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ELECTRON TRANSFER BETWEEN THE TWO PHOTOSYSTEMS

II. EQUILIBRIUM CONSTANTS

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(1) The equilibrium constants for the redox reactions occurring between Photosystem (PS) I donors were measured on chloroplasts, dark-adapted in the presence of sodium ascorbate and 3-(3',4'-dichlorophenyl)-1,1-dimethylurea (DCMU) and then illuminated by d.c. light. The equilibrium constant for the electron transfer between plastocyanin and P-700 is close to 1 and the overall equilibrium constant between cytochrome *f* and P-700 is about 2.3. As these equilibrium constants do not depend upon the intensity of the d.c. beam, the low values we measured cannot be due to kinetic limitations. (2) The equilibrium constants were measured also in the absence of DCMU using chloroplasts in oxidizing conditions (ferricyanide or far red illumination) illuminated by a saturating flash. During the course of the reduction of PS I donors by plastoquinol molecules formed by the flash, the equilibrium constants are higher than in the preceding conditions: the value for plastocyanin to P-700 is close to 5, and that for cytochrome *f* to P-700 is about 25. (3) The variations of these equilibrium constants are tentatively interpreted as being due to mutual electrostatic interactions between cytochrome *b* and *f* which are included in the same complex. This model implies that the perturbation of the redox properties of cytochrome *f* by a positive charge located on cytochrome *b* is identical to the perturbation of the redox properties of cytochrome *b* by a positive charge located on cytochrome *f*.

Introduction

The measurements of the equilibrium constants of the redox reactions involving the electron carriers between the two photosystems led to confusing and even contradictory results. Joliot et al. [1] measured in weak light a low equilibrium constant ($K = 3–10$) for the overall reaction between the PS II acceptor Q and the primary PS I donor P-700. This value contradicts the one which can be computed from the redox potentials of these two electron carriers. A high value of this equilibrium constant could be measured in the dark, since a fully oxidized Q can coexist with a fully reduced P-700. Kok et al. [2] reported an equilibrium constant close to 1 for the reaction between P-700 and the secondary PS I donors. Marsho and Kok [3] subsequently found much higher values for the 'dark' equilibrium constants between plastocyanin and P-700 and between cytochrome *f* and plastocyanin. Interestingly enough, these authors suggested that these values might be lower in the light.

After a flash given to chloroplasts preilluminated by far red light, Haehnel [4] measured a

Abbreviations: PS, Photosystem; cyt *f*, cytochrome *f*; cytochrome *b*, cytochrome *b*-563; PC, plastocyanin; DCMU, 3-(3,',4'-dichlorophenyl)-1,1-dimethylurea

high equilibrium constant between P-700 and its secondary donor. Using Chlorella, Bouges-Bocquet [5] measured a large equilibrium constant between plastocyanin and P-700 and an equilibrium constant of 2 between cytochrome *f* and plastocyanin. On the other hand, this author [6] proposed that cytochrome *f* was disconnected from plastocyanin for a period from 1 to 100 ms after a flash.

A survey of the literature on the problems of kinetics and equilibrium constants involving cytochromes *b* and *f* is presented in a review by Cramer and Whitmarsh [7].

In this paper, we present new experimental evidence showing that the equilibrium constants between cytochrome *f*, plastocyanin and P-700 vary in large proportions depending upon the experimental conditions. An interpretation based on the effect of electrostatic interactions between electron carriers is proposed.

Material and Methods

The experiments presented in this article were performed using the same material and methods as in Ref. 8.

Deconvolutions. In the green region of the spectrum, the deconvolutions for cytochrome *f*, plastocyanin and P-700 were made according to the procedures described in Ref. 8. In the blue region of the spectrum, $\Delta I/I$ (429 nm) was taken as a measure of P-700 (isobestic point of cytochrome *f*) and $\Delta I/I$ (421–439 nm) was taken as the measure of cytochrome *f*. At these two wavelengths, the absorption changes due to P-700 are about equal. This measurement does not eliminate the absorption changes due to cytochrome *b* which remain small in the conditions of Fig. 2.

Results

Equilibrium constants during the oxidation of PS I donors

The equilibrium constants of the electron transfer reactions between PS I donors were measured in the presence of DCMU, i.e., under conditions where there is no electron transfer between PS II and PS I. In order to eliminate the absorption change due to the reduction of Q (C550) [9], chloroplasts were preilluminated in the presence of hydroxylamine and then dark adapted for more than 3 min. In this condition, Q stays in its reduced form as its reoxidation is blocked by hydroxylamine [10]. Sodium ascorbate was added to reduce PSI donors slowly ($t_{1/2} \approx 15$ s); we therefore assume that in the conditions of our experiment, all the PS I donors are in the reduced state. In Figs. 1 and 2, the chloroplasts were illuminated in d.c. light and the spectral changes were measured either in the green or in the blue region. The characteristic spectrum of cytochrome *f* oxidation superimposed on the absorption increases due to plastocyanin (572 nm) and P-700 (512 nm) oxidations is seen in Fig. 1. In the blue region (Fig. 2), the spectrum mainly reflects cytochrome *f* (421 nm) and P-700 (430-nm) oxidations. In both cases, only minor changes can be attributed to cytochrome *b* reduction, which proves that there is no plastoquinol present prior to the illumination. The spectra in Figs. 1 and 2 show that during the course of the illumination, the

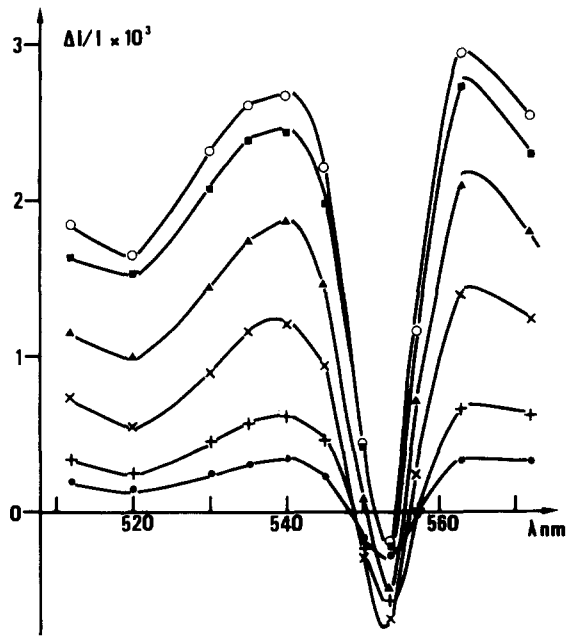

Fig. 1. Absorption changes in the green region of the spectrum measured for different times of red d.c. illumination. 30 μg/ml chlorophyll/3 mM hydroxylamine/50 μM DCMU/3 mM sodium ascorbate. Chloroplasts were first preilluminated and then dark-adapted for times longer than 3 min. Illumination times: (●), 10 ms; (+), 20 ms; (×), 50 ms; (▲), 100 ms; (■), 300 ms; (○), 2 s.

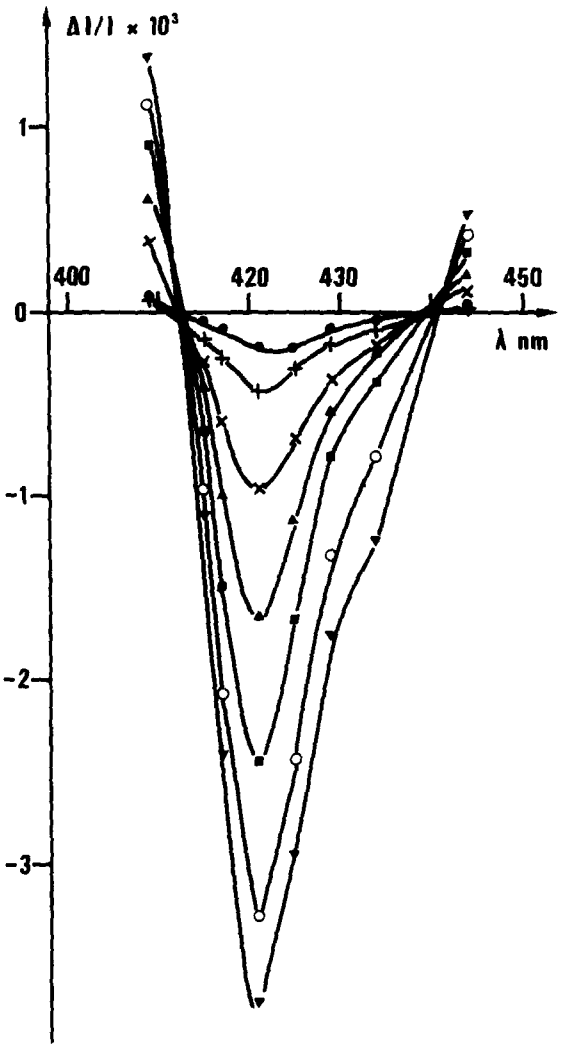

Fig. 2. Absorption changes in the blue region of the spectrum measured for different times of red d.c. illumination; 12 μg/ml chlorophyll/3 mM hydroxylamine/50 μM DCMU/3 mM sodium ascorbate. Chloroplasts were first preilluminated and then dark-adapted for times longer than 3 min. Illumination times: (●), 10 ms; (+), 20 ms; (×), 50 ms; (▲), 100 ms; (■), 200 ms; (○), 500 ms; (▼), 2.7 s.

redox states of the different components (P-700, plastocyanin, cytochrome *f*) vary in a rather parallel fashion.

In Fig. 3 are shown the kinetics of P-700 and cytochrome-*f* oxidations computed from Fig. 1. Both kinetics are close to an exponential, but the oxidation of cytochrome *f* is significantly faster than that of P-700.

Fig. 4 shows the redox state of cytochrome *f* vs.

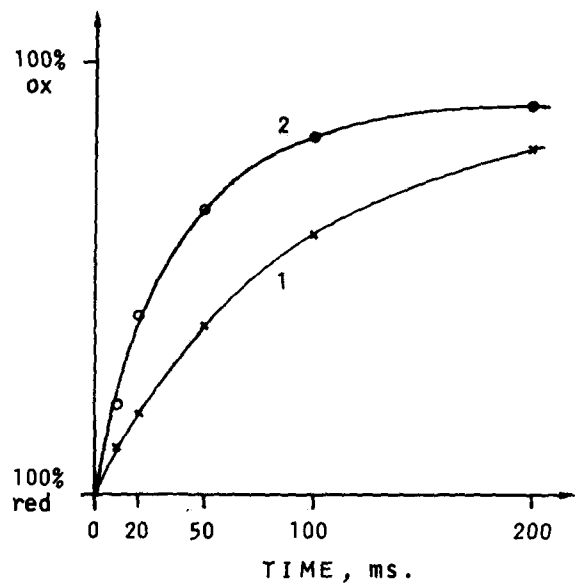

Fig. 3. Kinetics of P-700 (Curve 1, ×) and cytochrome *f* (Curve 2, ○) oxidation under d.c. illumination, computed from Fig. 1 after deconvolution.

the redox state of P-700 measured in the green and in the blue regions of the spectrum during the course of a d.c. illumination under different experimental conditions. The same relationship is observed when both components are detected either in the green or in the blue region, which confirms the validity of the deconvoluting procedure used in these experiments (see Materials and Methods). For the measurements performed in the blue region of the spectrum, three intensities of the actinic d.c. beam were used, varying by a factor 12.5. The half-times of PS I donors oxidation are approximately inversely proportional to the light intensity (for P-700, see legend of Fig. 4). Therefore, the relationship between the redox states of P-700 and cytochrome *f* does not depend upon the actinic light intensity in the explored range (Fig. 4). Our results establish that under our experimental conditions, the PS I donors are in equilibrium, i.e., that the rates of the involved dark reactions do not introduce any kinetics limitation. The value of the equilibrium constant between cytochrome *f* and P-700 is approx. 2.3. Similarly, during the course of the d.c. illumination, we computed from the data of Fig. 1 an equilibrium constant between plastocyanin and P-700 close to 1.

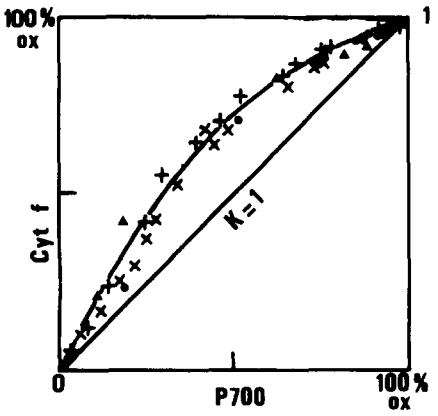

Fig. 4. Redox state of cytochrome *f* vs. redox state of P-700. The values were normalized assuming that both cytochrome *f* and P-700 are fully reduced in dark-adapted chloroplasts and fully oxidized at the end of d.c. illumination for the highest intensity of the d.c. beam. (▲), computed from Fig. 1; (●), computed from Fig. 2. Intensity of the d.c. illumination, in relative units $I = 1$; computed from an experiment similar to that of Fig. 2 for $I = 0.18$ (+) and $I = 0.08$ (×). The half-times for P-700 oxidation were 45 ms ($I = 1$), 270 ms ($I = 0.18$) and 700 ms ($I = 0.08$).

Electron transfer between PS II and PS I after flash illumination

In experiments shown on Fig. 5A and B, chloroplasts were continuously illuminated by a weak far red beam on which saturating actinic flashes were superimposed. The time interval between flashes (8 s) permitted the reoxidation of the PS I donors. The absorption changes were measured from 1.5 to 500 ms after each flash. From 1.5 to 60 ms, the two major features observed are the reductions of P-700 and of cytochrome *b*. Similar behaviour was observed on dark-adapted material in the presence of ferricyanide and after two preilluminating actinic flashes (see Fig. 2 in Ref. 8). The same experiment as the one shown on Fig. 5 was performed after a group of two flashes 3 ms apart (Fig. 6). In this last case, a larger amount of P-700 and plastocyanin is reduced than after a single flash. In addition, for times longer than 100 ms, a significant reduction of cytochrome *f* (554 nm) is observed.

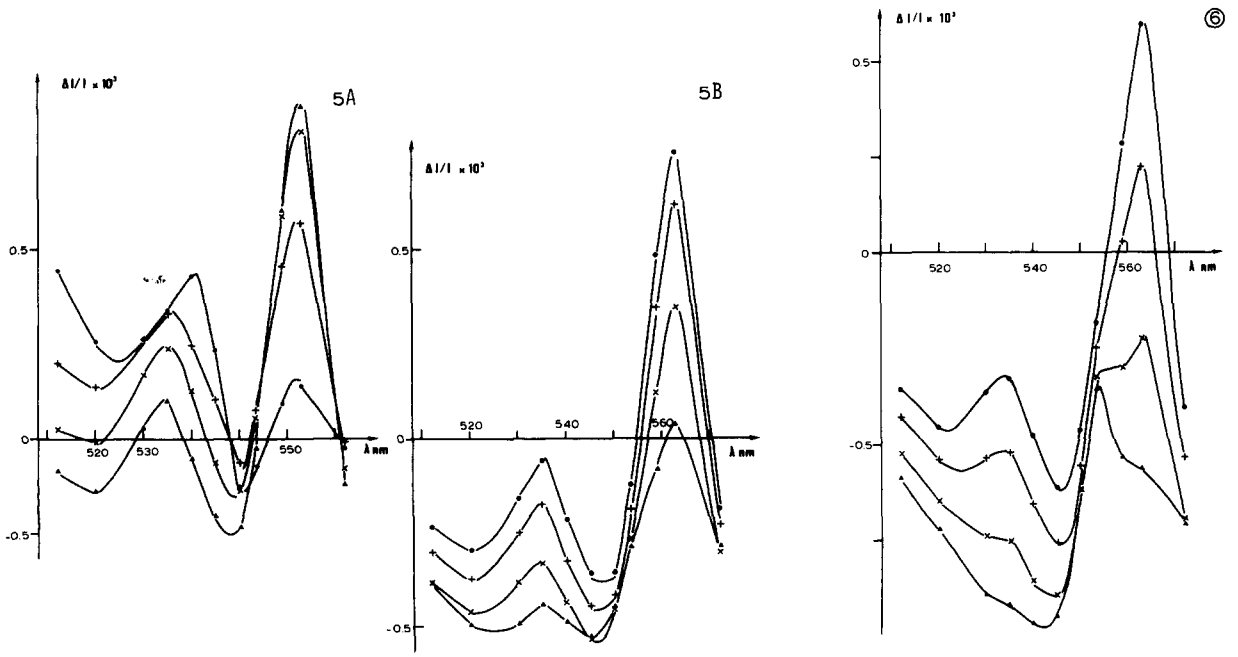

Fig. 5. Absorption changes in the green region induced by one saturating flash superimposed on a weak far red illumination. The intensity of the far red beam is about 0.5 photon per second per PS I center. 30 μg/ml chlorophyll. Detection times after the actinic flash: A: (●), 1.5 ms; (+), 6 ms; (×), 14 ms; (▲), 30 ms; B: (●), 60 ms; (+), 100 ms; (×), 200 ms; (▲), 500 ms.

Fig. 6. Absorption changes in the green region induced by a group of two saturating flashes 3 ms apart. 30 μg/ml chlorophyll. Detection times after the second actinic flash: (○), 60 ms; (+), 100 ms (×), 200 ms; (▲), 500 ms.

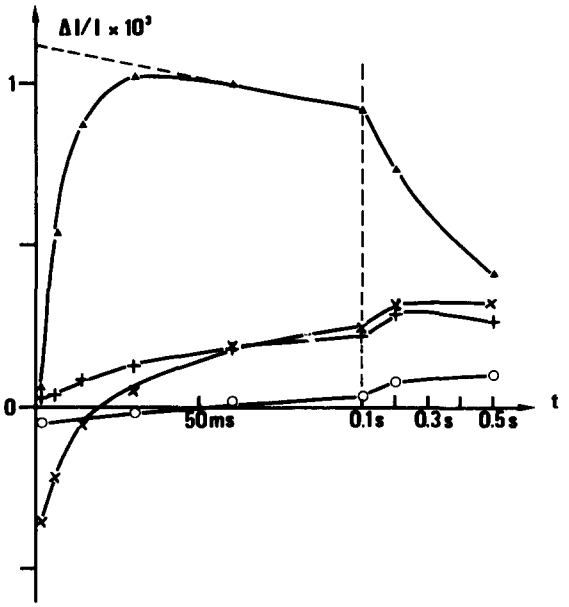

Fig. 7. Kinetics of the redox changes of cytochrome *b* and PS I donors measured after one flash superimposed on weak far red illumination computed from experiments Fig. 5. (×), P-700; (+), plastocyanin; (○), cytochrome *f*; (▲), cytochrome *b*. For each component, the absorption changes are plotted so that increasing values correspond to increasingly reduced state.

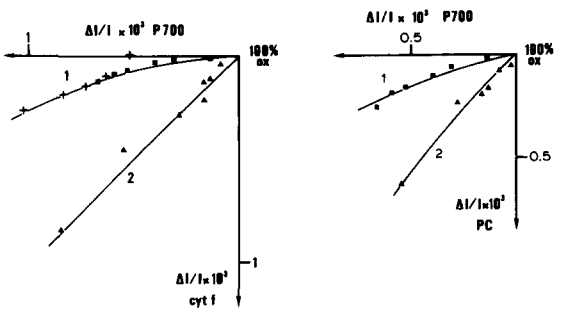

Fig. 8. $\Delta I/I$(cyt *f*) vs. $\Delta I/I$(P-700). Curve 1: (+), 0.1 mM ferricyanide. Dark-adapted chloroplasts illuminated by two saturating actinic flashes. Same experiment as Fig. 2 in Ref. 8. (■), chloroplasts illuminated by one actinic flash superimposed to far red d.c. illumination. Curve 2: (▲), computed from an experiment performed in the same conditions as Fig. 1.

Fig. 9. $\Delta I/I$(PC) vs. $\Delta I/I$(P-700). Same symbols and same conditions as in Fig. 8.

Fig. 7 shows the variations in the redox states of P-700, plastocyanin, cytochrome *f* and cytochrome *b* computed from the data shown on Fig. 5. 1.5 ms after the flash, the PS II electron acceptor Q is not yet totally reoxidized, and the spectrum shows a significant contribution of the C550 signal. For this detecting time, absorption changes due to cytochrome *f* were corrected on the basis of the C550 spectrum (11) and cytochrome *f* spectrum (12), taking into account the bandwidth (5 nm) of the detecting beam. After one flash, a small cytochrome *f* reduction is associated with a large reduction of P-700. A significant reduction of cytochrome *f* is observed only after a group of two flashes (Fig. 6). Unlike Haehnel [4], we observed then a lag time in the reduction of cytochrome *f*. Cramer et al. [13] have already observed a lag in the reduction of cytochrome *f*, although of small amplitude, immediately after a long flash which reduced a large fraction of the plastoquinone pool.

On Fig. 8, curve 1, $\Delta I/I$(cyt *f*) is plotted as a function of $\Delta I/I$ (P-700), computed from the data of Fig. 5. In this computation, we assume that 1.5 ms after the flash, P-700 and cytochrome *f* were fully oxidized. We plotted on the same figure the data obtained when chloroplasts, dark-adapted in the presence of 0.1 mM ferricyanide, were illuminated by a group of two flashes 100 ms apart (see Fig. 2 in Ref. 8). The relationship $\Delta I/I$(cyt *f*) $= f\Delta I/I$(P-700) is the same in the two experiments (curve 1). On the same figure, we also plotted (Fig. 8, curve 2) the data obtained when PS I donors are photooxidized in the presence of sodium ascorbate and DCMU (same conditions as in Fig. 1). Comparison of curves 1 and 2 of Fig. 8 shows that the equilibrium constant between cytochrome *f* and P-700 is at least 10-times higher when measured in oxidizing conditions than in reducing conditions.

The relationship $\Delta I/I$(PC) vs. $\Delta I/I$(P-700) is plotted in Fig. 9, using the same method of computation as in Fig. 8. As for the relationship between cytochrome *f* and P-700, the equilibrium constant between plastocyanin and P-700 is higher during the course of reduction of PS I donors than during their oxidation in the presence of DCMU. Nevertheless, this difference is less marked than in the case of cytochrome *f* and P-700.

Discussion

During the course of oxidation of PS I donors in the presence of DCMU, i.e., when no PS II-in-

duced formation of plastoquinol occurs, we measured a low value for the equilibrium constants between cytochrome *f*, plastocyanin and P-700 (cyt *f*/P-700, $K \approx 2.3$; plastocyanin/P-700, $K \approx 1$). We will first discuss the reliability of the determination of an equilibrium constant under the experimental conditions we used.

(1) We can exclude a limitation by the rate of electron transfer between these carriers, or a limitation by the rate of formation or dissociation of complexes between the cytochrome *b-f* protein, plastocyanin and PS I centers.

(2) Our method of measurement would be irrelevant if there were two pools of P-700, one connected to the secondary PS I donors with a high equilibrium constant, and the other one disconnected from the cytochrome *b-f* complex, due for instance to a leakage of the watersoluble plastocyanin in damaged thylakoids. The following points allow us to rule out this eventuality: (a) upon illumination by a short saturating flash, more than 80% of P-700 is re-reduced in less than 2 ms. Therefore, the fraction of disconnected P-700 – if any – would be lower than 20%; (b) the kinetics of P-700 oxidation would result from the superimposition of a slow kinetics of large amplitude with a well-developed lag (connected P-700) and of a fast exponential process of small amplitude (disconnected P-700). We did not observe such a complex behaviour in the kinetics of P-700 oxidation (Fig. 3, curve 1), which very likely excludes any heterogeneity in the P-700 pool. Besides, the absence of a lag in the kinetics of oxidation of P-700 and cytochrome *f* (Fig. 3, curve 2) proves that the equilibrium constant between cytochrome *f* and the FeS protein is close to 1 or that FeS remains oxidized in the presence of ascorbate. (c) After a flash given to chloroplasts in oxidizing conditions, no significant reduction of cytochrome *f* associated with the reduction of cytochrome *b* is observed; therefore, it can be excluded that a fraction of cytochrome *b-f* complexes is totally disconnected from P-700. It seems unlikely to us that a sufficiently fast equilibration among cytochrome *b-f* complexes could occur via the plastoquinone pool, through a possible connection between different thylakoid membranes.

Our results are fully consistent with previous conclusions drawn by Kok et al. [2] under similar conditions (DCMU). These authors observed exponential kinetics for P-700 oxidation, although a significant fraction of the secondary donors was in the reduced state; they concluded that the equilibrium constant between P-700 and the secondary donors was close to 1.

During the course of PS I donors reduction which occurs after a flash illumination, the equilibrium constant between cytochrome *f* and P-700 is 25, about 10-times higher than in the preceding case. An increase is observed also for the equilibrium constant between plastocyanin and P-700, although it has an approx. 5-times smaller amplitude.

The variations of the equilibrium constants observed suggest that the redox potentials of some of the carriers involved could well vary with the experimental conditions and several hypotheses can be proposed to interpret these variations: (a) the apoprotein of cytochrome *f* could exist under different conformational states to which would correspond different values of the redox potential; (b) the involved electron carriers are subjected to an electrical field due to the presence of charged species localized in their vicinity. This type of hypothesis has already been considered by many authors, and more specifically for the electron transfer between PS I donors, by Cramer et al. [13]. In our experiments, where gramicidin and KCl are present, we can exclude any effect of a delocalized membrane potential.

Our working hypothesis here is that cytochrome *b* and *f*, which are included in the same complex, are sufficiently close to influence one another by mutual electrostatic interactions. According to this hypothesis, the modifications of the redox properties of cytochrome *f* induced by the presence of a positive charge on cytochrome *b* would be equal to the modifications of the redox properties of cytochrome *b* induced by the presence of a postive charge on cytochrome *f*. In this discussion, we will consider the variation of the net charge of cytochrome *b* or *f* linked to the change in the redox state of these carriers and not the absolute charge of the proteins. We will arbitrarily call 'uncharged' the reduced form of cytochromes.

(1) Perturbation of cytochrome f by the redox state of cytochrome b. After a double flash given in the presence of ferricyanide (see Fig. 3B in Ref. 8),

or after a single flash superimposed on far red d.c. illumination, a large proportion of cytochrome *b* is in the reduced form. Under these conditions, the redox potential of cytochrome *f* is not perturbed, i.e., low.

Under the conditions of experiments Figs. 1 and 2, cytochrome *b* is in its oxidized form in the dark adapted chloroplasts. (We checked in the absence of DCMU that ascorbate did not reduce cytochrome *b*.) During the illumination, only a small fraction of cytochrome *b* is reduced (approx. 20%); thus, the majority of cytochrome *b-f* complexes includes an oxidized cytochrome *b*. According to our working hypothesis, the presence of a positive charge on cytochrome *b* would facilitate the fixation of a negative charge on cytochrome *f*, i.e., would make the redox potential of cytochrome *f* more positive. This could explain the low equilibrium constant between cytochrome *f* and P-700 measured under these conditions. A shift in the redox potential of more than 80 mV would correspond to the variation of the equilibrium constant we observed. The same type of electrostatic interactions could also perturb the equilibrium between plastocyanin and P-700. The largely hydrophilic environment of plastocyanin could explain why the shift in the redox potential is lower than that for cytochrome *f*. The variations of the equilibrium constants could be underestimated, as they are not measured by comparing the two limit conditions, cytochrome *b* fully oxidized or fully reduced: in experiments, Figs. 1 and 2, about 20% of cytochrome *b* was reduced at the end of the illumination period; in the flash experiment Figs. 5 and 6 and Fig. 2 in Ref. 8, a significant fraction (approx. 20–30%) of cytochrome *b* is oxidized.

(2) Perturbation of cytochrome b by the redox state of cytochrome f. During the dark period which follows a group of two flashes (Fig. 3B in Ref. 8) or after a single flash superimposed to far red d.c. illumination (Fig. 7), cytochrome *f* remains mainly oxidized. The positive charge on cytochrome *f* should, then, induce a shift of the redox potential of cytochrome *b* towards positive values, which would prevent the transfer of electrons to a carrier G [14] localized on the other side of the membrane. This would explain the long life-time of reduced cytochrome *b* and the absence of a slow electrogenic phase in oxidizing conditions.

The behaviour of the system is different under more reducing conditions, i.e., when a fraction of the plastoquinone pool and a majority of the PS I donors are already reduced. Bouges-Bocquet [6] observed that after a flash given to Chlorella, there was first a partial oxidation of cytochrome *f*, then an electrogenic phase *b* synchronous with the reduction of cytochrome *f*. No significant absorption changes due to cytochrome *b* are observed during this process. According to our model, a concerted reaction on a plastoquinol molecule occurs. This reaction leads to the reduction of cytochrome *b* and that of cytochrome *f* because primary and secondary donors P-700 and plastocyanin are already in the reduced form. Therefore, cytochrome *b* immediately returns to its low potential form, rapidly transferring its electron to an acceptor (G [14] or plastoquinone?) located on the outer face of the membrane. Thus, in reducing conditions, i.e., when cytochrome *b* remains in its low potential form most of the time, the electron transfer is limited chiefly by the rate of the concerted oxidation of plastoquinol occurring on the cytochrome *b*-FeS site.

We are aware of the fact that other interpretations can be proposed to explain the acceleration of the rate of cytochrome *b* oxidation observed under reducing conditions. In any case, this reductant-induced oxidation of cytochrome *b* presents a striking symmetry to the classic oxidant-induced reduction of cytochrome *b* which is an obvious prediction of our model.

If electrostatic interactions between electron carriers included in the same protein complex play an important role, our model can only represent a rough approximation of the actual situation, since the redox states of other electron carriers as the FeS protein, the second cytochrome *b* or plastocyanin should be also taken into account.

Acknowledgments

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