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MODIFICATION OF THE APPARENT REDOX REACTION BETWEEN CYTOCHROME *f* AND THE RIESKE IRON-SULFUR PROTEIN

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We have investigated the role of cytochrome *f* and the Rieske FeS protein in spinach chloroplasts using the quinone analogue 5-(*n*-undecyl)-6-hydroxy-4,7-dioxobenzothiazole (UHDBT). UHDBT inhibits electron transport at two different sites in spinach chloroplasts. Fluorescence yield measurements monitoring the redox state of Q, the first stable primary acceptor of Photosystem II, and polarographic measurements of electron transport show that at low concentrations UHDBT inhibits near Q. At higher concentrations UHDBT inhibits at a second site. Electron transfer from durohydroquinone to methyl viologen is inhibited (50% inhibition at 21 μM) but not the reaction dichlorophenolindophenol to methyl viologen. Spectroscopic measurements of the kinetics of cytochrome *f* show that UHDBT inhibits the dark reduction rate of the cytochrome following a 100 ms flash (50% inhibition at 15 μM). By contrast, the oxidation kinetics of cytochrome *f* following a single-turnover flash are altered little by UHDBT; the initial rates are indistinguishable, and the half-time increases from 220 μs in the control to 285 μs in the presence of 15 μM UHDBT, largely because the extent of the cytochrome *f* oxidation is enhanced 1.4-fold in the presence of the inhibitor. In a single-turnover flash in the absence of UHDBT, we observe 38–48% of the total cytochrome *f* turning over, while in the presence of UHDBT we observe 60–69% of the cytochrome turning over. We interpret these results in terms of a linear rapid donor pool to Photosystem I, FeS → cytochrome *f* → plastocyanin → P-700, in which UHDBT inhibits by interacting with the Rieske FeS center. We conclude that the enhanced extent of cytochrome *f* oxidation in the presence of UHDBT is due to the removal of the Rieske FeS center from the rapid donor pool. As a consequence, removal of a single electron from the pool results in a greater cytochrome *f* oxidation. These results indicate that the Rieske FeS center and cytochrome *f* equilibrate in a time period comparable to the oxidation time of the cytochrome.

Introduction

In chloroplasts electron transfer between the two photosystems is mediated by the cytochrome *bf*-FeS complex. The complex has been isolated from spinach [1] and is similar to the cytochrome *bc*-FeS complexes of mitochondria and photosynthetic bacteria [2,3]. It consists of at least five polypeptides including one cytochrome *f*, two b_6-type cytochromes, and one Rieske FeS center, and

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Abbreviations: DAD_{ox}, diiminodurene; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DQH_2, durohydroquinone; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; UHDBT, 5-(*n*-undecyl)-6-hydroxy-4,7-dioxobenzothiazole; PS, photosystem; Chl, chlorophyll.

has been shown to oxidize plastoquinol and reduce plastocyanin. The pathway of electrons and translocation of protons in this region is complicated, involving electron transfer from plastoquinol to plastocyanin; possibly a Q-cycle in which an electron cycles across the membrane translocating an additional proton [4]; and under some conditions PS I cyclic electron transport [5]. There have been several proposals describing the mechanism of quinol oxidation by the oxidoreductase [2–8]. Although opinions differ both over electron pathways and the indentity of the molecules undergoing redox reactions, the Rieske FeS center [9] is central to most of the recent electron-transfer and Q-cycle oxidoreductase schemes, despite the fact that the kinetic involvment of the center is especially difficult to characterize experimentally.

In mitochondria [10] and in photosynthetic bacteria [11] there is convincing evidence that the Rieske FeS center is a redox component in electron transfer. In chloroplasts there are fewer data. Light-induced oxidation of the Rieske center by Photosystem I followed by its dark reduction demonstrates that the Rieske center turns over in the light [12,13]. However, since the center can only be observed by its EPR signal at low temperatures, there is no direct measurement demonstrating whether it is kinetically competent to be a component in the PS I donor pool. It could simply equilibrate slowly with the rapid donors [12]. In view of the invisibility of the Rieske center at physiological temperatures we have attempted to determine its redox behavior by investigating its effect on cytochrome *f*, a component known to be in the donor pool. In chloroplasts, Koike et al. [14] showed that the addition of DBMIB increased the extent of cytochrome *f* oxidation by a short flash nearly 2-fold. They argued that a donor normally equilibrates rapidly with cytochrome *f*, but that in the presence of DBMIB the reduction of the cytochrome by this donor is blocked. They tentatively suggest that the Rieske center might be the donor involved. In the presence of DBMIB, removal of an electron from the smaller PS I donor pool by a single-turnover flash would result in a greater oxidation of the remaining donors, i.e., cytochrome *f* and plastocyanin. It is questionable, however, whether this explanation is adequate to account for their data, since they showed that DBMIB not only enhanced the extent but also induced biphasic kinetics in the oxidation of cytochrome *f*. In the simple linear scheme, DBMIB inhibition at the Rieske center would not be expected to induce biphasic kinetics in the oxidation of cytochrome *f* by P-700. Bowyer et al. [11] have shown that in the photosynthetic bacterium *Rhodopseudomonas sphaeroides*, bound cytochrome *c* equilibrated rapidly with a component inferred to be the Rieske center. This conclusion was substantiated by experiments using UHDBT, an electron-transfer inhibitor. UHDBT appears to bind to the Rieske center raising its midpoint potential from 280 to 350 mV and shifting the g_y value of its EPR spectrum from 1.90 to 1.89. They found that the addition of UHDBT to chromatophores increases the extent of cytochrome *c* oxidation in single-turnover flashes. The enhanced extent of the oxidation was accounted for by UHDBT blocking the Rieske center oxidation, thereby removing it from the high-potential donor pool, resulting in a greater cytochrome *c* oxidation.

In the present study we investigate the PS I reaction DQH_2 to methyl viologen [15]. This electron-transfer reaction is of particular interest because DQH_2 donates electrons prior to the cytochrome *bf*-FeS complex, thereby enabling us to investigate electron transport between quinol oxidation and P-700 reduction. We have focused on determining the light-induced behavior of cytochrome *f* and by inference the kinetic competency of the Rieske center. Our data using both single-turnover and long flashes show that the kinetics and extent of cytochrome *f* turnover are consistent with it functioning in a linear sequence as a rapid donor to P-700 [16,17]. Since the Rieske FeS protein cannot be seen under physiological conditions, we used the quinone analogue UHDBT to probe the interaction of the Rieske center with cytochrome *f*. In both mitochondria [18] and photosynthetic bacteria [11] UHDBT is an effective inhibitor of electron transport, specifically blocking the oxidation of the Rieske center. In the cytochrome *bf*-FeS complex isolated from spinach chloroplasts the plastoquinol-plastocyanin oxidoreductase activity with plastoquinol-1 and plastoquinol-9 was shown to be sensitive to UHDBT [1]. In chloroplasts we determined the effect of UHDBT on several electron-transport

reactions and found that there were at least two sites of inhibition *. In agreement with other workers [20,21], we found that UHDBT at submicromolar concentrations is a DCMU-like inhibitor, inhibiting both H_2O to methyl viologen and the PS II reaction H_2O to DAD_{ox}. This site of inhibition is verified in the present study by fluorescence yield measurements which monitor the kinetics of the reoxidation of Q after an actinic flash. At higher UHDBT concentrations we observed a second site of inhibition. The partial electron-transfer reaction DQH_2 to methyl viologen is inhibited by UHDBT [19,20], whereas the reaction DCIP/ascorbate to methyl viologen is not. Spectroscopic measurements of the light-induced oxidation and rereduction kinetics of cytochrome *f* localize the site of action of UHDBT between plastoquinol oxidation and cytochrome *f* reduction. These results indicate UHDBT inhibits electron transfer by interacting with the Rieske FeS center. We then proceeded to characterize the effect of UHDBT on the redox behavior of cytochrome *f* in a single-turnover flash. The data show that cytochrome *f* equilibrates rapidly with a lower potential component that we infer to be the Rieske FeS center. The equilibration time must be comparable to or faster than the oxidation time of cytochrome *f*, $t_{1/2} = 200–300$ μs, which is an order of magnitude faster than the rate-limiting step in overall electron transport.

Materials and Methods

Chloroplasts (naked lamellae) were isolated from spinach leaves purchased from local markets according to a procedure described elsewhere [22]. The experiments were done within 6 h of chloroplast preparation. The chlorophyll concentration was determined according to the method of Arnon [23]. Chloroplasts were suspended in a standard reaction medium containing 30 mM Tricine KOH (pH 8.0), 0.1 M sorbitol, 3 mM $MgCl_2$ and 20 mM KCl. Other additions are indicated in the figure legends. All experiments, unless otherwise indicated, were temperature controlled (±0.2°C) at temperatures between 16 and 18°C.

Electron transport was measured polarographically in a thermostatically controlled vessel using a Clark-type electrode. Actinic light was provided by a heat-filtered tungsten lamp filtered by a red-blocking filter (Corning CS 2-59). The intensity of the actinic light was saturating, ranging from 5 to $10 \cdot 10^5$ erg/cm^2 per s. UHDBT was a gift from B. Trumpower (Hanover, NH).

The relative fluorescence yield following an actinic flash was measured according to a method described by Joliot [24] and described in detail elsewhere [25]. The fluorescence, detected at right angles by a gated photomultiplier, was monitored by low-intensity, nonactinic flashes at varying times following a 300 ns laser flash.

Light-induced absorbance changes were measured using a home-built single-beam spectrophotometer (JW). Actinic light flashes were provided by a xenon flashlamp (FX-193, EG&G Electro-optics). The lamp was filtered by a red-blocking filter (Corning 2-58) and exhibited a half-peak width of 6 μs. These flashes were short enough to ensure but a single turnover of each PS I reaction center. This was demonstrated by comparing the extent of the fast phase of the absorbance change at 515 nm in PS I stimulated by the xenon flash and by a laser flash (Pase-R, model DL-1000, rhodamine 6G in ethanol, duration less than 1 μs). The extent of the absorbance increase was the same for both flashes (within 5%). Continuous actinic light was provided by a 250 W tungsten halogen lamp filtered by a hot mirror (Melles Griot 03 MHG 007), a heat-absorbing filter (Corning CS 1-75) and a red filter (Corning CS 2-58). The duration of the flash was controlled by Uniblitz shutters. Unless otherwise noted all actinic light was saturating to the extent that a 50% attenuation in intensity did not alter the extent or kinetics of the absorbance change. The monochromator was calibrated to within 0.3 nm using the 546.1 nm line of Hg. All experiments were done using a 1 cm^2 thermostatically controlled cuvette. Cytochrome *f* content was determined using a millimolar different extinction coefficient for the wavelength pair 553–540 nm of 20 $mM^{-1} \cdot cm^{-1}$.

* We have published elsewhere a preliminary report describing these results [19].

Results and Discussion

Inhibition by UHDBT near the PS II acceptor Q

In spinach chloroplasts UHDBT is an effective inhibitor of linear electron transport. The electron-transfer reaction from H_2O to methyl viologen is inhibited 50% at a concentration of 0.7 μM (data not shown). The site of inhibition, however, is different from the Rieske FeS site characterized in mitochondria and photosynthetic bacteria. This is evident from the observation that the reaction H_2O to DAD_{ox} was even more highly sensitive to UHDBT, 50% inhibition at a concentration of 0.2 μM (data not shown). Electron transport from H_2O to DAD_{ox} is driven by PS II and does not include the Rieske center, since the reaction is relatively insensitive to low DBMIB concentrations [26], an inhibitor that has been shown to act on the Rieske center [27]. The H_2O to DAD_{ox} reaction is more sensitive to UHDBT than is H_2O to methyl viologen because electron transport in the presence of DAD_{ox} is not limited by plastoquinol oxidation but by PS II [28]. These results indicate that UHDBT acts at or near Q in a DCMU-like manner. This site of inhibition is verified by observing the effect of UHDBT on the reoxidation kinetics of Q subsequent to an actinic flash. Oxidized Q is a potent quencher of chlorophyl fluorescence. Because of this the redox state of Q can be determined by measuring the relative fluorescence yield stimulated by a low-intensity flash at various time intervals after an actinic flash [29]. In the absence of UHDBT the relative fluorescence yield decreased within 2 ms of the actinic flash, indicating the rapid reoxisation of Q (Fig. 1). In the presence of UHDBT the fluorescence yield remained high for at least 5 ms, indicating that UHDBT blocks the reoxidation of Q in a manner similar to DCMU. These results confirm our earlier observation [19] and a similar conclusion by Oettmeier and co-workers [20] in which they showed that UHDBT displaces metribuzin, a DCMU-type

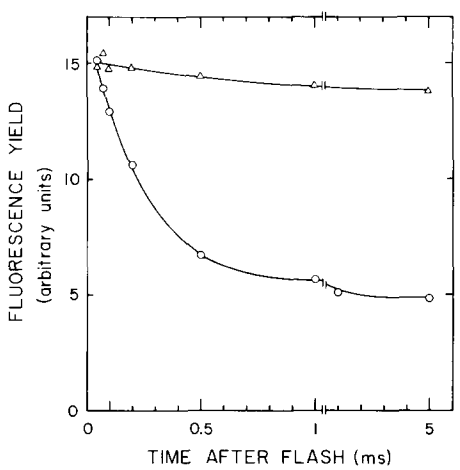

Fig. 1. Decay of chlorophyll fluorescence in chloroplasts following an actinic flash in the absence (○) and presence (△) of 1 μM UHDBT. In addition to the standard reaction medium the mixture contained 0.1 mM methyl viologen and chloroplasts equivalent to approx. 10 μg Chl/ml. Measurements were done at room temperature.

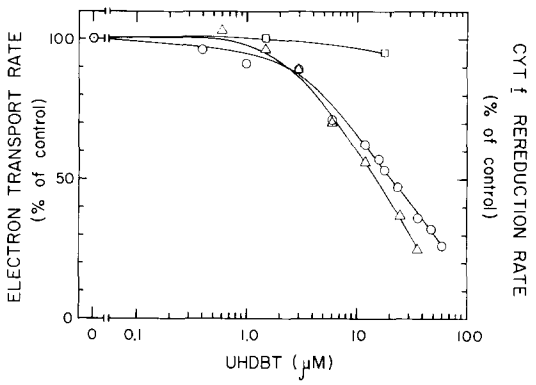

Fig. 2. Effect of UHDBT on electron transport and the rate of cytochrome *f* dark reduction in chloroplasts. (a) DCIP to methyl viologen (□———□). In addition to the standard reaction medium and UHDBT as indicated, the mixture contained 0.1 mM methyl viologen, 15 μM DCMU, 10 μM gramicidin, 0.25 mM DCIP, 1.5 mM ascorbate, 150 units superoxide dismutase/ml and chloroplasts equivalent to 20 μg Chl/ml. The control rate of electron transport was 465 μequiv./mg Chl per h. (b) DQH_2 to methyl viologen (○———○). In addition to the standard reaction mixture and UHDBT as indicated, the mixture contained 0.1 mM methyl viologen, 10 μM DCMU, 5 μM gramicidin, 0.5 mM DQH_2, 300 units superoxide disorutase/ml, and chloroplasts equivalent to 20 μg Chl/ml. The control rate of electron transport was 1130 μequiv./mg Chl per h. (c) Cytochrome *f* rereduction rate (△———△). The rate of cytochrome *f* rereduction is defined as the inverse half-time in seconds of the dark reduction of cytochrome *f* following a 100 ms actinic flash. The kinetics were determined by measuring the change in absorbance at 554–540 nm as a function of time (see Fig. 4). The control rate was 42 s^{-1}. In addition to the standard reaction medium and UHDBT as indicated, the mixture contained 0.1 mM methyl viologen, 10 μM DCMU, 5 μM gramicidin, 0.5 mM DQH_2 and chloroplasts equivalent to 20 μg Chl/ml.

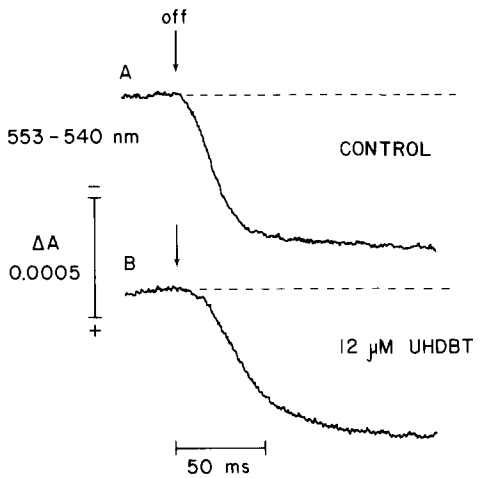

Fig. 3. Kinetics of the dark reduction of cytochrome *f* monitored at 553–540 nm in chloroplasts following a 100 ms actinic flash in the absence and presence of UHDBT. The traces shown are the average of 64 runs. The repetition rate of the flashes was 0.3 Hz. The response time of the instrument was 1 ms. The half-bandwidth was 2 nm. (A) In addition to the standard reaction medium, the mixture contained 0.1 mM methyl viologen, 10 μM DCMU, 5 μM gramicidin, 0.5 mM DQH_2 and chloroplasts equivalent to 20 μg Chl/ml. (B) As in A plus 12 μM UHDBT.

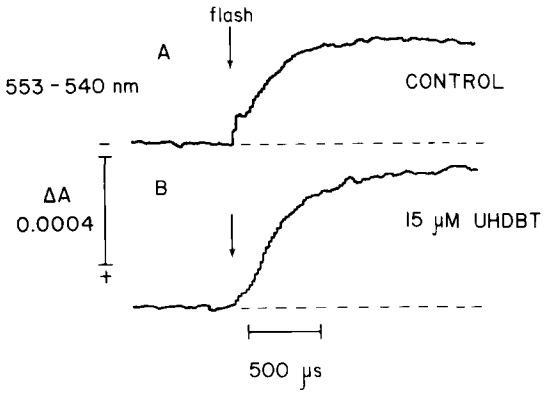

Fig. 4. Kinetics of the oxidation of cytochrome *f* monitored at 553–540 nm in chloroplasts induced by a single-turnover flash. The initial small absorbance decrease observed in the control is similar to that observed by Haehnel et al. [33] which was attributed to formation of a carotenoid triplet. The traces shown are the average of 512 runs using eight samples. The repetition rate of the flashes was 0.3 Hz. The response time of the instrument was 30 μs. The half-bandwith was 2 nm. (A) In addition to the standard reaction medium, the mixture contained 0.1 mM methyl viologen, 10 μM DCMU, 5 μM gramicidin, 0.5 mM DQH_2 and chloroplasts equivalent to 25 μg Chl/ml. (B) As in A plus 15 μM UHDBT.

inhibitor, from spinach thylakoid membranes. This site of inhibition is sufficient to account for most of the data in Ref. 21, where it was shown that UHDBT at concentrations below 1 μM inhibits the reaction H_2O to methyl viologen but not the reaction DQH_2 to methyl viologen (see below).

Cytochrome f redox kinetics and inhibition by UHDBT at the Rieske FeS protein

At higher concentrations UHDBT inhibits linear electron transport at a second site. The reaction DQH_2 to methyl viologen is driven by PS I and is sensitive to low concentrations of DBMIB [19] but insensitive to DCMU, indicating that the electron-transfer pathway includes the cytochrome *bf*-FeS complex. The steady-state electron-transport data in Fig. 2 show that the DQH_2 to methyl viologen reaction was inhibited 50% at a UHDBT concentration of 21 μM. In the reaction DCIP to methyl violgen, which does not include the cytochrome *bf*-FeS complex, UHDBT had no noticeable effect. Using chloroplast fragments from a mutant of *Chlamydomonas reinhardii* Oettmeier and co-workers [20] also found the reaction DQH_2 to methyl viologen to be sensitive to UHDBT.

In the dark cytochrome *f* is reduced. In continuous light the cytochrome is oxidized by PS I, and reduced in the dark by electrons from the plastoquinol pool. Although the rereduction kinetics are complex, dependent on the components in the PS I donor pool and the oxidation rate of the plastoquinol pool [16], in the reaction H_2O to methyl viologen it has been shown that the rate of cytochrome *f* rereduction following a long flash is linearly proportional to the rate-limiting step in steady-state electron transport [17,30]. Hence, slowing the rate-limiting step results in a decrease in the rate of cytochrome *f* dark reduction. We observed the same relationship when DQH_2 is used as the electron donor and PS II is blocked by DCMU. In the reaction DQH_2 to methyl viologen the dark reduction rate of cytochrome *f*, defined as the inverse half-time of the decay, was slowed by the addition of UHDBT (Fig. 3), whereas the oxidation was rapid, and little affected by UHDBT (Fig. 4). Following a 100 ms flash the rereduction half-time of cytochrome *f* in the control was 20 ms

and in the presence of 12 μM UHDBT the half-time was 35 ms. The absorbance increase following a 100 ms flash plotted as a function of wavelength both in the absence and presence of UHDBT shows that the change was due to the α-band of cytochrome *f* (data not shown). In the presence of DQH_2 we typically observed one cytochrome *f* turning over per 700 Chl molecules. This value is approx. 90% of the total cytochrome *f* observed either turning over in the light in the reaction H_2O to methyl viologen or in chemical titrations [17]. In an unexplained observation, we found that following a 20 ms flash the slowing of cytochrome *f* rereduction by UHDBT was less. At 12 μM UHDBT the rate was decreased by about 20% following a 20 ms flash (data not shown) compared to a 45% decrease following a 100 ms flash.

The effect of various concentrations of UHDBT on the dark rereduction rate of cytochrome *f* is shown in Fig. 2. The sensitivity of the rate to UHDBT (50% inhibition at 15 μM) was similar to that of steady-state electron transport. The fact that 15 μM UHDBT inhibited by 50% the rereduction rate of cytochrome *f*, while the light-induced oxidation remained much more rapid than the rate-limiting step *, indicates that the site of inhibition by UHDBT is on the quinol side of cytochrome *f*. The data shown here limit the site of inhibition, at higher UHDBT concentrations, between quinol oxidation and cytochrome *f* reduction. In view of these results and the EPR data in mitochondria and photosynthetic bacteria demonstrating an interaction between the Rieske center and UHDBT, we conclude that the site of UHDBT inhibition in chloroplasts is the Rieske protein. The most probable mechanism of inhibition, suggested by Trebst [31] for DBMIB, is that UHDBT and quinol compete for a binding site at the Rieske protein.

The assignment of UHDBT blocking at the Rieske center is adequate to account entirely for the results presented here, and adds to an increasing amount of data indicating that the Rieske center is an obligatory component in electron transport, and that the pathway of electrons from plastoquinol (PQ) to P-700 is: PQ → FeS → cytochrome *f* → plastocyanin → P-700. However, the kinetic competency of the Rieske center has yet to be established. The only work touching on this question is that of Koike and co-workers [14] discussed above, which is complicated by the fact that DBMIB not only increased the extent of cytochrome *f* oxidation but also induced biphasic oxidation kinetics. We have attempted to determine the extent of the Rieske center and cytochrome *f* interaction by examining the influence of UHDBT on the redox behavior of cytochrome *f* in single-turnover flashes.

Apparent redox reaction between cytochrome f and the Rieske FeS center

The proportion of cytochrome *f* observed to undergo oxidation in a single-turnover flash would be expected to depend upon the components in the PS I donor pool, primarily the stoichiometries and midpoint potentials of each of the donors and their rates of oxidation by P-700 and rereduction

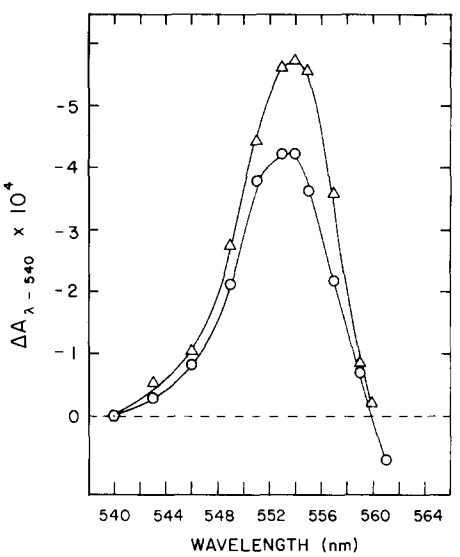

Fig. 5. Wavelength dependence of the absorbance change induced by a single-turnover actinic flash in chloroplasts. Experimental conditions were as described in Fig. 5 except the time constant was 100 μs. All absorbance changes were determined using 540 nm as the reference wavelength. (○) Control, (△) 15 μM UHDBT.

* In the presence of 15 μM UHDBT the half-time for one electron through P-700 in saturating light is approx. 5 ms. This half-time is determined using an electron-transport rate of 620 μequiv./mg Chl per h and a P-700/Chl stoichiometry of 600 (see Ref. 17 for details).

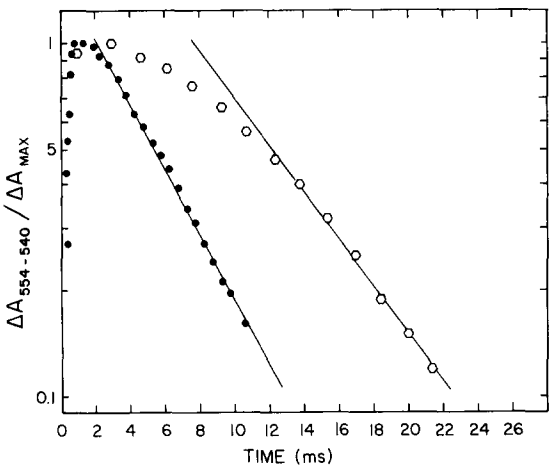

Fig. 6. Semilogarithmic plot of the reduction of cytochrome *f* following a single-turnover flash. Conditions as in Fig. 5. (●) Control, (○) 2 μM UHDBT.

by plastoquinol. Because of this, the light-induced redox change in cytochrome *f* can be used as a sensitive measure of the redox state of its neighbors. For example, altering the rate of electron transfer to or from cytochrome *f*, or a change in the midpoint potential of a component in rapid equilibrium with cytochrome *f*, can have a pronounced effect on the extent of cytochrome *f* oxidation. This can be seen in Fig. 4 where the light-induced oxidation kinetics of cytochrome *f* are shown in the absence and presence of UHDBT. The wavelength dependence of the absorbance change shows that at 554–540 nm the change is due primarily to a decrease in absorbance of the α-band of cytochrome *f* (Fig. 5). In the reaction DQH_2 to methyl viologen, a single-turnover flash results in the oxidation of 38–48% of the total cytochrome *f* (total cytochrome *f* 1 : 650 Chl [17]. The addition of 15 μM UHDBT increases the extent of the oxidation 1.4 ± 0.1-fold. The half-time of cytochrome *f* oxidation was increase slightly by UHDBT from 220 μs in the control to 280 μs in the presence of UHDBT.

These data indicate that a component in rapid equilibrium with cytochrome *f* is altered by addition of UHDBT. The most probable component is the Rieske FeS protein. Two possible mechanisms arise immediately. (i) UHDBT binds to the Rieske center, decreasing its oxidation rate so that it is slower than the oxidation rate of cytochrome *f*. This would have the effect of removing the lowest potential component from the donor pool, at least on the time scale of cytochrome *f* oxidation (200–300 μs). As a consequence, photooxidation of P-700 and the subsequent removal of an electron from the smaller donor pool results in an enhanced oxidation of cytochrome *f*. In fact, assuming the midpoint potential of plastocyanin is 10–20 mV higher than that of cytochrome *f*, that their stoichiometry is 1 : 1 and that the Rieske center is removed from the donor pool, then using the Boltzmann energy distribution the expected extent of cytochrome *f* oxidation due to a single-turnover flash is 55–60%. If, instead, the stoichiometry of plastocyanin to cytochrome *f* is 2 : 1 then under the same conditions the expected extent of cytochrome *f* oxidation is 39–45%. In the presence of UHDBT we observed 55–68% of cytochrome *f* turning over. Because of the uncertainty in the midpoint potentials of plastocyanin and cytochrome *f* these data cannot be used to determine precisely their stoichiometry. However, in view of the large extent of cytochrome *f* oxidation observed in the presence of UHDBT, we can say it is likely that either the stoichiometry is equal to or less than 2 : 1, or the difference in midpoint potential of the two components is greater than 30 mV. The enhanced extent of cytochrome *f* oxidation in the presence of UHDBT sets an upper limit of several hundred microseconds on the time of electron donation from the Rieske center to cytochrome *f*. The rate of cytochrome *f* oxidation after a single-turnover flash depends primarily on the rate of equilibration of the cytochrome with plastocyanin and with the Rieske center. If UHDBT blocks the oxidation of the Rieske center, in principle it is possible to establish an approximate value for the equilibration time between the Rieske center and cytochrome *f* by comparing the oxidation kinetics of cytochrome *f* in the absence and presence of UHDBT; the difference between the traces of Fig. 4 suggests a $t_{1/2}$ in the range of 500–800 μs for the change occurring after 200 μs. However, the data shown here are not adequate to pursue this analysis to shorter times, and we cannot exclude the possibility that a faster equilibration occurs. (ii) Alternatively, UHDBT could bind to the Rieske center, stabilizing the reduced form

so that the midpoint potential increases. If in the presence of UHDBT the midpoint potential of Rieske center was comparable to that of cytochrome *f*, then the hole created by removal of a single electron would be shared equally by the Rieske center and cytochrome *f*. In photosynthetic bacteria, Bowyer et al. [1] have shown that the midpoint potential of the Rieske center is raised approx. 70 mV upon the addition of UHDBT. Inasmuch as we observed oxidation of up to 68% of cytochrome *f* in a single-turnover flash, this second explanation would require that the midpoint potential of the Rieske center in the presence of UHDBT be measurably greater than that of cytochrome *f*. It should be noted that the two explanations are not mutually exclusive.

There are other notable features concerning the oxidation of cytochrome *f*. The extent of cytochrome *f* oxidation in a single-turnover flash in the reaction DQH_2 to methyl viologen was typically 40% of the total cytochrome. This extent was substantially greater than that observed in the reaction H_2O to methyl viologen, where typical values are 20% or less of the total cytochrome *f* (see Ref. 32). To account for the enhanced oxidation we offer the ad hoc explanation that DQH_2 binds to the reduced Rieske center, raising the FeS center redox potential. Increasing the midpoint potential of the Rieske center would result in a greater single-flash oxidation of cytochrome *f*.

Another anomalous result was that at a UHDBT concentration (e.g., 2 μM) that has little effect on steady-state electron transport, the extent of cytochrome *f* oxidation in a single-turnover flash is increased 1.4-fold and the half-time of the rereduction was more than twice as long (data not shown). We pointed out earlier that it is surprising that 2 μM UHDBT would slow the rereduction following a single-turnover flash over 2-fold and have only a 15% effect on the cytochrome rereduction rate following a 100 ms flash [19]. However, examination of the rereduction kinetics in a semilogarithmic plot shows that if we discount the delay, the actual half-time for the decay increases from 3.3 ± 0.3 ms in the control to 4.4 ± 0.4 ms in the presence of 2 μM UHDBT (Fig. 6). These half-times are consistent with the rate of steady-state electron transport under the same conditions, where we calculate half an electron through each cytochrome *bf*-FeS complex in 3–4 ms.

There is a great deal of interest recently in the partitioning of PS II and PS I between appressed and nonappressed thylakoid membrane [34]. Studies indicate that the cytochrome *bf*-FeS complex is evenly distributed over the two membrane phases [35]. In order to explain electron transfer between the two photosystems it has been suggested that plastocyanin is a mobile carrier, capable of electron transfer over several hundred ångstroms on a millisecond time scale. In assessing the feasibility of this transfer process, the transfer time commonly used has been the rate-limiting step in overall electron transport, i.e., on the millisecond time scale. Our results, in the presence of UHDBT, show that approx. 65% of cytochrome *f* can be oxidized with a half-time of 285 μs. This observation places more severe constaints on the diffusion time of plastocyanin, since significantly more than one-half of cytochrome *f* must be oxidized by P-700 with a half-time under 300 μs. It should be noted that this constraint is not necessarily forbidding for the diffusion of a molecule the size of plastocyanin in an aqueous phase [36].

These data provide evidence for inhibition of linear electron transport by UHDBT at the Rieske FeS center. In addition, they show that the cytochrome *f* equilibrates with a lower potential component, most probably the Rieske center, within a time scale of a few hundred microseconds. Inasmuch as slowing the turnover of the Rieske center with UHDBT slows the reduction of cytochrome *f*, the results are entirely consistent with a linear PS I donor pool: FeS → cytochrome *f* → plastocyanin → P-700 at a stoichiometry of FeS : cytochrome *f* : P-700 of 1 : 1 : 1. Consideration of alternative explanations that exclude the Rieske center would necessitate introducing a new component into the pool, or considering more complex interactions between the known components.

Addendum

Subsequent to the completion of this work, we became aware of a recent publication by Prince et al. [37] in which they demonstrate light-driven electron transfer from *R. sphaeroides* reaction centers to a cytochrome *bf*-FeS complex isolated

from spinach. The data show a light-induced reduction of cytochrome b_6 occurring within 35 ms. The reduction is dependent upon a redox compoment with an apparent midpoint potential of 280 mV that is presumably the Rieske FeS center.

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