CYTOCHROME *f* FUNCTION IN PHOTOSYNTHETIC ELECTRON TRANSPORT

J. WHITMARSH AND W. A. CRAMER, *Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907*

ABSTRACT The questions of whether the stoichiometry of the turnover of cytochrome *f*, and the time-course of its reduction subsequent to a light flash, are consistent with efficient function in noncyclic electron transport have been investigated. Measurements were made of the absorbance change at the 553-nm α-band maximum relative to a reference wavelength. In the dark cytochrome *f* is initially fully reduced, oxidized by a 0.3-s flash, and reduced again in the dark period after the flash. In the presence of gramicidin at 18°C, the dark reduction was characterized by a half-time of 25–30 ms, stoichiometries of cytochrome *f*:chlorophyll and P700:chlorophyll of 1:670 and 1:640, respectively, and a short time delay. The time delay in the dark reduction of cytochrome *f*, which is expected for a component in an intermediate position in the chain, becomes more apparent in the presence of valinomycin and K^+. Under these conditions the half-time for cytochrome *f* dark reduction is 130–150 ms, and the delay is $\simeq$20 ms. The measured value for the activation energy of the dark reduction of cytochrome *f* (11 $\pm$ 1 kcal/mol) is the same as that for noncyclic electron transport in steady-state light. A sigmoidal time-course for the reduction of cytochrome *f* has been calculated for a simple linear electron transport chain. The kinetics for reduction of cytochrome *f* predicted by the calculation, in the presence of valinomycin and K^+, are in reasonably good agreement with the experimental data. There is an appreciable amount of data in the literature to document complex properties of cytochrome *f* after illumination with short flashes, and evidence for complexity in a light-minus-dark transition. The data presented here, obtained after a long flash that should establish steady-state conditions, either fulfill or are consistent with the basic criteria for efficient function of cytochrome *f* in noncyclic electron transport.

INTRODUCTION

Since the initial work of Duysens et al. (1) supporting the hypothesis that cytochrome *f* is involved in electron transfer between photosystem II and photosystem I that is coupled to phosphorylation (2), there have been numerous experimental results interpreted within the framework of this concept. It has been proposed that electron transfer to cytochrome *f* from a pool of reduced plastoquinone is linked to the release of protons that are subsequently used in phosphorylation. In a simple linear pathway, electrons from cytochrome *f* would be transferred to plastocyanin and then to the reaction center of photosystem I (P700) (3). Evidence for this scheme is provided by the following observations: (*a*): Cytochrome *f* is reduced by photosystem II and oxidized by photosystem I with a time-course that appears to be compatible with the kinetics of other components of the electron transport chain (4–9). (*b*) The midpoint potential of cytochrome *f* in vitro (10) is between those of plastoquinone and P700. (*c*) Cytochrome *f* has been found to be present at a stoichiometry of approximately one per electron transport chain (11–14), consistent with the measurement of two electron equivalents between plastoquinone and P700 (11, 15). (*d*) Inhibition between plastoquinone

and cytochrome *f* by the plastoquinone analogue 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB) blocks the photoreduction of cytochrome *f* but not its photooxidation (14). (*e*) Inhibition or removal of plastocyanin blocks the photooxidation of cytochrome *f* (16–19). The oxidation rate constant of parsley cytochrome *f* by plastocyanin is much larger than the rate constant for oxidation of other *c* type cytochromes by plastocyanin (20). The reduction of plastocyanin by plastoquinol-1 is catalyzed by chloroplasts in a DBMIB-sensitive pathway that reduces cytochrome *f* (21). (*f*) Electron transport between the two photosystems is blocked in a mutant lacking cytochrome *f* (16). (*g*) In subsaturating red light the oxidation state of cytochrome *f* is dependent upon the addition of ADP and phosphate, ATP, or uncouplers (22, 23).

Although these data provide an extensive basis for considering cytochrome *f* to function in the noncyclic electron transport chain between plastoquinone and plastocyanin, there are several experimental observations involving the kinetics and amplitude of the redox changes of cytochrome *f*, as well as its equilibration with plastocyanin and P700, that have been interpreted in terms of a branched or parallel pathway (11, 13, 24–31).

Of particular interest are recent measurements of Haehnel on cytochrome *f* turnover with submillisecond time resolution that implied cytochrome *f* was substantially less efficient in carrying electrons from plastoquinone to P700 than would be expected on the basis of a stoichiometry to P700 of 1:1, and that it did not appear kinetically to be in a linear pathway (26, 27). These conclusions were based on the following observations: (*a*) The amount of cytochrome *f* turning over with respect to P700, in a group of short flashes, was $\simeq$0.5:1. (*b*) The kinetics of dark reduction of cytochrome *f* did not exhibit the delay expected if the first electrons leaving the plastoquinone pool were transferred to P700 and plastocyanin. (*c*) The photooxidation of cytochrome *f* was faster than plastocyanin, which is inconsistent with plastocyanin functioning as an intermediate between cytochrome *f* and P700. It was proposed from these data that, in the dark, cytochrome *f* is involved in a side path from plastoquinone and does not transfer electrons to P700, whereas, in the light, cytochrome *f* transfers electrons between plastoquinone and P700 in parallel with plastocyanin, with plastocyanin carrying the larger flux of electrons. It is necessary to consider these observations and conclusions regarding the electron transport properties of cytochrome *f* in terms of the data summarized above which imply that cytochrome *f* functions in the noncyclic electron transport chain in a linear pathway.

In the present study we examine the extent of the light-induced absorbance change of cytochrome *f* and the kinetics of its dark reduction using an actinic flash sufficiently long that steady-state conditions are reached. The kinetic results and the rate-limiting step of electron transport are compared to a theoretical calculation that predicts the kinetic behavior of plastoquinone, cytochrome *f*, plastocyanin, and P700 subsequent to an actinic flash.

METHODS

Chloroplast preparation

Spinach leaves gathered from a controlled climate facility were used to isolate chloroplasts according to the technique of Ort and Izawa (32). The chloroplasts were used for measurements between 1 and 5 h after preparation. The chlorophyll concentration was determined according to the method of Arnon (33).

Experimental

Electron transport rates were measured polarographically with a Clark-type oxygen electrode (5331; Yellow Springs Instrument Co., Yellow Springs, Ohio). The reaction mixture (2 ml) was contained in a 1-cm-diam water-jacketed vessel. The actinic light, which was saturating ($>10^6$ ergs/cm^2 · s), was provided by two 500-W tungsten-halogen lamps filtered by a $CuSO_4$ solution and a Corning CS 2-59 blocking filter (Corning Glass Works, Corning N.Y.). To measure electron transport at temperatures below 15°C it was necessary to use a high sensitivity teflon membrane (5937; Yellow Springs Instrument Co.), and to calibrate the electrode at each temperature. The Tricine/NaOH buffer (Tricine, Sigma Chemical Co., St. Louis, Mo.) was titrated so that the pH at each temperature was 7.8.

Cytochrome chemical difference spectra were measured with a single-beam scanning instrument on-line to a mini-computer (34). To determine the concentration of cytochrome *f* more accurately, cytochrome *b*-559 was converted to a lower potential state by treatment with NH_2OH in the light (35). The oxidation state of P700 was measured by using a DW-2 spectrophotometer (American Instrument Co., Silver Spring, Md.).

Light-induced cytochrome absorbance changes were measured by using a single-beam spectrophotometer. Signals from a photomultiplier (EMI Gencom, Inc., Plainview, N.Y.) were amplified and then recorded and added together in a digital signal averager (NS-570; Tracor Northern, Middleton, Wisc.) with a verticle scale resolution of 12 bits. The data in the signal averager was then transferred to a mini-computer. The curve smoothing operation, used for the data shown in Figs. 6 and 9, involved addition of one-half of the value of the two neighboring address points to each address point and subsequent division by two. The time constant of the instrument was 2 ms or less as indicated in the figure legends. The photomultiplier was protected from scattered actinic light by a Balzers DT-G interference filter (Balzers High-Vacuum Corp., Santa Ana, Calif.) and a Corning 4-96 filter. The actinic flash was provided by a 150-W tungsten-halogen lamp filtered by a glass heat filter and a Corning CS 2-58 blocking filter. The intensity was 3–4 · 10^5 ergs/cm^2 · s. To obtain an accurate and repeatable flash duration, two Uniblitz electronic shutters (Vincent Associates, Rochester, N.Y.) were used in tandem so that before a flash one was open and the other was closed. Timing circuitry was designed in order to control the shutter on/off times. At a flash duration of 0.3 s the jitter was <0.2 ms. The closing time of the shutter was 1.6 ms. The arrows in Figs. 1, 3, 4, and 6 indicate the time at which the shutter was completely closed. Although the photomultiplier was guarded from magnetic noise by a μ-metal shield, it was necessary to remove the electronic shutters from the vicinity of the tube by using a 1-cm-diam, 55-cm-long light guide between the actinic light and the sample. The optical path length was 1 cm and a ground glass scattering plate was placed between the sample and photomultiplier.

Differential absorbance changes were determined in the following manner: If n flashes were to be recorded at a particular wavelength, λ_1, with a reference wavelength, λ_2, then $n/2$ flashes at λ_1 were stored in channel 1, n flashes at λ_2 were stored in channel 2, and finally $n/2$ flashes at λ_1, were stored in channel 1 again. For the next sample the order of λ_1 and λ_2 was reversed. Several samples were generally averaged in this way, after which the signal in channel 2 was subtracted from that in channel 1 and recorded. Spectra were measured in a similar manner. For each wavelength contributing to the spectrum, a measurement was also made of the absorbance change at 553 nm using the same sample, and the ratio of the total absorbance change at λ minus 540 nm to that at 553 minus 540 nm was plotted.

Before light-induced absorbance measurements the chloroplast sample was illuminated with actinic light to eliminate any changes unique to the first few flashes. All chloroplast samples were thermostatted and were discarded after 5 min.

The monochrometer was calibrated using the 546.1-nm mercury line to within 0.3 nm.

RESULTS

It is known that in chloroplasts light-induced absorbance changes in the spectral region from 540 to 570 nm consist of a superposition of several components. In fact, at 553 nm it is possible

to measure changes due to cytochrome *f*, cytochrome *b*-559, cytochrome b_6, the electrochromic band shift, C550, P700, and light scattering (36). Therefore, to investigate cytochrome *f* oxidation-reduction kinetics, and to minimize other contributions, we used the differential spectrophotometric technique described in Methods, with the reference wavelength set close to the peak of the cytochrome *f* α-band. Even then the extent of the electrochromic band shift was still significant and it was necessary to add either gramicidin, or valinomycin and K^+, to accelerate its decay. Under these conditions the contribution of the electrochromic band shift compared to the cytochrome *f* absorbance change during steady-state illumination was negligible.

An example of a typical light-induced absorbance change observed in chloroplasts at 553–540 nm in the presence of gramicidin (2 μM) is shown in Fig. 1. At the onset of the 0.3-s red actinic flash there is a rapid absorbance decrease that corresponds to the oxidation of cytochrome *f* which, under saturating light, is more rapid than the opening time of the shutter (data not shown). After the actinic flash is turned off there is an absorbance increase that corresponds to the reduction of cytochrome *f*. The absorbance increase observed in the dark is shown as a function of wavelength in Fig. 2. The spectrum exhibiting a peak at 553–554 nm, and an asymmetry, is similar to the reduced-minus-oxidized α-band spectrum of isolated cytochrome *f* (30). The shoulder on the short wavelength side of the spectrum raises the possibility that part of the absorbance increase is due to C550 (37). If one assumes that the asymmetry in the spectrum of Fig. 2 arises totally from C550, then its contribution to the absorbance change at 553 nm would be $\simeq$12%. The amplitude of the C550 absorbance change may be decreased by the presence of gramicidin (38). In fact, in the presence of an

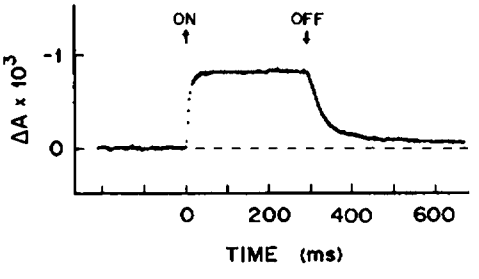


FIGURE 1

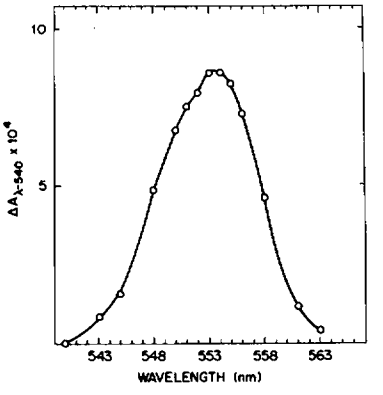


FIGURE 2

FIGURE 1 Kinetics of the absorbance change (ΔA) at 553–540 nm induced by illumination of spinach chloroplasts in the presence of 2 μM gramicidin. The trace shown is the average of 360 runs during which the sample was changed 18 times. The repetition rate of the actinic flash was 0.2 Hz. The time constant was 1 ms. The half-bandwidth of the measuring beam was 3 nm. The reaction mixture was kept at 18°C and contained 30 mM Tricine/NaOH (pH 7.8), 0.2 M sucrose, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM methylviologen, 2 μM gramicidin, and 25 μg chlorophyll/ml. For further details see Materials and Methods.

FIGURE 2 Wavelength dependence of the absorbance increase (ΔA) observed in the dark subsequent to a 0.3-s actinic flash in chloroplasts in the presence of 2 μM gramicidin. The experimental conditions were the same as described in Fig. 1 except the half-bandwidth was 2 nm. Details of the measurements are given in Materials and Methods.

excess of ferricyanide to oxidize cytochrome *f*, and gramicidin, the amplitude of a rapid light-induced absorbance change at 553–540 nm that could be attributed to C550 was found to be <10% of the absorbance change observed without ferricyanide (data not shown). The spectrum of the absorbance decrease (cytochrome *f* oxidation) shown in Fig. 1 was the same as that for reduction (Fig. 2) except that it was typically 5–10% larger. Experiments done using flash durations of either 50 ms or 1 s gave similar results (data not shown).

In view of the discrepancy discussed in the Introduction between the amount of cytochrome *f* observed turning over in flash experiments and that observed under steady-state light or in chemical difference spectra we have investigated this point in some detail. Table I (39–41) shows the amount, on a chlorophyll basis, of cytochrome *f* reduced in the dark subsequent to a 0.3-s actinic flash at rates comparable to those shown in Fig. 1 and 6. This amount can be compared to the concentration of cytochrome *f* and high potential (HP) cytochrome *b*-559 (i.e., reducible by hydroquinone) observed in chemical difference spectra and also P700. The stoichiometries of cytochrome *f*:P700:cytochrome *b*-559 HP:chlorophyll were ≃1:1:2:650. The range in cytochrome *f*:chlorophyll stoichiometry was 1:640–720.

The details of the kinetics of reduction of cytochrome *f* in the presence of gramicidin can be seen more clearly with an expanded time scale (Fig. 3) and in a semilog plot of absorbance vs. time (Fig. 4). The half-time for the dark reduction of cytochrome *f* was 27 ms (Fig. 4). This value ranged from 25 to 30 ms at 18°C.

The dependence upon temperature from 5 to 24°C of the rate of electron transport under steady-state, saturating light, and the rate of the dark reduction of cytochrome *f* are shown in Fig. 5. The activation energy for the rate-limiting step in electron transport was 11 ± 1 kcal/mol. For the dark reduction of cytochrome *f*, it was also 11 ± 1 kcal/mol. The similar temperature dependences of the rate-limiting step and the rate of cytochrome *f* reduction does not in itself provide evidence for involvement of cytochrome *f* in the noncyclic electron transport chain. Clearly two reactions may have the same activation energy without sharing in

TABLE I
CONCENTRATIONS OF CYTOCHROME *f*, CYTOCHROME *b*-559 (HP) P700, RELATIVE TO CHLOROPHYLL

Component	Measurement	Ratio
		mol:mol of chlorophyll
Cytochrome *f*	Chemical difference (FeCy-hydroquinone)	1:630
Cytochrome *f*	Light-dark reduction (+ gramicidin)	1:670
Cytochrome *f*	Light-dark reduction (+ valinomycin and K^+)	1:650
Cytochrome *b*-559 HP	Chemical difference (FeCy-hydroquinone)	2:610
P700	Red light reduced–far-red light oxidized	1:640

The reduced-minus-oxidized extinction coefficients used were: cytochrome *f*, $\epsilon_{553-540} = 20\ mM^{-1} \cdot cm^{-1}$; cytochrome *b*-559, $\epsilon_{559-570} = 16\ mM^{-1} \cdot cm^{-1}$; P700, $\epsilon_{702-720} = 58\ mM^{-1} \cdot cm^{-1}$ (30,39). The cytochrome concentration determined from chemical difference spectra involved a modification of the formula given by Heber et al. (40). The P700 concentration was determined according to the method described by Givan and Levine (41). The concentrations used were: ferricyanide (FeCy), 0.125–0.5 mM; hydroquinone (HQ), 1.5–3 mM; gramicidin, 1–2 μM; valinomycin, 0.8 μM.

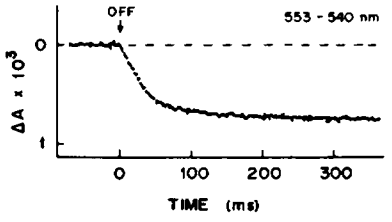


FIGURE 3

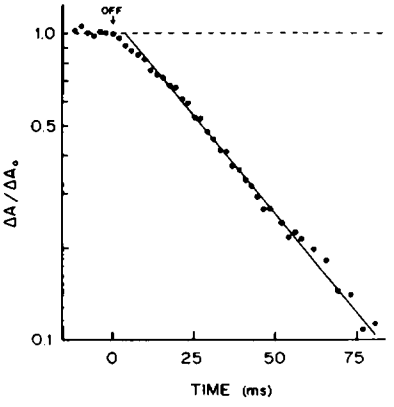


FIGURE 4

FIGURE 3 Expansion of the absorbance increase (ΔA) observed in the dark shown in Fig. 1.
FIGURE 4 Plot on a logarithm scale of the absorbance increase shown in Fig. 3 vs. time. ΔA_0 is the maximum observed absorbance change. The data were taken from the digital readout of the signal averager. The straight-line was generated by a least-squares polynomial fit of the data points from 8 to 60 ms after the flash.

a common pathway. However, had the temperature dependences been substantially different it would have been difficult to consider a simple linear chain including cytochrome *f*. In our measurements the half-time for the dark reduction of cytochrome *f* was typically 5–7 times longer than the half-time calculated for the rate-limiting step (Fig. 5).

In the semilog plot of the time-course of cytochrome *f* reduction, a short delay was consistently observed (Fig. 4), although the small extent makes its significance questionable. It should be kept in mind that a short time delay or small degree of sigmoidal kinetics may be masked due to the superposition of a small absorbance change of independent origin (13, 30). Because a residual steady-state electrochromic band shift and/or a C550 absorbance change at 553–540 nm would cause an increase in absorbance in the dark, either of these effects could result in abolishing or altering the possible sigmoidal nature of cytochrome *f* reduction. Conversely, a small absorbance change in the opposite direction could induce sigmoidal kinetics at 553–540 nm that may not exist for cytochrome *f* reduction alone. Hence, the absence or presence of a small delay must be interpreted with caution.

To investigate the possibility of sigmoidal kinetics for cytochrome *f* dark reduction more thoroughly, we sought first of all to minimize any contribution from C550 by using 545 nm as the reference wavelength, and, secondly, to overcome the kinetic limitations of our instrument, we used valinomycin with K^+, rather than gramicidin, to remove the steady-state electrochromic band shift. Because valinomycin does not act as an uncoupler (42), the electron transport kinetics are several times slower than in the presence of gramicidin. A typical example of the absorbance increase observed at 553–545 nm in chloroplasts in the presence of valinomycin (0.8 μM), subsequent to a 0.3-s flash of red light, is shown in Fig. 6. The wavelength dependence of the absorbance change (Fig. 7) indicates that it results from the reduction of cytochrome *f*. In Fig. 6 it can be seen that the dark reduction of cytochrome *f* exhibits sigmoidal kinetics with a half-time of 140 ms and a delay ≃20 ms. One difference

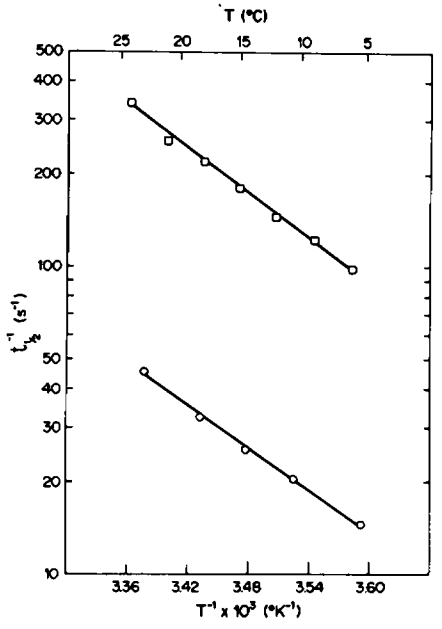


FIGURE 5

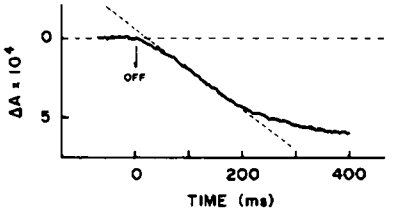


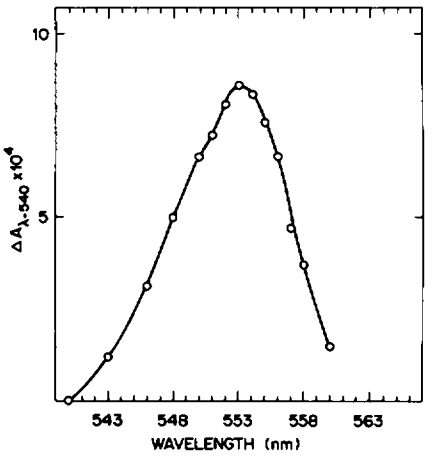
FIGURE 6

FIGURE 5 Arrhenius plots of cytochrome *f* dark reduction rate (○) after a 0.3-s flash and the rate of electron transport (□) under steady-state conditions, in the presence of 2 μM gramicidin. The rate of cytochrome *f* reduction is given as the reciprocal of the half-time (t) of the absorbance increase at 552–540 nm. The rate of electron transport is the reciprocal of the time for one-half of an electron to go through one electron transport chain assuming 600 chlorophyll molecules per chain. For electron transport measurements the reaction mixture contained 30 mM Tricine/NaOH (pH 7.8 at each temperature [T]), 0.2 M sucrose, 10 mM KCl, 2 mM $MgCl_2$, 0.1 M methylviologen, 1 mM NaN_3, and 25 μg chlorophyll/ml. The conditions were the same for cytochrome *f* reduction measurements except NaN_3 was not present.

FIGURE 6 Kinetics of the absorbance increase at 553–545 nm subsequent to a 0.3-s flash in chloroplasts in the presence of 0.8 μM valinomycin. The trace shown, which has been curve smoothed once, is the average of 800 runs during which the sample was changed 20 times. The flash frequency was 1 Hz. The half-bandwidth of the measuring beam was 2 nm. The time constant was 2 ms. The reaction mixture was kept at 18°C and contained 30 mM Tricine/NaOH (pH 8.0), 0.2 mM sucrose, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM methylviologen, 0.8 μM valinomycin, and 25 μg chlorophyll/ml.

between the experimental situation in the presence of gramicidin and in the presence of valinomycin and K^+ is that a pH gradient exists in the latter case and valinomycin causes a small inhibition in the rate of electron transport (42). A pH gradient would slow the rate-limiting step and as a consequence increase the time delay. Observation of a time delay does not in any case depend upon the existence of a pH gradient inasmuch as it can be observed in the presence of gramicidin and diphenyl ether herbicides that inhibit electron transport.[1] The latter experiments imply that the delay is not a consequence of an interaction of valinomycin with electron transport components participating in the rate-limiting step (42).

[1]Bugg, M. W., J. Whitmarsh, C. E. Riecke, and W. S. Cohen. 1979. Inhibition of energy-linked reactions by diphenyl ether herbicides: II localization of the site of inhibition. Manuscript submitted for publication.



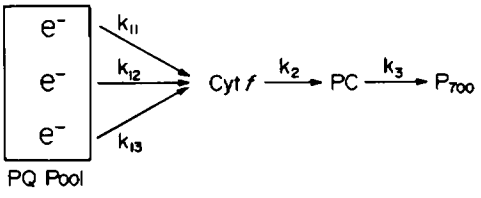


FIGURE 7

FIGURE 8

FIGURE 7 Wavelength dependence of the absorbance increase observed subsequent to a 0.3-s flash in chloroplasts in the presence of 0.8 μM valinomycin. The experimental conditions were as described in Fig. 6. Details of the measurements are given in Materials and Methods.

FIGURE 8 Electron (e^-) transport chain used for predicting the redox reactions of plastoquinone (PQ), cytochrome (cyt) *f*, plastocyanin (PC), and P700 after illumination. For a discussion see text and footnote 2.

To gain insight into the meaning of the sigmoidal kinetics of cytochrome *f* reduction, we have developed a theoretical calculation relating the kinetics, after a light flash, for reduction of the components in the irreversible, linear, electron transport chain shown in Fig. 8. Qualitatively, the kinetic behavior of the chain can be described as follows. The rate-limiting step is considered to be between plastoquinone and cytochrome *f*. During a relatively long flash of actinic light, the active plastoquinone pool is reduced by electrons from photosystem II, and cytochrome *f* and plastocyanin are oxidized, because the oxidation by P700 is much faster than the rate of reduction by plastoquinone. When the light is turned off, electrons from plastoquinone reduce the three oxidized components, P700, plastocyanin, and cytochrome *f* in the following order. The first electron out of the plastoquinone pool is transferred to P700 with a half-time comparable to the rate-limiting step of electron transport. Subsequent transfer of electrons in a sequential manner is accompanied by a delay that increases with the number of electrons transferred. Thus the second electron transferred from plastoquinone reduces plastocyanin with a small delay and the third electron transferred reduces cytochrome *f* with a longer delay. Details of the implications and limitations of the calculation, including the effect of possible additional components (43–45) in the chain between plastocyanin and P700 will be given elsewhere.[2] The results of the calculation are that the half-time for the reduction of cytochrome *f* should be at least three times slower than that for the rate-limiting step, dependent upon the relative rate constants of the three electrons out of the plastoquinone pool. Furthermore, the predicted delay in the dark reduction of cytochrome *f* is much shorter than had been previously suggested (26). Fig. 9 shows a comparison of the kinetics of the dark

[2]Whitmarsh, J., and W. A. Cramer. 1979. The rate-limiting step in photosynthesis: theoretical time dependence of oxidation reduction reactions subsequent to a light flash. Manuscript in preparation.

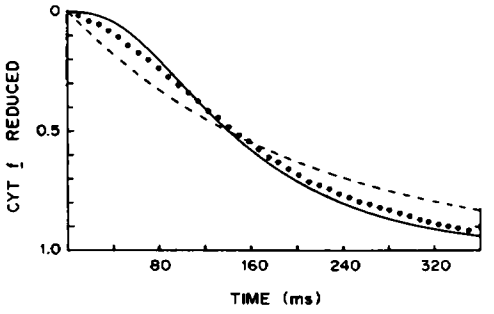


FIGURE 9 The kinetics of the dark reduction of cytochrome (cyt) *f*. (*a*) (solid line) The kinetics predicted by the calculation described in footnote 2. The trace shown is for the following first order rate constants (per second): $k_{11} = 30, k_{12} = 20, k_{13} = 10, k_2 = 6{,}940, k_3 = 9{,}900$. (*b*) (dashed line) The kinetics for first order decay with a half-time of 140 ms. (*c*) (circles) Experimental data for chloroplasts in the presence of valinomycin (0.8 μM). The data were taken from a trace that had been curve smoothed. The experimental conditions were as described in Fig. 6.

reduction of cytochrome *f* (*a*) predicted for the model shown in Fig. 8, and for first-order kinetics, and (*b*) measured experimentally. The experimental results are in fair agreement with the theoretical prediction. Comparison of the experimental data in Fig. 9 with the curve describing first-order decay emphasizes the sigmoidal nature of the dark reduction of cytochrome *f*.

DISCUSSION

It can be argued that much of the evidence cited above (4–23) for efficient function of cytochrome *f* in the noncyclic electron transport chain does not establish kinetic competence of the cytochrome in this pathway. To demonstrate that a given electron transport component, whose properties and chemical stoichiometry have been determined in the dark, can function with full efficiency in the light, it is necessary to prove that the kinetic parameters for oxidation and reduction changes are compatible with those of the known reactions of the pathway, and that the stoichiometry of the component can be accounted for in the amplitude of the light-induced absorbance changes. Haehnel (26, 27) found that the stoichiometry of cytochrome *f* reduced with an appropriate half-time after reduction of the plastoquinone pool was very small, ≃ 1 cytochrome *f* for each 1,200–1,500 chlorophylls. It was implied that the dark reduction of 40–60% of the cytochrome *f* was too slow to be measured on an appropriately fast time scale and was therefore not kinetically competent to function in the main pathway. The missing cytochrome *f* was, however, not accounted for in terms of absorbance changes on any time scale. It was proposed that cytochrome *f* may function in a side path, or parallel to plastocyanin in a relatively inefficient pathway to P700, with transfer to P700 disconnected in the dark. In contrast to the above observations, the data presented here show that the stoichiometry of cytochrome *f* observed to be oxidized in the light and reversibly reduced in the dark, with a half-time of 25–30 ms, is one cytochrome *f* per 670 chlorophylls. This stoichiometry is similar to that measured for P700. The amplitude of these changes is compatible with the concept of efficient participation of one cytochrome *f* per chain in the noncyclic pathway.

The absence of a delay in the dark reduction of cytochrome *f* has been cited as evidence against its involvement in electron transfer between plastoquinone and P700 (13, 26, 27). In measurements done at subzero temperatures, a time delay was not observed for cytochrome *f* reduction, measured in the absence of gramicidin (13). It was noted, however, that the delay could have been missed because of an independent absorption change, and no additions were made to abolish the electrochromic band shift. In our data the sigmoidal kinetics in the reduction of cytochrome *f* observed in the presence of valinomycin (Figs. 6 and 9) are clearly observable. The calculation of Haehnel for the expected time delay for cytochrome *f* reduction had indicated a delay comparable to the measured half-time for cytochrome reduction (26). However, our calculation indicates a much shorter delay.[2] The purpose of the calculation is mainly to obtain an estimate of the expected delay in the dark reduction of cytochrome *f* and the ratio of the rate of cytochrome *f* reduction to the rate-limiting step. The model is simple inasmuch as it does not take into account reversibility of electron transfer, particularly important between plastocyanin and cytochrome *f*, nor the possibility of additional components between plastoquinone and P700 (43–45, 46). The virtues of the model are that it can be solved and that the time-course predicted for the oxidation of plastoquinone and the reduction of P700, plastocyanin, and cytochrome *f* appear to be in close agreement with experimental data. The results of the calculation indicate that our experimental observations concerning cytochrome *f* and the rate-limiting step are consistent with the linear chain shown in Fg. 8. This analysis does not exclude the possibility that other models, including reversibility for example, may fit the data better, but rather is used to argue that the extent and kinetics of the observed cytochrome *f* reduction are reasonable in terms of its participation in noncyclic electron transport (Fig. 8). If the model were altered to include reversibility between cytochrome *f* and plastocyanin, we would expect a smaller delay. If the scheme of Fig. 8 were modified by the single change of inserting another component between plastocyanin and P700 (43–45), we would predict a longer delay. We have not included in our model the Fe-S component, inferred from oxidized minus reduced electron paramagnetic resonance spectra to function between plastoquinone and cytochrome *f* (46), because light-induced changes have not yet been reported.

Haehnel (27) concluded that plastocyanin could not transfer electrons between cytochrome *f* and P700 inasmuch as the half-time for oxidation of cytochrome *f* was observed to be much faster (<40 μs) than the half-time (200 μs) observed for oxidation of plastocyanin by system I. In contrast to these results, the half-times for oxidation of cytochrome *f* and plastocyanin in *Chlorella* have recently been reported to be 100 and 70–150 μs, respectively, with a delay in cytochrome *f* oxidation that would be expected if there were an intermediate in the pathway of its oxidation (9). It was inferred from these data that cytochrome *f* and plastocyanin function in a linear pathway to P700 during a flash, but that the transfer from cytochrome *f* to plastocyanin is interrupted during a period of 1–100 ms after the flash (9, 29). The basis for the latter inference is that the rate of cytochrome *f* reduction in the dark was greater than that of plastocyanin. The possible interruption or lack of equilibration between cytochrome *f* and its acceptor in the dark was measured after illumination consisting of one or more short flashes (26, 27, 29). The conditions of our experiment allow assay of cytochrome *f* under conditions closer to those of steady-state illumination. There is precedent for believing that the properties of cytochrome *f* may be different in the light and dark (11, 26, 27, 29, 34). The

properties of cytochrome *f* described in this work after illumination with a long flash that should establish steady-state conditions imply an efficient connection of cytochrome *f* in the main chain. It is possible that the different conclusion reached by Haehnel regarding the properties of cytochrome *f* is a consequence of the use of different illumination conditions.

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