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THE ROLE OF THE RIESKE IRON-SULFUR CENTER AS THE ELECTRON DONOR TO FERRICYTOCHROME c_2 IN *RHODOPSEUDOMONAS SPHAEROIDES*

JOHN R. BOWYER [a,*], P. LESLIE DUTTON [b], ROGER C. PRINCE [b] and ANTONY R. CROFTS [a]

[a] *Department of Physiology and Biophysics, University of Illinois, Urbana, IL 61801 (U.S.A.) and* [b] *Department of Biochemistry and Biophysics, Johnson Research Foundation, University of Pennsylvania, Philadelphia, PA 19104 (U.S.A.)*

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Summary

The Rieske iron-sulfur center in the photosynthetic bacterium *Rhodopseudomonas sphaeroides* appears to be the direct electron donor to ferricytochrome c_2, reducing the cytochrome on a submillisecond timescale which is slower than the rapid phase of cytochrome oxidation ($t_{1/2}$ 3—5 μs). The reduction of the ferricytochrome by the Rieske center is inhibited by 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) but not by antimycin. The slower (1—2 ms) antimycin-sensitive phase of ferricytochrome c_2 reduction, attributed to a specific ubiquinone-10 molecule (Q_z), and the associated carotenoid spectral response to membrane potential formation are also inhibited by UHDBT. Since the light-induced oxidation of the Rieske center is only observed in the presence of antimycin, it seems likely that the reduced form of Q_z (Q_zH_2) reduces the Rieske center in an antimycin-sensitive reaction. From the extent of the UHDBT-sensitive ferricytochrome c_2 reduction we estimate that there are 0.7 Rieske iron-sulfur centers per reaction center.

UHDBT shifts the EPR derivative absorption spectrum of the Rieske center from g_y 1.90 to g_y 1.89, and shifts the $E_{m,7}$ from 280 to 350 mV. While this

* To whom correspondence should be addressed. Current address: Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03755, U.S.A.

Abbreviations: UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole; DBMIB, 2,5-dibromo-3-methyl-6-isopropylbenzoquinone; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; PES, *N*-ethyl phenazonium ethosulfate; PMS, *N*-methyl phenazonium methosulfate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Mops, morpholinopropane sulfonic acid; $(BChl)_2$, bacteriochlorophyll dimer; $(BChl)_2^+$, the oxidized form of the dimer.

latter shift may account for the subsequent failure of the iron-sulfur center to reduce ferricytochrome c_2, it is not clear how this can explain the other effects of the inhibitor, such as the prevention of cytochrome *b* reduction and the elimination of the uptake of H^+_{II}; these may reflect additional sites of action of the inhibitor.

Introduction

Light-driven electron flow in the photosynthetic bacteria is initiated in photochemical reaction centers, where the photon elicits the oxidation of a bacteriochlorophyll dimer and the reduction of a bacteriopheophytin molecule. In turn, the latter reduces the primary quinone which is also tightly associated with the reaction center and remains with it on isolation of the reaction center from the membrane. Thus within a few hundred picoseconds of the arrival of the photon the reaction center has an oxidized bacteriochlorophyll dimer [$(BChl)^+_2$] and a reduced primary quinone (Q^-_I) (see Refs. 1 and 2 for recent reviews). The direct electron donor to the $(BChl)^+_2$ is a *c*-type cytochrome (see 3), but the fate of the electron on Q^-_I is less clear. Nevertheless, the reaction center drives a cyclic electron transport system, and the electron on Q^-_I eventually returns to the ferricytochrome c_2 by passing through a ubiquinone-cytochrome *b*-c_2 (Q-b/c_2) oxidoreductase [3—6]. The Q-b/c_2 oxidoreductase has been studied in some detail in the closely related bacteria *Rhodopseudomonas sphaeroides* and *Rhodopseudomonas capsulata*, and it seems that it is functionally very similar to the ubiquinone-cytochrome *b*-c_1 oxidoreductase of mitochondria (e.g., see Ref. 7).

A functionally unique molecule of ubiquinone-10 (Q_z, formerly known as Z) plays a central role in the Q-b/c_2 oxidoreductase. It is not visible spectroscopically, so its properties have been determined by its effects on ferricytochrome c_2 reduction [8—11] and by differential ubiquinone extraction experiments [12—15]. In *Rps. sphaeroides* the interaction of Q_z with ferricytochrome c_2 appears to be rate limited by a collisional, second order reaction [9,10] with the kinetics indicating a stoichiometry of 0.8 Q_z molecules per reaction center [9]. Under optimal conditions the reduction of ferricytochrome c_2 by Q_z has an apparent halftime of 1—2 ms [8—10].

Antimycin abolishes the 1—2 ms reduction of ferricytochrome c_2 by Q_z, and under these inhibited conditions three turnovers of the reaction center are required to convert all the ferrocytochrome c_2 into a stably oxidized form [16]. Approximately half of the total ferrocytochrome is stably oxidized on the first flash, and the extent of oxidation on each flash fitted a model where a pair of cytochromes c_2 were electrostatically attached to each reaction center [16,17]. Such a situation was apparently analogous to the case of *Chromatium vinosum*, where two cytochromes *c*-555 are bound to each reaction center, both in situ and in detergent-solubilized reaction center preparations. However, this model has required reappraisal in the light of recent observations using the 'ubiquinone analog' 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) [20,21]. The presence of UHDBT increases the amount of cytochrome c_2 stably oxidized after a single flash, suggesting that it inhibits an antimycin-

insensitive ferricytochrome c_2 reduction which is normally so rapid that it is undetectable. The source of the antimycin-insensitive, UHDBT-sensitive electrons, designated J, appeared to be approximately equipotential with cytochrome c_2 ($E_{m,7}$ 295 mV), and Bowyer et al. [22] suggested that it might be the Rieske iron-sulfur center ($E_{m,7}$ 285 mV [23]). This was not unprecedented, for Evans et al. [24] had previously reported the light-induced oxidation of the Rieske iron-sulfur center in *C. vinosum*, and similar results were obtained in *Rps. sphaeroides* [23]. However, these experiments had used continuous illumination, and the possibility remained that the observed oxidation was the result of slow equilibration reactions of little physiological significance.

In this paper, we examine whether the Rieske iron-sulfur center is indeed the direct reductant of ferricytochrome c_2 in a UHDBT-sensitive reaction, and whether Q_zH_2 reduces the oxidized Rieske center in an antimycin-sensitive reaction. A preliminary report of this work has been presented [28].

Materials and Methods

Rhodopseudomonas sphaeroides Ga was grown and chromatophores were prepared as described in Ref. 16 for the EPR and H^+ binding experiments, and as described in Ref. 29 for the other measurements. EPR measurements were made with a Varian E-109 spectrometer equipped with a flowing helium cryostat [23]. Chromatophore samples for EPR analysis were frozen within 10 s of removal from the anaerobic vessel in a 5 : 1 mixture of 2-methylbutane and cyclohexane at liquid nitrogen temperature. When samples were illuminated before freezing, this was done within 10 s of removal from the vessel, and the samples were frozen within 700—1000 ms. Flash illumination was provided either by a 20 ns flash from a Q-switched ruby laser (Apollo Lasers Inc., 6365 Arizona Circle, Los Angeles, CA 90045) or by a 6 μs Xenon flash. Continuous illumination was provided by a focused 8 V, 5 A microscope lamp 10 cm from the sample.

Flash-induced optical changes were measured as described previously [22] using either a 24 μs Xenon flash or a 300 ns laser flash (Phase-R DL 1200). Flash-induced proton binding was measured optically as described in Ref. 30, using a 6 μs Xenon flash.

The control of the state of reduction of the chromatophores was achieved using redox potentiometry as previously described [16]. Valinomycin was routinely added to prevent build-up of a membrane potential, and UHDBT, a generous gift from Drs. Karl Folkers and Thomas Porter of the University of Texas at Austin, was added as an ethanolic solution.

Ferrocytochrome c_2 oxidation and $(BChl)_2$ oxidation were measured at 551—542 nm ($\epsilon_{red\text{-}ox}$ = 18.1 $mM^{-1} \cdot cm^{-1}$) and 542 nm ($\epsilon_{ox\text{-}red}$ = 10.8 $mM^{-1} \cdot cm^{-1}$), respectively. Reaction center oxidation was measured at 542 nm because absorption changes due to cytochrome c_2 and cytochrome b are negligible at this wavelength, and the background absorption due to the light harvesting pigments is low. The extinction coefficients were based on those obtained by Dutton et al. [16] after correction for the slightly different measuring wavelengths used here. The degree of saturation of the flash was determined at high E_h when there are no electron donors available to re-reduce $(BChl)_2^+$ [16,20].

Results

Electron paramagnetic resonance spectroscopy

Fig. 1 shows the results of an experiment designed to determine whether the Rieske iron-sulfur center was involved in electron flow after a single turnover of the reaction center. Samples of chromatophores were poised at a redox potential (E_h 200 mV) where the Rieske center and cytochrome c_2 were reduced and Q_z was oxidized before illumination, and antimycin was added to slow electron flow around the Q-b/c_2 oxidoreductase. A single laser flash caused the oxidation of more than 40% of the Rieske iron-sulfur center under

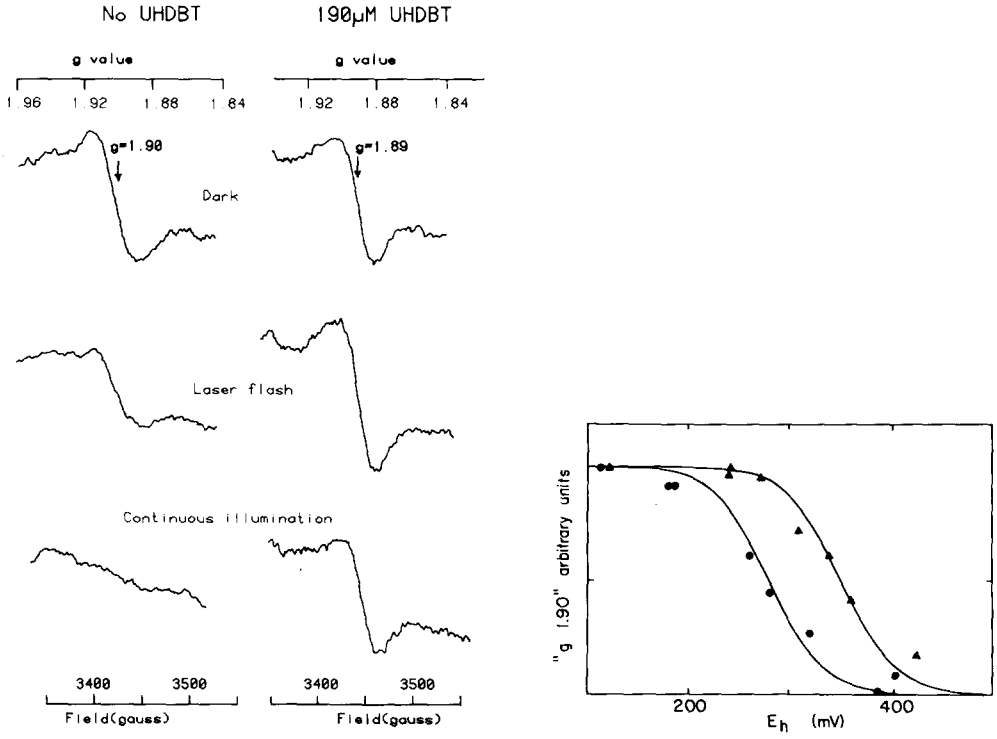

Fig. 1. Effect of illumination and UHDBT on the g_y signal of the Rieske iron-sulfur center. Chromatophores were suspended to 11.2 μM reaction center in 20 mM Mops, 100 mM KCl, pH 7.0 containing 500 μM potassium ferrocyanide, 30 μM $FeCl_3$, 1 mM EDTA, 30 μM FCCP, 5 μM valinomycin, and 30 μM antimycin. The redox potential was 200 ± 10 mV. Spectrometer settings: sample temperature 12 K; microwave power 20 mW; modulation amplitude, 16 gauss; microwave frequency, 9.134 GHz. Illuminated samples were frozen about 700 ms after excitation by a ruby laser flash or by 10 s of continuous illumination.

Fig. 2. The effect of UHDBT on the oxidation reduction midpoint potential of the Rieske iron-sulfur center. Chromatophores were suspended to 9 μM reaction center in 100 mM Mops, 100 mM KCl, pH 7.0, 50 μM DAD and PMS (●), and plus 300 μM UHDBT (▲). EPR spectrometer settings: sample temperature, 18 K; microwave power, 5 mW; modulation amplitude, 16 gauss, microwave frequency, 9.134 GHz. The points show the relative concentration of reduced iron-sulfur center (measured from the amplitude difference between the high field trough and low field peak of the g_y 1.90 band) as a function of redox potential, in the presence and absence of UHDBT. Superimposed are theoretical curves for one electron carriers with midpoint reduction oxidation potentials of 280 mV and 350 mV.

these conditions (Fig. 1), and continuous illumination resulted in its complete oxidation. As shown on the right of the figure, UHDBT eliminated all light-induced oxidation.

UHDBT also shifts both the derivative absorption spectrum of the Rieske center and its midpoint oxidation reduction potential. As shown in Fig. 1, the g_y value of the Rieske center is shifted from g 1.90 to g 1.89 by the addition of UHDBT. However, this shift disappeared between 0 and −100 mV at pH 7, possibly because of a change in the redox state of the UHDBT ($E_{m,7}$ −40 mV; 2 electron, 2 proton carrier (measured in collaboration with Haggerty, J. and Trumpower, B.L.)). The shift in the derivative spectrum is accompanied by a shift in the $E_{m,7}$ of the Rieske center from 280 mV to 350 mV (Fig. 2).

The incomplete oxidation of the Rieske iron-sulfur center in Fig. 1 was not due to an insufficiently bright flash since the result was unaffected by a 20-fold decrease in flash intensity. Up to 80% of the total concentration of the Rieske center could be oxidized by two closely spaced flashes. This incomplete oxida-

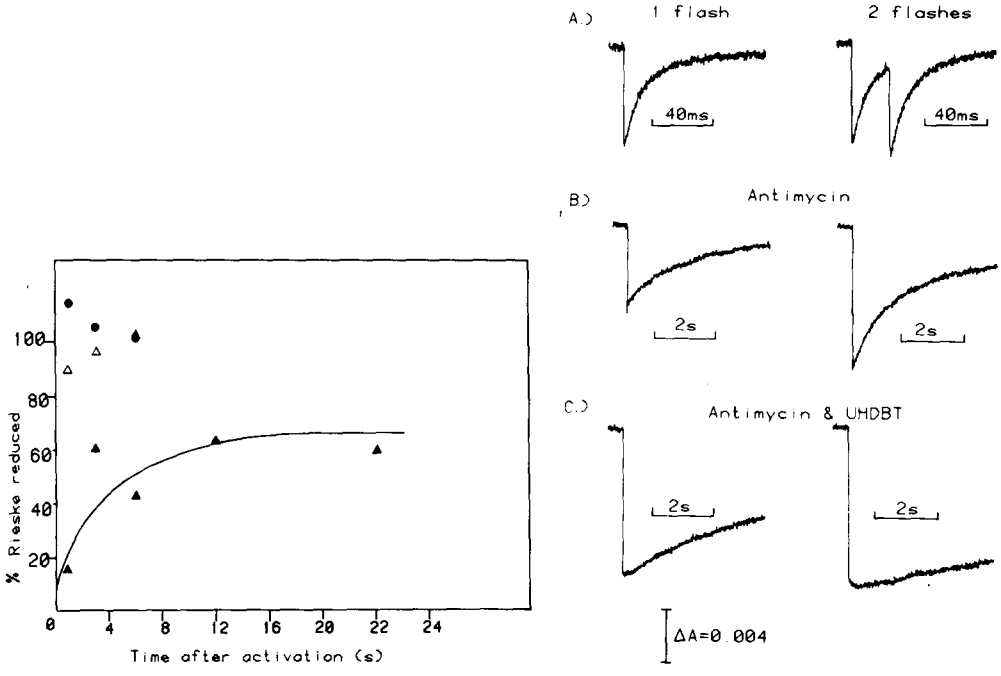

Fig. 3. The effect of varying the time between flash excitation and freezing on the extent of Rieske center oxidation. Chromatophores were subjected to 2 Xenon flashes with 25 ms between flashes, and then frozen after the times indicated. (▲) Conditions as in the legend to Fig. 1. (△) Conditions as in the legend to Fig. 1 but with no antimycin, and the redox potential was 110 ± 5 mV. (●) Conditions as in the legend to Fig. 1 but with no antimycin.

Fig. 4. The relaxation kinetics of cytochrome c_2 oxidized by flash excitation under conditions similar to those used in the EPR experiments. Chromatophores were suspended to 0.7 μM reaction center in 50 mM Mops, 100 mM KCl, 1 mM $MgCl_2$, pH 7.0 containing 30 μM $FeCl_3$, 1 mM EDTA, 500 μM potassium ferrocyanide, 10 μM FCCP and 2 μM valinomycin. Antimycin was added where indicated to 2 μM, and UHDBT to 25 μM. Chromatophores were subjected to 2 Xenon flashes, with 25 ms between flashes. The traces in (A) are an average of 2, instrument response time 50 μs. Those in (B) and (C) were not averaged and the instrument response time was 200 μs.

tion could be the result of some re-reduction in the 700—1000 ms between illumination and freezing, and Fig. 3 shows that this could indeed be occurring, although the extent of re-reduction reached a steady state within less than 12 s (and possibly less than 3 s) of the flash, and did not increase further for tens of seconds. Fig. 3 also shows that in the absence of antimycin, no oxidation of the Rieske iron-sulfur center could be detected either with Q_z reduced (E_h 108 mV) or with Q_z oxidized (E_h 200 mV), indicating that re-reduction occurred within the 700—1000 ms period between flash and freezing.

Fig. 4 shows the behavior of cytochrome c_2 under conditions similar to those used in the EPR experiments. In the absence of antimycin, the flash-oxidized cytochrome was almost completely re-reduced within 40 ms of the second flash, but this was dramatically slowed by antimycin. The addition of UHDBT slowed the reduction even further, and as reported earlier by Bowyer et al. [20,22], the amount of stably oxidized cytochrome c_2 after the first turnover is markedly increased by UHDBT (Figs. 6, 8 and 9).

Optical spectroscopy

a. Redox potential dependence of the antimycin-insensitive ferricytochrome c_2 re-reduction at pH 7.0

The points in Fig. 5 show a redox titration of the difference between the extent of cytochrome c_2 oxidation 40 ms after a Xenon flash in the presence of antimycin, with and without UHDBT. If it is assumed that the difference in extent reflects UHDBT-sensitive electron transfer from the Rieske center to cytochrome c_2, the maximal extent of antimycin-insensitive cytochrome c_2 re-reduction indicates a stoichiometry of 0.6—0.7 Rieske centers per reaction center, assuming E_m values of 285 mV for the Rieske center and 295 mV for cytochrome c_2.

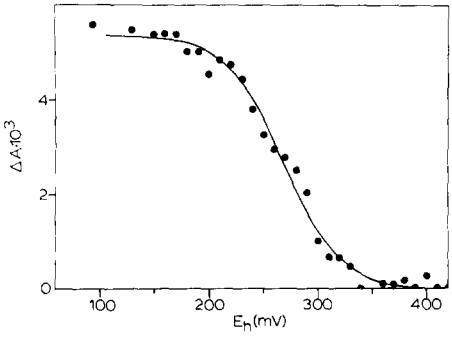

Fig. 5. The redox potential dependency of the effect of UHDBT on cytochrome c_2 oxidation. Chromatophores were suspended to approximately 1.1 μM reaction center in 50 mM Mops, 100 mM KCl, pH 7.0 containing 2 μM antimycin and 2 μM valinomycin. Redox mediators used were at least 200 μM potassium ferrocyanide and 7 μM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) (at redox potentials above 300 mV), and 7 μM each of DAD, 1,4-naphthoquinone, 1,2-naphthoquinone and 2-hydroxy-1,4-naphthoquinone (at redox potentials below 300 mV). The points (•) show the difference between the extent of cytochrome c_2 oxidation 40 ms after a Xenon flash in the presence and absence of 40 μM UHDBT, as a function of redox potential. Superimposed is a computer-fitted theoretical Nernst curve for a one-electron carrier of midpoint potential 270 mV.

Superimposed on the data is a computer-fitted Nernst curve for a one electron carrier of E_m 270 mV, close to that of the Rieske center. The fit of the experimental data to a Nernst curve is somewhat fortuitous, since the proximity of the E_m values of the Rieske center and cytochrome c_2 should lead to some deviation, depending on the E_m values, stoichiometry and relative mobility of the redox centers. Nevertheless, the redox potential range over which the putative re-reduction titrates is consistent with the proposed role of the Rieske center.

b. Optical spectroscopy at alkaline pH

The oxidized form of the Rieske iron-sulfur center exhibits a pK at pH 8.0 [25], while cytochrome c_2 has no significant pK that affects its E_m between

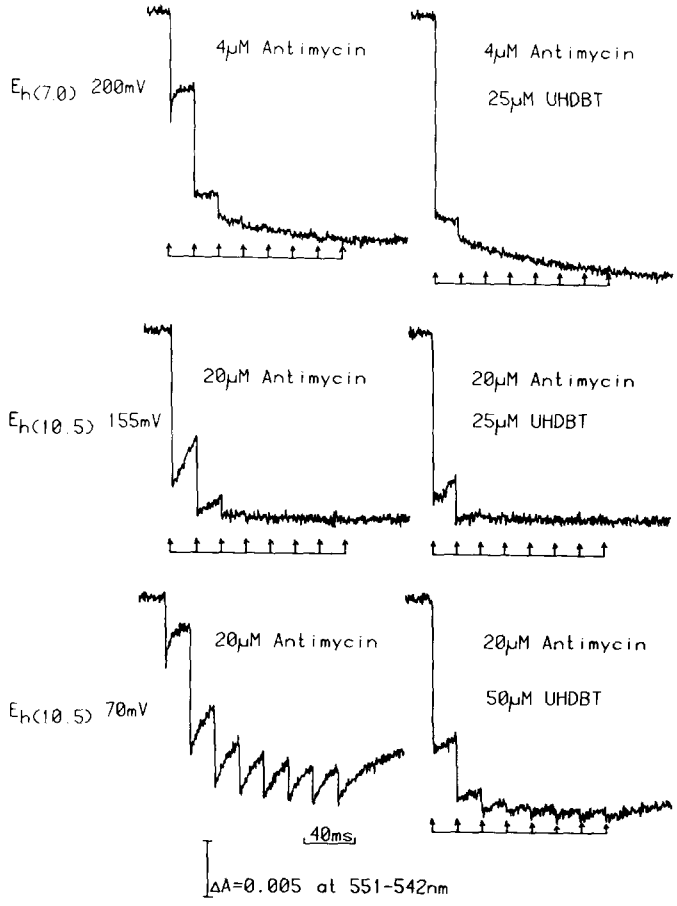

Fig. 6. The effect of pH on the pattern of cytochrome c_2 oxidation on multipulse excitation. At pH 7.0, chromatophores were suspended to 1.2 μM reaction center in 50 mM Mops, 100 mM KCl containing 10 μM each of DAD, 1,2-naphthoquinone, 1,4-naphthoquinone, and 2-hydroxyl-1,4-naphthoquinone, and 1 μM valinomycin. The redox potential was 200 ± 5 mV. Antimycin and UHDBT were added as indicated. Chromatophores were subjected to 8 Xenon flashes with 20 ms between each flash (indicated by arrows). At pH 10.5, chromatophores were suspended to 1.2 μM reaction center in 20 mM glycine, 100 mM KCl, 1 mM $MgCl_2$. Other additions were as at pH 7.0, and nigericin was also added to 1 μM. Other conditions are shown in the figure. Signals were not averaged, and the instrument response time was 200 μs.

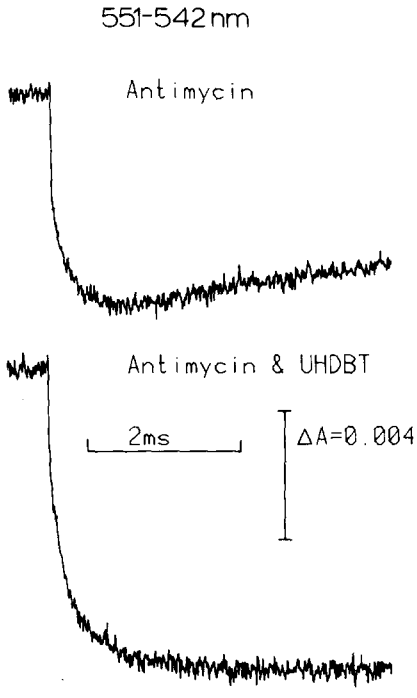

Fig. 7. The kinetics of cytochrome c_2 oxidation. Chromatophores were suspended to 0.7 μM reaction center in 50 mM Mops, 100 mM KCl containing 5 μM each of PMS and PES, 10 μM each of DAD, 1,4-naphthoquinone, 1,2-naphthoquinone and 2-hydroxy-1,4-naphthoquinone, 1 mM potassium ferrocyanide, 2 μM valinomycin and 1 μM nigericin. The redox potential was 180 ± 5 mV. Antimycin was added where indicated to 2 μM and UHDBT to 25 μM. The cytochrome c_2 changes were induced by a single Xenon flash and are an average of 8 with an instrument response time of 5 μs.

pH 5 and 11, at least in vivo [26,27]. At pH 10.5 the E_m of the Rieske center is therefore approximately 135 mV, and one would expect UHDBT to have little effect at E_h values more positive than this. This is shown in Fig. 6; at potentials where cytochrome c_2 is reduced but the Rieske center is oxidized (E_h 155 mV, pH 10.5), UHDBT has little effect. When both the Rieske center and cytochrome c_2 are reduced (E_h 200 mV, pH 7; E_h 70 mV, pH 10.5) UHDBT has a substantial effect.

c. The kinetics of electron flow from the Rieske iron-sulfur center to ferricytochrome c_2

The traces in Fig. 7 (see also Fig. 3 in Ref. 22) show that UHDBT does not affect the fast phase of cytochrome c_2 oxidation ($t_{1/2}$ about 3 μs) representing about 40% of the total oxidation [31], but clearly affects the slower phase ($t_{1/2}$ 200—400 μs) [16,22,31]. This indicates that the Rieske iron-sulfur center re-reduces the cytochrome c_2 at a rate similar to the slower phase of cytochrome oxidation. Because oxidation of cytochrome c_2 is occurring over the same period as its re-reduction, kinetic parameters for the reaction between the iron-sulfur center and cytochrome c_2 cannot be directly determined from the data in Fig. 7.

d. Extents of cytochrome c_2 oxidation and $(BChl)_2^+$ re-reduction in the presence of UHDBT

If ferrocytochrome c_2 is the sole direct reductant of $(BChl)_2^+$, there should be a precise matching of the extent of ferrocytochrome c_2 oxidation and $(BChl)_2^+$ reduction following flash excitation when all electron transport to ferricytochrome c_2 is inhibited. Dutton et al. [16] found such a match in the presence of antimycin at pH 7 and E_h 200 mV, and came to the conclusion that there were two molecules of cytochrome c_2 associated with each reaction center. In contrast, Bowyer et al. [20—22] found only a poor correlation unless UHDBT was added to the sample. This discrepancy was one of the major reasons for the experiments reported here, and we have attempted to determine whether any difference in interpretation or experimental approach might provide an explanation.

Bowyer et al. [22] estimated the extent of ferrocytochrome c_2 oxidation in the presence of antimycin after a transient 'spike' in the cytochrome c_2 oxidation kinetics (about 5 ms after the flash; see Fig. 8). They interpreted the spike as representing an overshoot of cytochrome c_2 oxidation during re-reduction of cytochrome c_2 via an antimycin-insensitive, UHDBT-sensitive pathway by a

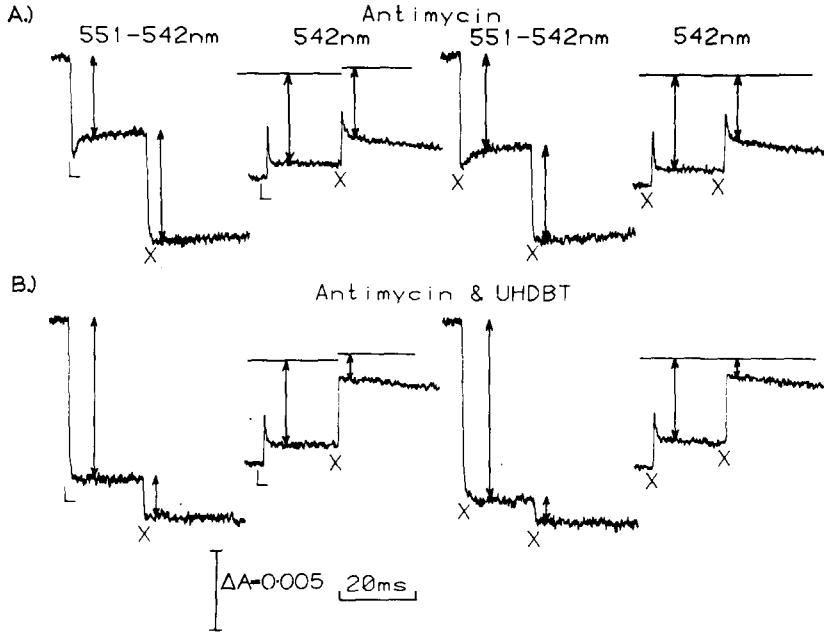

Fig. 8. The extents of cytochrome c_2 oxidation and reaction center reduction on flash-excitation. Chromatophores were suspended to 0.67 μM reaction center in 50 mM Mops, 100 mM KCl, pH 7.0 containing 10 μM DAD, 2 μM valinomycin and 2 μM antimycin. UHDBT was added as indicated to 25 μM. The redox potential was 200 ± 5 mV. Chromatophores were subjected to two flashes 20 ms apart. The first flash was provided either by a dye laser (L) or by a Xenon flash lamp (X). The second flash was always provided by the Xenon flash lamp. The signals were not averaged. The instrument response time was 50 μs. The changes at 551—542 nm show the cytochrome c_2 change, those at 542 nm show the reaction center change. The horizontal lines drawn above the changes at 542 nm represent the full extent of oxidation of the reaction center achieved by the flash, as estimated from measurements made with cytochrome c_2 oxidized before excitation. The vertical arrows indicate where absorbance changes were measured.

previously uncharacterized component, J. However, Dutton et al. [16] measured the extent of cytochrome c_2 oxidation at about 1 ms after the flash (at the peak of the spike in slower kinetic measurements), considering that any subsequent reduction of the cytochrome c_2 represented antimycin-insensitive non-specific reduction by redox mediators or 'endogenous reductant'. They also carried out their detailed matching experiment at E_h 240 mV where the Rieske center is only 85% reduced, and therefore the transient overshoot in the cytochrome c_2 oxidation kinetics was less marked and the extent of rapid antimycin-insensitive cytochrome c_2 re-reduction somewhat smaller (Figs. 4 and 6 in Ref. 16). Thus the experimental conditions and analysis used by Dutton et al. [16] were not optimized to demonstrate the existence of an antimycin-insensitive donor to cytochrome c_2.

Although the analysis of Bowyer et al. [22] indicated that the correlation between the extent of cytochrome c_2 oxidation and $(BChl)_2^+$ re-reduction was considerably improved by the addition of UHDBT to antimycin-treated chromatophores, they found that the amount of ferricytochrome c_2 formed was slightly greater than the amount of $(BChl)_2^+$ re-reduced. In these experiments, the flash duration was 24 μs at half maximal intensity, and not 5 μs as previously reported. A simple cause of the extra ferrocytochrome c_2 oxidation could be that the intensity of the exciting flash had not fallen to a negligible level before significant $(BChl)_2^+$ reduction and reduced primary acceptor (Q_I^-) oxidation had occurred. This would result in a second turnover of some of the reaction centers, and in further oxidation of any remaining ferrocytochrome c_2. Fig. 8 and Table I compare the extents of ferrocytochrome c_2 oxidation and $(BChl)_2^+$ re-reduction following excitation by either a 24 μs Xenon flash or a 300 ns laser flash. They show that, in the presence of UHDBT, use of the shorter laser flash resulted in both a diminution in the extent of ferrocytochrome c_2 oxidation (after allowing for any difference in flash saturation) and an apparent increase in the extent of reaction center re-reduction, as would be expected if the Xenon flash caused some 'double hits'. Even when using laser excitation, there was an apparent over-oxidation of ferrocytochrome c_2 (Table I). It is possible that an additional oxidant for ferrocytochrome c_2 other than $(BChl)_2^+$ is generated following excitation in the presence of UHDBT, but it

TABLE I

ANALYSIS OF THE RESULTS SHOWN IN FIG. 8

	Ferrocytochrome c_2 oxidized (nM)	$(BChl)_2^+$ re-reduced (nM)
Antimycin present		
Flash 1 Laser	276	525
Flash 2 Xenon	376	400
Flash 1 Xenon	318	540
Flash 2 Xenon	323	359
Antimycin and UHDBT present		
Flash 1 Laser	556	501
Flash 2 Xenon	139	143
Flash 1 Xenon	621	477
Flash 2 Xenon	83	98

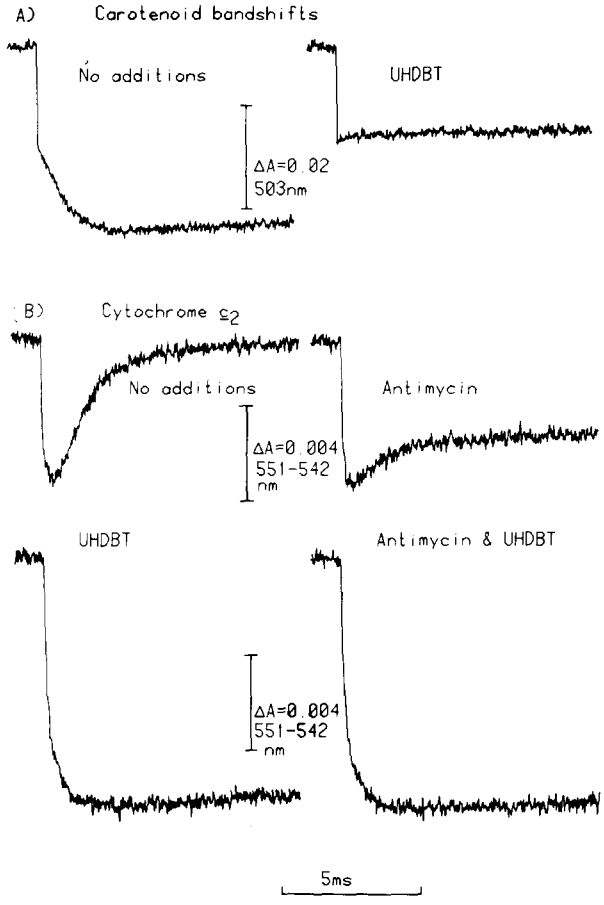

Fig. 9. Antimycin-like effects of UHDBT. Chromatophores were suspended to 0.7 μM reaction center in 50 mM Mops, 100 mM KCl, pH 7.0 containing 5 μM each of PMS and PES, 10 μM each of DAD, 1,4-naphthoquinone, 1,2-naphthoquinone and 2-hydroxyl-1,4-naphthoquinone, 2 μM valinomycin and 1 μM nigericin. The redox potential was 100 ± 3 mV. Antimycin was added where indicated to 2 μM, and UHDBT to 25 μM. Chromatophores were subjected to one Xenon flash. The changes recorded at 503 nm show predominantly the carotenoid response; those at 551—542 nm show the cytochrome c_2 change.

seems more likely that a systematic error in the measurements is responsible. As reported previously, the matching was poor in the presence of antimycin alone [22] (Table I and Fig. 8). The correlation on the second flash was poor for all the measurements made. This in part reflects the fact that a laser could not be used for the second excitation, and also the fact that the extent of ferrocytochrome c_2 oxidation on the second flash in the presence of UHDBT was small.

e. Other effects of UHDBT

UHDBT causes some effects that are similar to those of antimycin. It inhibits the flash-induced binding of H^+_{II} [32,33], but not H^+_I, and, as shown in Fig. 9, UHDBT also inhibits the third phase of the carotenoid bandshift, which seems to be linked to the reduction of ferricytochrome c_2 by Q_zH_2 [13] (via the

Rieske center). UHDBT also inhibits all ferricytochrome c_2 reduction in the absence of antimycin (Fig. 9). In addition, UHDBT inhibits cytochrome b_{50} reduction, but this will be discussed in more detail elsewhere.

Discussion

We interpret our results as showing that the Rieske iron-sulfur center functions between the special ubiquinone (Q_z) of the ubiquinone-cytochrome b-c_2 oxidoreductase and cytochrome c_2. The inhibition by UHDBT (but not antimycin) of both Rieske center oxidation and cytochrome c_2 reduction, and the redox potential-dependency of the antimycin-insensitive phase of cytochrome c_2 re-reduction, indicate that the Rieske center is the direct electron donor to cytochrome c_2. Confirmation will require matching the kinetics of Rieske center oxidation and ferricytochrome c_2 reduction in chromatophores depleted of Q_z. The shift in the E_m of the Rieske center caused by the addition of UHDBT may account for the subsequent failure of the Rieske center to reduce ferricytochrome c_2. However, UHDBT has the additional effect of inhibiting cytochrome b_{50} reduction and the binding of H^+_{II}, and it is not clear how a binding of the inhibitor to the Rieske iron-sulfur center should affect these reactions. Indeed, UHDBT has the effect of disconnecting the reaction center and cytochrome c_2 from the Q-b/c_2 oxidoreductase.

In the light of the experiments reported here and earlier [22], it seems clear that only a single cytochrome c_2 is functionally associated with each reaction center, even though substantially more cytochrome c_2 may be present inside the chromatophore lumen [16].

Role of the Rieske center in other systems

The direct electron donor to ferricytochrome c_2 thus seems to be the Rieske iron-sulfur center. Such a role for the Rieske center is in accord with recent developments in the study of mitochondrial electron flow. Trumpower et al. [7,35,36] have demonstrated that the Rieske center is necessary for electron flow from succinate to cytochrome c_1, but is not necessary for the reduction of cytochrome b unless antimycin is present. Further indications of an important role for the Rieske center were reported by Leigh and Chance [37]; a rapid oxidation of the Rieske center was observed following photodissociation of carbon monoxide from cytochrome oxidase at −20°C. There are several lines of evidence that the Rieske center plays the role of an electron carrier between plastoquinone and cytochrome f in chloroplasts [38–41], and the results of Koike et al. [42] using 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB) bear a noteworthy resemblance to those reported here with UHDBT. They showed that DBMIB approximately doubled the extent of cytochrome f oxidation by a single flash, and also slowed the subsequent reduction. The extra oxidation in the presence of DBMIB had slower kinetics, and they interpreted their observations to indicate that DBMIB blocked the reduction of ferricytochrome f by the Rieske center. DBMIB apparently shifts the EPR spectrum of the chloroplast Rieske center to g 1.95 [43], and also inhibits duroquinol reduction of the Rieske center [38]. However, DBMIB does not block either the antimycin- or UHDBT-sensitive phases of ferricytochrome c_2

reduction in chromatophores, although it does inhibit cytochrome *b* reduction at redox potentials above 200 mV (Bowyer, J.R., unpublished observations, and see Ref. 44), so there are some subtle differences between the chloroplast and bacterial systems.

One other candidate has been suggested as the direct electron donor to ferricytochrome c_2. A detailed analysis of the absorption changes in *C. vinosum* led van Grondelle et al. [34] to conclude that a soluble *c* type cytochrome with $E_{m,7.0}$ of 260 ± 10 mV was the direct reductant of cytochrome *c*-555. If UHDBT blocked the reduction of ferricytochrome c_2 by a soluble cytochrome, the total steady-state level of cytochrome *c* oxidation on multiple flash excitation in the presence of antimycin would be greater in the absence of UHDBT than in its presence, assuming that antimycin blocked re-reduction of the soluble cytochrome. This is clearly not the case. Difference spectra of the flash-induced change (not illustrated) show that the extra absorption decrease recorded at 551—542 nm in the presence of UDHBT is indeed an oxidation of cytochrome c_2. However, the peak position of the 550 nm band of cytochrome c_2 is somewhat variable and is always shifted 1—2 nm to the red of the isolated cytochrome, implying some heterogeneity in the cytochrome c_2 content. This will be discussed in more detail in a forthcoming paper.

Location of the Rieske center within the membrane

At pH 7.0, in the presence of antimycin A to inhibit phase III of the carotenoid spectral shift, UHDBT barely affects the remaining carotenoid response (not shown, but compare Fig. 9 with data in Refs. 13 and 17). This indicates that the Rieske center is close to the inside of the chromatophore membrane, near to cytochrome c_2, if indeed the Rieske center is the direct electron donor to cytochrome c_2. A position closer to the outside of the membrane would be expected to result in a blue shift of the carotenoid spectrum concomitant with electron flow (at pH values below the p*K* on the oxidized form at pH 8, Ref. 25) from the Rieske center to ferricytochrome c_2, so that an increase in the red shift would occur when UHDBT was added to antimycin-inhibited chromatophores; this is not seen. A position for the Rieske center near the inner side of the membrane in *C. vinosum* is also indicated in measurements of the effects of the impermeant paramagnetic gadolinium cation [44].

Relative mobility of the redox centers

In the chromatophore it appears that the Rieske center, Q_z, and the antimycin binding site all occur at a stoichiometry of 0.7 + 0.1 per reaction center [9,33], and by analogy with the mitochondrial cytochrome b-c_1 complex, it seems likely that the three components are associated in a discrete complex in the chromatophore membrane. The antimycin-sensitive phase of ferricytochrome c_2 reduction attributed to electron transfer from Q_zH_2 has second order kinetics [9,10]. Our results suggest that this phase is rate-limited either by the reaction between Q_zH_2 and oxidized Rieske center, or between the Q_z semiquinone generated in that reaction and its reaction partner (see Ref. 9). The overall second order kinetics of Q_zH_2 oxidation indicate that the reactants in one or both of the above reactions are mobile with respect to one another. Mobility between Q_zH_2 and the Rieske center might be mediated either by a

direct mobility of the two components or by direct two-electron transfer between Q_z species in separate complexes. There is evidence that one molecule of Rieske protein may serve multiple cytochrome b-c_1 segments in the mitochondrial system, since electron transfer in resolved succinate-cytochrome c reductase proceeds at a rate which is not diminished in direct proportion to the loss of the Rieske center (Ref. 46, and interpreted in Ref. 7).

Some limited mobility of the Rieske center, and indeed cytochrome c_2, in the photosynthetic system may also occur, sufficient to allow full reduction of $(BChl)_2^+$ and ferricytochrome c_2 even though the stoichiometry of reactants may be less than unity. This limited mobility would explain the observation that a small fraction of the $(BChl)_2^+$ re-reduction in the presence of antimycin is UHDBT-sensitive (Fig. 8 and Ref. 20).

Finally, the demonstration of a phase in the oxidation kinetics of cytochrome c_2 which is not seen in the absence of UHDBT necessitates a reappraisal of previous observations, which we shall list below.

(a) Extinction coefficients of $(BChl)_2^+$ and cytochrome c_2. These were previously obtained under conditions in which the Rieske center was either completely oxidized and thus could not act as a rapid electron donor, or was dissociated from the chromatophore membrane by addition of detergent. Thus the published values [16] still appear valid. However, it is worth recalling how the original extinction coefficients were determined. The addition of detergent to the chromatophore suspension to allow the access of the external mammalian cytochrome c standard caused an apparent increase in the extinction coefficient of the $(BChl)_2^+$. Although this was ascribed to a hypochromic effect, an alternative explanation is that it is due to optical 'sieve effects'. These may not have a linear dependency on concentration, and may make the original extinction coefficient inappropriate for other concentrations of chromatophores. Experiments are in progress to evaluate this possibility.

(b) Kinetics of Q_zH_2 oxidation. The kinetics of Q_zH_2 oxidation have been followed by analysis of the re-reduction kinetics of cytochrome c_2 [9—11]. Since it now appears that Q_zH_2 re-reduces cytochrome c_2 via the Rieske center, the behavior of Q_zH_2 will have to be reevaluated. The Rieske center re-reduces cytochrome c_2 with an estimated $t_{1/2}$ of <1 ms. If we assume that the 1—2 ms phase of cytochrome c_2 re-reduction is rate-limited by the reaction between Q_zH_2 and the Rieske center, and not by the reduction of ferricytochrome c_2 by the latter, then the slow phase of cytochrome c_2 reduction gives a measure of the kinetics of the proposed Rieske center reduction by Q_zH_2. However, since the concentration of the Rieske center is apparently similar to that of cytochrome c_2 [20—22], this reappraisal of the nature of the actual reactants does not significantly change the measured parameters of the reaction, nor the overall interpretation that the reaction between Q_zH_2 and ferricytochrome c_2 is mediated by a collisional process of apparent second order rate constant of $(3.0 \pm 1.8) \times 10^9\ M^{-1} \cdot s^{-1}$ with 0.8 ± 0.1 molecules of Q_z per reaction center.

(c) The number of antimycin binding sites per reaction center. The titer was obtained by several methods, some of which appear to be independent of any involvement of the Rieske center [33].

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References

1 Dutton, P.L., Prince, R.C. and Tiede, D.M. (1978) Photochem. Photobiol. 28, 939—949
2 Hoff, A.J. (1979) Phys. Rep. 54, 75—200
3 Dutton, P.L. and Prince, R.C. (1978) in The Photosynthetic Bacteria (Clayton, R.K. and Sistrom, W.R., eds.), pp. 525—570, Plenum Press, New York
4 Crofts, A.R. and Saphon, S. (1978) in Frontiers of Physico-chemical Biology (Pullman, B., ed.), pp. 459—483, Academic Press, New York
5 Crofts, A.R. and Wood, P.M. (1978) Curr. Top. Bioenerg. 7, 175—244
6 Dutton, P.L. and Prince, R.C. (1978) in The Bacteria (Ornston, L.N. and Sokatch, J.R., eds.), Vol. 6, pp. 524—584, Academic Press, New York
7 Trumpower, B.L. and Katki, A.G. (1979) in Membrane Proteins in Energy Transduction (Capaldi, R.A., ed.), pp. 89—200, Marcel Dekker, New York
8 Prince, R.C. and Dutton, P.L. (1977) Biochim. Biophys. Acta 462, 731—747
9 Prince, R.C., Bashford, C.L., Takamiya, K., van den Berg, W.H. and Dutton, P.L. (1978) J. Biol. Chem. 253, 4137—4142
10 Crofts, A.R., Crowther, D., Bowyer, J. and Tierney, G.V. (1977) in Structure and Function of Energy Transducing Membranes (van Dam, K. and van Gelder, B.F., eds.), pp. 133—155, Elsevier/North-Holland, Amsterdam
11 Crofts, A.R. and Bowyer, J. (1977) in The Proton and Calcium Pumps (Azzone, G.F., Avron, M., Metcalfe, J.C., Quagliarello, E. and Siliprandi, N., eds.), pp. 55—64, Elsevier/North Holland, Amsterdam
12 Bowyer, J.R., Baccarini-Melandri, A., Melandri, B.A. and Crofts, A.R. (1978) Z. Naturforsch. 33c, 704—711
13 Bashford, C.L., Prince, R.C., Takamiya, K.-I. and Dutton, P.L. (1979) Biochim. Biophys. Acta 545, 223—235
14 Takamiya, K., Prince, R.C. and Dutton, P.L. (1978) in Frontiers of Biological Energetics (Dutton, P.L., Leigh, J.S. and Scarpa, A., eds.), Vol. 1, pp. 183—190, Academic Press, New York
15 Takamiya, K., Prince, R.C. and Dutton, P.L. (1979) J. Biol. Chem. 254, 11307—11311
16 Dutton, P.L., Petty, K.M., Bonner, H.S. and Morse, S.D. (1975) Biochim. Biophys. Acta 387, 536—556
17 Jackson, J.B. and Dutton, P.L. (1973) Biochim. Biophys. Acta 325, 102—113
18 Parson, W.W. (1969) Biochim. Biophys. Acta 153, 248—259
19 Case, G.D. and Parson, W.W. (1971) Biochim. Biophys. Acta 253, 187—202
20 Bowyer, J.R. and Crofts, A.R. (1978) in Frontiers of Biological Energetics (Dutton, P.L., Leight, J.S. and Scarpa, A., eds.), Vol. 1, pp. 326—333, Academic Press, New York
21 Crofts, A.R. (1979) in Light-Induced Charge Separation in Biology and Chemistry (Gerischer, H. and Katz, J.J., eds.), pp. 389—407, Dahlem Konferenzen, Berlin
22 Bowyer, J.R., Tierney, G.V. and Crofts, A.R. (1979) FEBS Lett. 101, 207—212
23 Prince, R.C., Lindsay, J.G. and Dutton, P.L. (1975) FEBS Lett. 51, 108—111
24 Evans, M.C.W., Lord, A.V. and Reeves, S.G. (1974) Biochem. J. 138, 177—183
25 Prince, R.C. and Dutton, P.L. (1976) FEBS Lett. 65, 117—119
26 Prince, R.C. and Dutton, P.L. (1977) Biochim. Biophys. Acta 459, 573—577
27 Prince, R.C. and Bashford, C.L. (1979) Biochim. Biophys. Acta 547, 447—454

28 Bowyer, J.R., Dutton, P.L., Prince, R.C., Porter, T.H., Folkers, K. and Crofts, A.R. (1979) in Abstracts of the International Workshop on Membrane Bioenergetics (I.U.B. Symp. 93), Detroit, MI
29 Bowyer, J.R., Tierney, G.V. and Crofts, A.R. (1979) FEBS Lett. 101, 201—206
30 Petty, K.M. and Dutton, P.L. (1976) Arch. Biochem. Biophys. 172, 335—345
31 Overfield, R.E., Wraight, C.A. and DeVault, D. (1979) FEBS Lett. 105, 137—142
32 Petty, K.M., Jackson, J.B. and Dutton, P.L. (1979) Biochim. Biophys. Acta 546, 17—42
33 Van den Berg, W.H., Prince, R.C., Bashford, C.L., Takamiya, K., Bonner, W.D. and Dutton, P.L. (1979) J. Biol. Chem. 254, 8594—8604
34 Van Grondelle, R., Duysens, L.N.M., van der Waal, J.A. and van der Waal, H.N. (1977) Biochim. Biophys. Acta 461, 188—201
35 Trumpower, B.L. (1976) Biochem. Biophys. Res. Commun. 70, 73—80
36 Trumpower, B.L. and Edwards, C.A. (1979) FEBS Lett. 100, 13—16
37 Leigh, J.S. and Chance, B. (1974) Fed. Proc. 33, Abstr. 376
38 White, C., Chain, R.K. and Malkin, R. (1978) Biochim. Biophys. Acta 502, 127—137
39 Malkin, R. and Posner, H.B. (1978) Biochim. Biophys. Acta 501, 552—554
40 Trebst, A., Harth, E. and Draber, W. (1970) Z. Naturforsch. 25b, 1157—1159
41 Whitmarsh, J. and Cramer, W.A. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4417—4420
42 Koike, H., Satoh, K. and Katoh, S. (1978) Plant Cell Physiol. 19, 1371—1380
43 Chain, R.K. and Malkin, R. (1979) Arch. Biochem. Biophys. 197, 52—56
44 Evans, E.H. and Gooding, D.E. (1976) Arch. Microbiol. 111, 171—174
45 Case, G.D. and Leigh, J.S. (1974) in Proc. 11th Rare Earth Res. Conf., Traverse City, MI (Haschke, J.M. and Eick, H.A., eds.), Vol. 2, pp. 706—715, U.S. Atomic Energy Commission, Oak Ridge
46 Yamashita, S. and Racker, E. (1969) J. Biol. Chem. 244, 1220—1227