xrightarrow{k_x} \tfrac{1}{2}PQ^{2-} \xrightarrow[H^+]{k'_H} \tfrac{1}{2}PQH_2 \xrightarrow[H^+]{k'_2} \text{Chl-}a_I$$

The differential equations of this first-order reaction sequence are easily integrated for the initial conditions also assumed for the calculation in Fig. 1: After the flash at $t = 0$ *X*-320 is reduced by one electron equivalent per light reaction, plastoquinone and chlorophyll a_I are fully oxidized. In addition, equal numbers of the two light reactions and equilibrium constants $\gg 1$ for all electron transfer steps are presumed. This results in the following equation for the electron equivalents of reduced chlorophyll a_I per light reaction [Chl-a_I] as a function of the time t:

$$[\text{Chl-}a_1] = 1 - \left(\frac{k'_H \cdot k'_2 \cdot \exp(-k_x \cdot t)}{(k'_H - k_x)(k'_2 - k_x)} + \frac{k_x \cdot k'_2 \cdot \exp(-k'_H \cdot t)}{(k_x - k'_H)(k'_2 - k'_H)} + \frac{k_x \cdot k'_H \cdot \exp(-k'_2 \cdot t)}{(k_x - k'_2)(k'_H - k'_2)} \right) \quad (1)$$

Calculation of the time courses of reduced chlorophyll a_1 at decreasing pH values from 8 to 6 have to take into account the decrease of k_x from 1260 s^{-1} to 850 s^{-1} shown in Fig. 5, of k_2' from 43 s^{-1} to 30 s^{-1} as reported by Rumberg and Siggel [34] (see also above), and the increase of $k'_H = k_H \cdot c_{H^+} \cdot k_H$, the second-order rate constant of the protonation reaction, was chosen as $3 \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$ to give the best fit of the lag of the calculated chlorophyll a_1 reduction with the experiments at pH 8. The resulting half time of 2.3 ms for the proton uptake at pH 8 agrees with the experimentally detected half time of the proton uptake reaction at this pH measured by Ausländer and Junge [31]. Thus, the proton uptake seems to contribute alone to the lag in addition to the oxidation of *X*-320. The lag of the calculated kinetics as a function of pH is plotted in Fig. 3 with the dashed line. The assumed reaction sequence causes a much greater dependence of the lag on the pH than actually found experimentally. The discrepancy indicates that at least one additional reaction step, which is not accelerated by increasing proton concentration, precedes the rate-limiting step.

Influence of temperature and viscosity on chlorophyll a_1 reduction

With regard to a fast proton uptake with a rate of diffusion controlled reactions

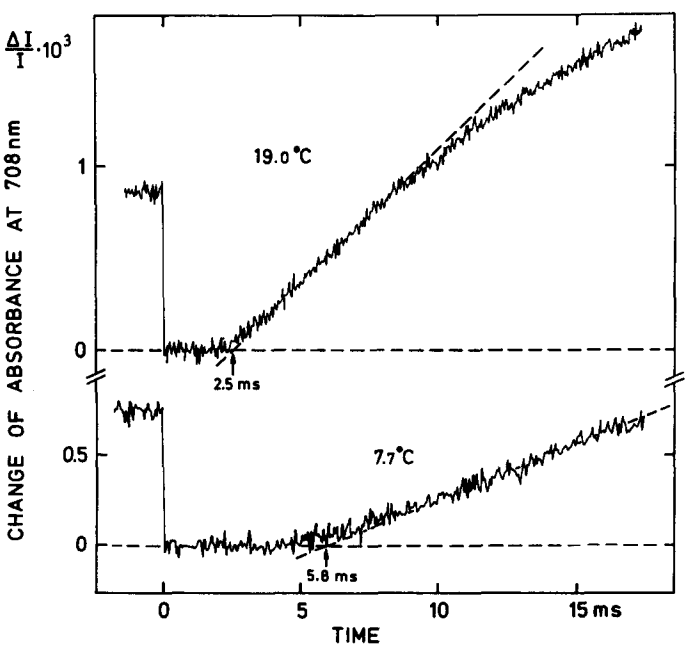


Fig. 6. Absorbance change of chlorophyll a_1 at 708 nm as a function of time induced by a flash at continuous far-red illumination at pH 7.2. The temperature during the measurement was 19.0 °C (above) and 7.7 °C (below). The dashed lines indicate the tangents at the point of inflection of the transients. Average over 256 signals.

the influence of temperature on chlorophyll a_I reduction has been investigated and is shown in Fig. 6. A decrease of the maximal rate of reduction of chlorophyll a_I by decreasing the temperature from 19 °C to 7.7 °C is indicated by the tangent at the point of inflection of the transients. The ratio of the two slopes is 2.4. Because the maximal slope and the rate constant of the rate-limiting step are proportional to each other (see below), the activation energy of the rate-limiting step can be estimated: $E_A \approx 13\ \text{kcal} \cdot \text{mol}^{-1}$. This value is similar to the value of $10-12\ \text{kcal} \cdot \text{mol}^{-1}$ reported by Cox [36] for the reduction of 2,6-dichlorphenolindophenol above 0 °C. The lag of chlorophyll a_I reduction is not directly related to the rate constants. However, as a first approximation, the increase of the lag from 2.5 ms at 19 °C to 5.8 ms at 7.7 °C indicates a decrease of the over-all reaction rate from light Reaction II to the rate-limiting step by a factor greater than two. At least one reaction step with a higher activation energy ($E_A > 10\ \text{kcal} \cdot \text{mol}^{-1}$) than that of diffusion controlled reactions ($E_A \approx 4\ \text{kcal} \cdot \text{mol}^{-1}$) should be engaged in this reaction sequence.

A proton uptake from the outer medium is expected to be slowed down by an increase of the viscosity. In order to determine the influence of viscosity on chlorophyll a_I reduction the viscosity of the reaction mixture was increased by additions of the high polymer ficoll, which causes negligible changes of osmotic pressure. The first half time for the reduction of chlorophyll a_I is not affected. The time of the lag, however, is slightly decreased from 2.4–2.5 ms at 1 cP (standard reaction mixture at pH 7.2) (cf. Fig. 6 above) to 2.3 ms at 8 cP, and to 2 ms at 40 cP (data not shown).

Derivation of the time course of plastohydroquinone formation

The time course of plastohydroquinone formation should indicate more details of the proton uptake reaction. It has, as yet, not been measured. However, it can be derived from the reduction kinetics of chlorophyll a_I if the following experimental results are used:

(1) The re-oxidation of plastohydroquinone (PQH_2) exhibits first-order kinetics after one flash or a group of a few flashes [37, 4, 5]:

$$-\frac{d[PQH_2]}{dt} = k_2' \cdot [PQH_2] \tag{2}$$

The interpretation of the exponential decay as pseudo first-order [4] does not affect the following derivation.

(2) The initial rates of the plastohydroquinone re-oxidation and of the chlorophyll a_I^+ (Chl-a_I^+) reduction are proportional to each other at increasing concentrations of plastohydroquinone [5]

$$\tfrac{1}{2}\frac{d[\text{Chl-}a_I^+]}{dt} \sim \frac{d[PQH_2]}{dt} \tag{3}$$

(3) After a flash following far-red preillumination, electrons are quantitatively transferred from H_2O via plastoquinone to chlorophyll a_I during the first half time of chlorophyll a_I reduction [33]. Therefore, the sign of proportionality in Eqn. 3 can be replaced by a sign of equality.

Thus, by combining these relations, the rate of chlorophyll a_I^+ reduction turns out to be proportional to the concentration of plastohydroquinone:

$$-\frac{d[\text{Chl-}a_I^+]}{dt} = 2 \cdot k_2'[PQH_2] \quad (4)$$

This relation can only be valid when the level of reduced chlorophyll a_I is less than 50 %, i.e. when the amount of reduced plastocyanin is still negligible due to the equilibrium constant of 10–20 [24, 7] between these two electron carriers.

The time course of the chlorophyll a_I absorbance change induced by two flashes can be used to examine Eqn. 4 and is shown in Fig. 7. The reduction kinetics after the first flash showing a lag of 2.4 ms (pH 7.2) have been discussed above. The second flash, fired 12 ms after the first one, completely oxidizes chlorophyll a_I again. However, 12 ms after a flash, plastoquinone is still reduced by more than 0.5 electrons per light Reaction II ($t_{\frac{1}{2}} \approx 20$ ms) [4, 5]. This concentration of plastohydroquinone should not be changed in the flash. According to Eqn. 4 the reduction rate of chlorophyll a_I immediately after the second flash should be different from zero and should be equal to the rate immediately before this flash. Both expectations are in agreement with the about equal slopes of the dotted tangents in Fig. 7. In addition, the reduction rate of chlorophyll a_I increases after the second flash over that after the first flash as indicated by the dashed tangents at the points of inflection. This is consistent with the accumulation of electrons in the plastoquinone pool remaining from the first flash and produced in the second flash [4, 5]. The points of inflection of the time courses occur at approximately equal times after the flashes. This indicates the same reaction mechanism of plastohydroquinone formation after both flashes.

The reduction rate of chlorophyll a_I contains the information about the concentration of plastohydroquinone. This enables one to derive the time course of plastohydroquinone formation. According to Eqn. 4, it is the first derivative of the chlorophyll a_I reduction kinetics in the lag phase region. Fig. 8 shows the first deriva-

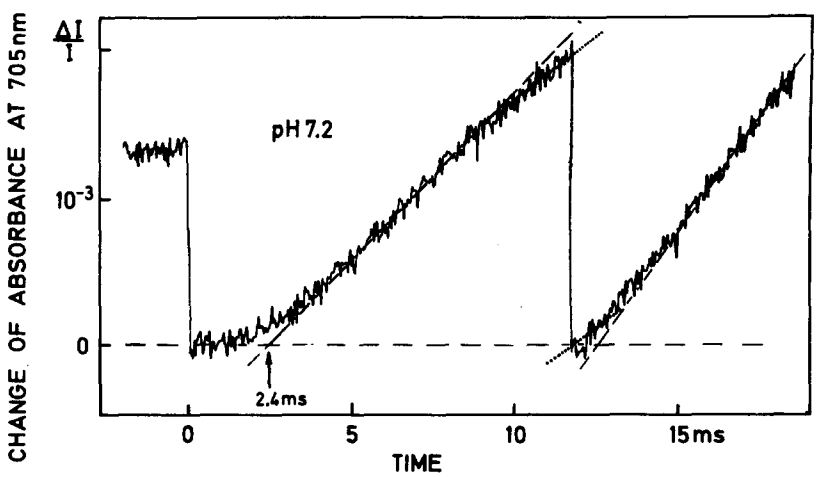


Fig. 7. Absorbance change of chlorophyll a_I at 705 nm as a function of time induced by two flashes spaced at 12 ms after far-red preillumination for 4.5 s with an intensity of $3 \cdot 10^4$ ergs $\cdot$ cm$^{-2} \cdot$ s^{-1}. The far-red light was switched off 0.1 s before the first flash. Dashed lines: tangents at the point of inflection. Dotted lines: tangents immediately before and after the second flash. Average over 256 signals.

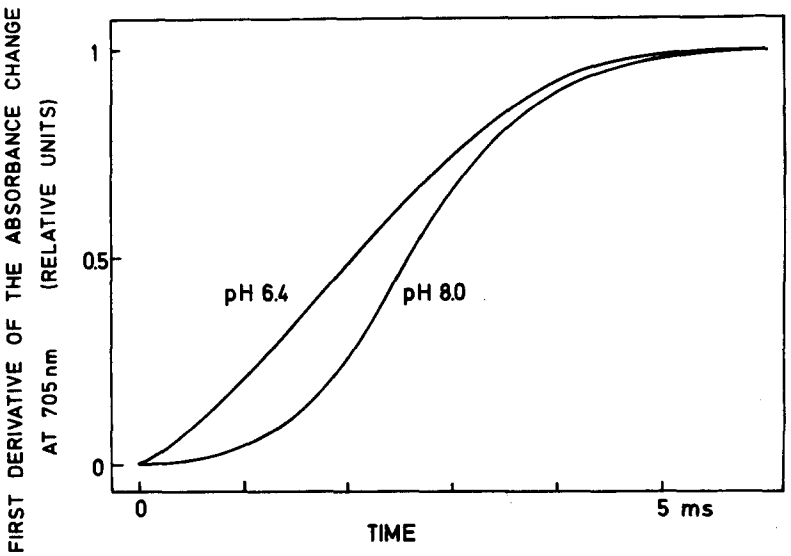


Fig. 8. Time course of the formation of plastohydroquinone derived from the initial time course of the absorbance changes of chlorophyll a_I at 705 nm. The experimental conditions for the measurement of the absorbance changes are the same as in Fig. 2 below. Both traces are the average from three different measurements. The assumptions for the derivation are given in the text.

tives of the reduction kinetics of chlorophyll a_I at pH 8.0 and 6.4 obtained point by point from the slope of the hand-smoothed transients. Due to the low signal to noise ratio (50–100) of the chlorophyll a_I transients the uncertainty of the derived plastohydroquinone kinetics at pH 8.0 and 6.4 is small enough to state the following properties, supported by transients measured at intermediate pH values (data not shown):

(1) The concentration of plastohydroquinone increases with sigmoidally shaped kinetics to its maximal value 5–6 ms after a flash. The half time of the rising phase is about 1–2 ms and seems to be slightly decreased at decreasing pH.

(2) The lag phase of the plastohydroquinone formation is diminished from about 1.5 ms to 0.3 ms as the pH is decreased from 8 to 6.4. This is evidence for a fast proton uptake reaction preceding the formation of plastohydroquinone.

It should be emphasized that this derivation concerns the formation of plastohydroquinone that is oxidizable by the electron carriers of System I via the rate-limiting step. It may be necessary to differentiate between this and the plastohydroquinone formed first.

The decreasing rate of reduction of chlorophyll a_I at decreasing pH, mentioned above, may be due to a decrease in the rate constant of the rate limiting step k_2' [34] as well as in the plastohydroquinone concentration (cf. Eqn. 4). However, an almost equal concentration of plastohydroquinone after one flash at the pH values used is favoured by the approximately constant activity of light Reaction II, indicated by the amplitudes of X-320 in Fig. 4, and the great difference between k'_2 and the other rate constants. The normalization of the first derivatives in Fig. 8 to the slope of the tangents at the point of inflection accounts for this reasoning.

DISCUSSION

The lag phase of the reduction kinetics of chlorophyll a_I is inevitable for a component located at the end of a chain of oxidized electron carriers. Its finding is in support of this location of chlorophyll a_I participating in linear electron transport between the two light reactions. Since not only a loss of electrons but also redox equilibria would increase the lag, it gives the upper limit of the electron transfer time over all forward reactions from the primary acceptor of light Reaction II to the rate-limiting step between the light reactions. This allows clear restrictions on possible mechanisms.

On the reaction of the primary acceptor of light Reaction II

Evidence has been presented for showing that the decay of the absorbance changes of the primary acceptor of light Reaction II, *X*-320, is not due to a proton uptake. The decay of the *X*-320 absorbance is tentatively attributed to the formation of a plastohydroquinone dianion from two plastosemiquinone anion radicals. This may be supported by the different finding of Renger [38] of an approximately pH independent half time of 0.6 ms of oxygen evolution in the second flash of a double flash.

A two electron accumulating secondary acceptor of light Reaction II, presumable a special plastoquinone B different from the pool, is indicated by oscillations with a periodicity of two after increasing numbers of flashes of the methylviologen reduction by electrons from light Reaction II [18], of the fluorescence [19] and of the oxygen yield under special conditions [20]. These experiments do not support the hypothesis of a cooperation of two light Reactions II during dismutation of two *X*-320 [4, 39]. Oscillations should be completely damped at our excitation conditions (cf. ref. 40). However, consistent with the proposed mechanism [18,19] and with respect to the amplitude after one flash, the decay of the *X*-320 absorbance should be contributed to the formation of B^{2-} fron $X\text{-}320^-$ and B^-:

$$X\text{-}320^- \text{–} B^- \rightarrow X\text{-}320\text{–}B^{2-}$$

A superposition of these and minor absorbance changes arising from different absorbance spectra of the plastosemiquinone anion radicals of the primary and secondary acceptor may occur during simultaneous transfer of the first electron:

$$X\text{-}320^- \text{–} B \rightarrow X\text{-}320\text{–}B^-$$

The slight increase of the relaxation time of the *X*-320 absorbance changes at an increase in the proton concentration by a factor of 100 in Fig. 5 is not yet understood. Changes in redox potentials or in the structural relationship of the electron carriers could occur. The rising phase of the derived kinetics for plastohydroquinone formation seen in Fig. 8 seems to exhibit a similar increase.

Derived kinetics of plastohydroquinone formation

Kinetics of reaction components can generally be derived from the time courses of other components if the order of reaction of each species participating is known. The rate constants and the oxidation-reduction equilibria in linear electron transport allow the rather simple approach to the kinetics of plastohydroquinone described above.

The derived kinetics for plastohydroquinone formation (see Fig. 8) provides evidence for two new consecutive reactions in linear electron transport, a protonation step followed by an additional step of 1–2 ms indicated by the rising phase. If protonation directly yields neutral plastohydroquinone the consecutive additional reaction step does not change the type of molecules but makes plastohydroquinone available to the electron carriers of System I. The described derivation would be the only approach to the kinetics of the latter process because spectroscopic measurements could only detect the total neutral plastohydroquinone.

With respect to the indirect information obtained from the lag of chlorophyll a_I reduction it is not possible to discriminate between a contribution to proton uptake from reduced plastoquinone [28] or from a special proton pump molecule as has been suggested for the proton release to the inner phase [41]. However, the above suggested protonation of plastohydroquinone is strongly supported not only by high pK values of other hydroquinones [42] but also by the difference spectrum of the absorbance changes of plastoquinone which shows a good agreement with the difference spectrum of neutral plastohydroquinone minus plastoquinone [8]. Although the spectrum of the plastohydroquinone dianion is not known, its difference spectrum should exhibit a distinct absorbance at around 320 nm due to a bathochromic shift of the peak and an increase of the extinction coefficient compared to the neutral form [42]. Thus, in more detailing previous formulations [1, 4, 37], the concentration of protonated plastohydroquinone should determine the rate of electron transfer via the rate-limiting step.

Kinetic limitations to proton uptake mechanisms

As pointed out above a reaction not accelerated at decreasing pH contributes to the lag in addition to proton uptake and the reoxidation of X-320. To derive limitations to proton uptake directly coupled to linear electron transport, we have calculated the time courses of plastohydroquinone and chlorophyll a_I from the simplest model that incorporates these experimental findings. It is the sequence assumed for Eqn. 1 expanded by a pH independent step with a half time of 1 ms between the proton uptake and the rate-limiting step. If k_H is chosen as $10^{11}\ M^{-1} \cdot s^{-1}$, the calculated time courses of plastohydroquinone at pH 6.4 and 8 as well as the pH dependence of the lag of chlorophyll a_I reduction can be designated as a first approximation to the experimental results in Figs. 3 and 8. $10^{11}\ M^{-1} \cdot s^{-1}$ would, however, be the lower limit of a rate constant for protonation of any electron carrier engaged in linear electron transport. Neutral plastohydroquinone should be formed from the dianion after two protonation steps. Therefore, corresponding combinations of the rate constants of two consecutive protonations with the higher one of at least $1.5 \cdot 10^{11}\ M^{-1} \cdot s^{-1}$ have to be postulated.

These estimated rate constants are larger than the rate constants of the fastest protonations of organic molecules in aqueous solutions of $5 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$ [43]. An explanation for the discrepancy would be that the proton concentration at the site of the proton uptake reaction in the membrane is at least a factor of 3 higher than in the medium, even in the presence of high concentrations of gramicidin. Another, as yet indistinguishable, possibility may be a faster diffusion controlled reaction of protons in the plane of the membrane compared to that in solution. A reduction of dimensionality has been shown by Richter and Eigen to effectively enhance reaction rates [44].

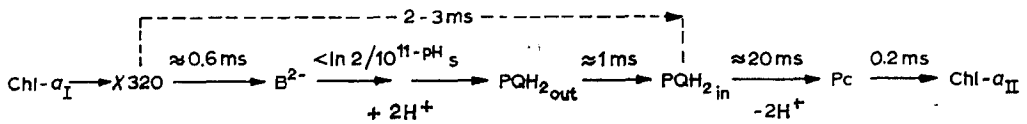
The half time of 2 ms for proton uptake detected with pH indicator dyes by Ausländer and Junge [31] seems to be too slow. At pH 8, a half time shorter than 0.7 ms would be necessary to be consistent with our results. A residual permeability barrier may delay the dissociation of the indicator dye [31]. Experiments in which the viscosity of the medium is increased from 1 to 40 cP show no increase of the lag phase for chlorophyll a_I reduction. This would be possible if ficoll is not able to approach the proton uptake site, the site probably being separated from the outer medium by a diffusion barrier as pointed out by Junge and Ausländer [30]. During the experiments this barrier could cause a lower proton activity at the site of the proton uptake than in the outer medium. Therefore, the actual rate constant of the protonation may even be larger than the lower limit estimated above.

On the electron transport mechanism between the light reactions

There are several possibilities for molecular realization of each of the discriminated reaction steps. The protonation of the secondary acceptor could be involved in one possible mechanism consistent with the results. This would be favoured if the electron carrier were a special plastoquinone [18–20]. Another possible mechanism would be a fast two electron transfer from the secondary acceptor to the plastoquinone pool followed by proton uptake by a plastohydroquinone dianion.

A candidate for the reaction step, having a half time of about 1 ms and not being accelerated by protons, is the diffusion of neutral plastohydroquinone in the pool. In the case of protonation of the secondary acceptor an additional contribution by the simultaneous transfer of two electrons and two protons to the pool is possible. A vectorial diffusion of neutral plastohydroquinone from the outer to the inner side of the hydrophobic core of the thylakoid membrane would not need a special proton transfer protein for a separate transfer of electrons and protons as suggested by Siggel [41]. In the latter model the diffusion would be in the plane of the membrane [45]. To discriminate between combinations of these possibilities through sets of differential equations, integrated by a digital computer, too many rate constants are needed. They are, as yet, unavailable.

However, without claiming to be complete the following sequence may illustrate the discriminated reaction steps located between the light reactions of chlorophyll a_{II} (Chl-a_{II}) and chlorophyll a_I (Chl-a_I) and their half times. The half time of proton uptake at the pH of the medium should be shorter than $\ln 2/10^{11-pH}$ s. $PQH_{2_{out}}$ stands for the primary protonated electron carrier and $PQH_{2_{in}}$ symbolizes the neutral plastohydroquinone available to the electron carriers of System I, probably to plastocyanin (Pc).



An electron transfer time of 15 ms from the secondary acceptor to the plastoquinone pool has been suggested by Joliot [46]. An engagement of this step in linear electron transport is not compatible with the time of the lag and the amount of plastoquinone reducible by flashes spaced at 1.6 ms or at shorter times [4, 5].

Another reaction in linear electron transport, not consistent with our spectroscopic evidence, is a proton uptake by the primary acceptor of light Reaction II assumed from measurements of luminescence [47, 48]. In reinterpreting the decay kinetics of luminescence measured by Zankel [49], Lavorel proposed a half time of 35 μs for this reaction [48]. The large absorbance change expected for this fast protonation of the plastosemiquinone anion radical ($\Delta\varepsilon_{320} = 11.2\ \mathrm{mM}^{-1} \cdot \mathrm{cm}^{-1}$ [35]), should be seen in Fig. 4 at pH 8 when a half time of 110 μs is expected instead of 35 μs at pH 7.5 [49]. In addition, this protonation is proposed to be followed by the formation of neutral plastohydroquinone with the 0.6 ms half time [48]. However, the difference spectrum of the decay kinetics of *X*-320 [8] does not correspond to the difference spectrum of neutral plastosemiquinone [35] minus neutral plastohydroquinone [50] and the amplitude after one flash would be at least four times smaller than that of *X*-320 ($\Delta\varepsilon \approx 2.4\ \mathrm{mM}^{-1} \cdot \mathrm{cm}^{-1}$ between 320 and 335 nm).

Further investigation of the lag at improved signal to noise ratios and perhaps the finding of absorbance changes of plastohydroquinone with a sigmoidal rise in the predicted time range may yield more information on the proton uptake and the electron transfer mechanisms located between the light reactions. However, other possibilities of electron transfer, e.g. involving *b*-cytochromes as have appeared in literature [51–53], must be accommodated to the kinetic limits posed by our observations.

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