Biochimica et Biophysica Acta, 545 (1979) 429—444

BBA 47625

THE INVOLVEMENT OF IRON AND UBIQUINONE IN ELECTRON TRANSFER REACTIONS MEDIATED BY REACTION CENTERS FROM PHOTOSYNTHETIC BACTERIA

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(Received June 29th, 1978)

Key words: Ubiquinone; Bacteriochlorophyll; Iron content; Electron transfer; Bacterial photosynthesis

Summary

Reaction centers from *Rhodopseudomonas sphaeroides* strain R-26 were prepared with varying Fe and ubiquinone (Q) contents. The photooxidation of *P*-870 to *P*-870$^+$ was found to occur with the same quantum yield in Fe-depleted reaction centers as in control samples. The kinetics of electron transfer from the initial electron acceptor (I) to Q also were unchanged upon Fe removal. We conclude that Fe has no measurable role in the primary photochemical reaction.

The extent of secondary reaction from the first quinone acceptor (Q_A) to the second quinone acceptor (Q_B) was monitored by the decay kinetics of *P*-870$^+$ after excitation of reaction centers with single flashes in the absence of electron donors, and by the amount of *P*-870 photooxidation that occurred on the second flash in the presence of electron donors. In reaction centers with nearly one iron and between 1 and 2 ubiquinones per reaction center, the amount of secondary electron transfer is proportional to the ubiquinone content above one per reaction center. In reaction centers treated with $LiClO_4$ and *o*-phenanthroline to remove Fe, the amount of secondary reaction is decreased and is proportional to Fe content. Fe seems to be required for the secondary reaction. In reaction centers depleted of Fe by treatment with SDS and EDTA, the correlation between Fe content and secondary activity is not as good as that found using $LiClO_4$. This is probably due in part to a loss of primary photochemical activity in samples treated with SDS; but the correlation is still not perfect after correction for this effect.

The nature of the back reaction between *P*-870$^+$ and Q_B^- was investigated

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Abbreviations: I, the initial electron acceptor between *P*-870 and quinone, thought to be bacteriopheophytin; *P*-870, the primary electron donor bacteriochlorophyll in reaction centers; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; Q, quinone.

using stopped flow techniques. Reaction centers in the $P\text{-}870^+$ Q_B^- state decay with a 1-s half-time in both the presence and absence of *o*-phenanthroline, an inhibitor of electron transfer between Q_B^- and Q_B. This indicates that the back reaction between $P\text{-}870^+$ and Q_A^- is direct, rather than proceeding via thermal repopulation of Q_A^-. The $P\text{-}870^+$ Q_B^- state is calculated to lie at least 100 mV in free energy below the $P\text{-}870^+$ Q_A^- state.

Introduction

Reaction center preparations isolated from photosynthetic bacteria contain about one equivalent of non-heme Fe [1—3]. The Fe initially was thought to serve as the electron acceptor in the primary photochemical reaction, which is the oxidation of a bacteriochlorophyll complex called *P*-870. The main evidence that the Fe participated in the primary electron transfer reaction was that reduction of the acceptor generated a broad ESR signal that seemed to be due to the Fe [4,5]. However, photochemically active preparations with low iron contents were obtained, and a narrower ESR spectrum identified as that of the ubiquinone anion radical was observed [6,7]. The broad ESR signal apparently results from magnetic interactions between the quinone radical and the Fe. More recent experiments have convincingly identified the electron acceptor as a quinone (ubiquinone in *Rhodopseudomonas sphaeroides* and menaquinone in *Chromatium vinosum*) [8—14], while the function of iron has remained abscure.

The reduced quinone acceptor (Q_A^-) transfers an elctron in 10—200 μs [15–18] to another acceptor (Q_B), which also appears to be a quinone [18–20]. The kinetics of this secondary reaction can be monitored by double-flash experiments in the presence of an electron donor [15–17], by measuring absorbance changes at 750 mm [18], or by studying the competition between the secondary reaction and reverse electron transfer from Q_A^- to $P\text{-}870^+$ [17]. Recent evidence suggests that Q_B is functionally distinct from the large ubiquinone pool that is present in chromatophores [21].

The suggestion has been made that iron is involved in electron transfer from Q_A^- to Q_B^-, although no strong evidence has been presented to support this idea [2]. Two observations that agree with this view are that the Fe chelator *o*-phenanthroline inhibits electron transfer between the two quinones [16,22, 23], and that either Q_A or Q_B can couple magnetically to the iron [20,24,25]. There is, however, no evidence that the effect of *o*-phenanthroline results from chelation of the Fe. This report presents further evidence that supports the assignment of iron to a function between the two quinones. A preliminary report on part of this work has appeared [26].

Materials and Methods

Reaction centers of *Rps. sphaeroides* strain R-26 were prepared essentially as described by Clayton and Wang [27]. It was necessary to purify the reaction centers further by DEAE cellulose chromatography to obtain iron contents of one Fe per reaction center. The reaction centers were adsorbed on a 2.5 × 10 cm column of DEAE sephacel (Pharmacia) equilibrated with 0.1% lauryl dimethyl-

amide oxide, 10 mM Tris (pH 8.0), 10 μM EDTA, and washed with several column volumes of the same buffer followed by 50 ml 0.07 M NaCl in the same buffer solution. The reaction centers were then eluted by increasing the NaCl concentration to 0.15 M, dialyzed vs. 0.025% lauryl dimethylamine oxide, 10 mM Tris (pH 8.0), 10 μM EDTA, and concentrated using an Amicon model 52 ultrafilter with a PM-10 membrane. Reaction centers prepared in this way had an A_{280}/A_{800} ratio of about 1.3, and contained 0.8—1.0 Fe/reaction center and slightly more than one Q per reaction center. Reaction centers with $\approx$1.8 Q/reaction center were prepared by decreasing the lauryl dimethylamine oxide concentration from 1.5 to 0.1% during the ammonium sulfate fractionation.

Reaction centers were depleted of iron by incubating a reaction center sample ($A_{800} \approx 10$) in 0.025% lauryl dimethylamine oxide, 10 mM Tris (pH 8.0), 0.75 M $LiClO_4$, and 1.0 mM *o*-phenanthroline for 2 h at room temperature (Okamura, M., personal communication), followed by low speed centrifugation, and dialysis vs. 0.025% lauryl dimethylamine oxide, 10 mM Tris (pH 8.0), and 10 μM EDTA. For some experiments the detergent was changed to 0.05% Triton X-100 by further dialysis. The recovery of the reaction centers usually was about 60%. Dialysis was shown to reverse completely the effects of treating reaction centers with *o*-phenanthroline in the absence of $LiClO_4$. The absorption spectrum of iron-depleted reaction centers was quite similar to that of untreated samples, except that the *P*-870 of the reaction centers was partially oxidized by the treatment (reversibly) and the 760 nm absorption band due to bacteriopheophytin was increased slightly. Other differences in the absorption spectra were a narrowing and slight red shift of the absorption band at 535 nm, and a slight blue shift of the 870 nm band. There also was a small absorbance increase at 690 nm, indicative of bacteriochlorophyll degradation products.

Iron depletion also was achieved by incubating reaction center samples in low concentrations of SDS (0.05—0.25%, w/v) in 10 mM Tris (pH 8.0), 0.025% lauryl dimethylamine oxide, and 1 mM EDTA, for one or more days at room temperature [7]. This procedure caused similar spectral shifts, but less oxidation of *P*-870, and smaller absorbance increases at 760 and 690 nm. Recovery by this procedure was greater than 90%.

Reaction center concentrations were determined from the absorption at 800 nm, after dialysis to remove solubilized iron and $LiClO_4$ or SDS. An extinction coefficient of 288 $mM^{-1} \cdot cm^{-1}$ (ref. 28) was used.

Iron content was determined using a Perkin Elmer 403 graphite furnace atomic absorption spectrophotometer. Concentrated standard solutions were prepared in 1% HNO_3 and the final dilution (10 times) was made into the Tris-lauryl dimethylamide oxide buffer solution. This procedure gave the standards the same surface tension characteristics of the reaction center samples, but kept the pH low enough to avoid iron precipitation. Iron contents quoted are accurate to $\pm$5%.

Ubiquinone 50 was purchased from Sigma and used from a 5 mg/ml stock solution in ethanol. Control experiments indicated that ethanol alone had no effect. Ubiquinone analyses were performed essentially as described by Takamiya and Takamiya [29], using a differential extinction coefficient (oxi-

dized minus reduced) of 12.25 $mM^{-1} \cdot cm^{-1}$ at 275 nm [30].

Flash-induced absorbance changes were measured at room temperature, using the single beam laser spectrophotometer described previously [15,31]. Excitation in most cases was provided by 20 ns ruby laser pulses. For the quantum yield experiments shown in Fig. 1, the ruby laser pulses were used to pump a dye laser that lases at 834 nm. Flash intensity was measured by a vacuum photodiode that was calibrated against a ballistic thermopile. For the experiments of Fig. 4, the ambient redox potential was controlled as described previously [32]. The experiments reported in Figs. 1, 4 and 8 were recorded photographically from oscilloscope traces; a Biomation 802 transient digitizer coupled to a PDP-8 computer served as the data acquisition and analysis system for the remainder. The computer utilizes UW Focal as language [33]. For the experiments in which *P*-870 decay kinetics were used as an assay of secondary electron transfer activity, the data were converted from transmittance to absorbance by the computer and then fit with a two-component exponential expression by an iterative, nonlinear least-squares program. The analysis program required no initial estimates of relative amplitudes or decay constants, and achieved close fits to the data in all cases.

A Durrum stopped-flow apparatus coupled to a Keithley electrometer and storage oscilloscope was used for the experiments shown in Fig. 8. White light for preillumination was obtained from a 100 W tungsten bulb and filtered through a Corning 1-69 heat filter. Broad-band infrared flashes were obtained from a 10 μs Xe flash filtered through a Corning 2600 filter. Because of the design of the apparatus, only part of the unmixed solutions was exposed to the preillumination. This part was maximized by using a relatively large volume of sample for each pulse (1 ml). The dead time between mixing and measuring was about 2 ms; the dead time between preillumination and measuring was somewhat longer than this.

Picosecond kinetics were measured as previously described [34].

Results

Primary reaction

Fig. 1a shows a light saturation curve for *P*-870 photooxidation in reaction centers at room temperature. The abscissa gives the incident flash intensity on a logarithmic scale. Fig. 1b shows theoretical saturation curves predicted for reactions with quantum yields of 1 and 0.1. The saturation curve is given by the cumulative one-hit Poisson distribution, and its position along the intensity axis is a measure of the quantum yield. The data of Fig. 1a indicate that the quantum yield of *P*-870 photooxidation is the same in control and iron-depleted reaction centers.

The experimental curves of Fig. 1a are not precisely the same shape as the theoretical curves of Fig. 1b; they approach saturation more gradually at high light intensities. This distortion is probably due to the fact that the laser pulses do not have uniform intensity across the surface of the cuvette. Hot and cold spots would result in the observed saturation behavior. Departure from the theoretical curves also could reflect heterogeneity of the reaction center population. It appears not to be due to dichroism induced by the linearly polar-

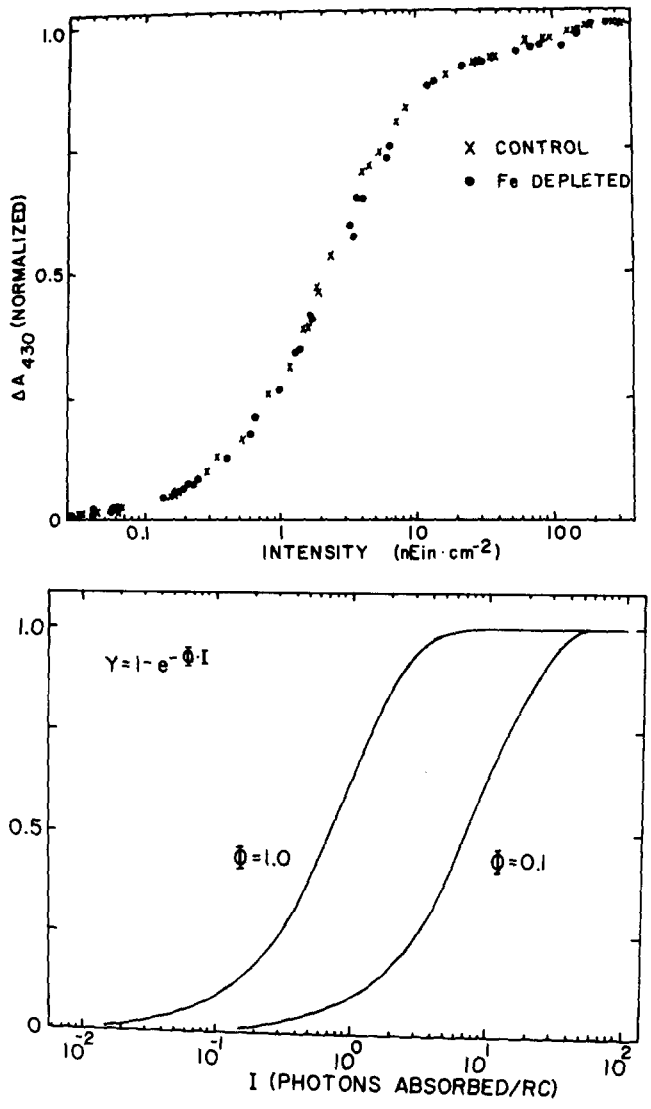

Fig. 1. (a) Light saturation curve for control and iron-depleted reaction centers. Flashes were 20 ns in duration at 834 nm. Flash intensity was varied using calibrated neutral density filters. Flash-induced absorbance changes at 430 nm were measured as described in Materials and Methods. X, control, 2.6 μM reaction centers in 0.05% Triton X-100, 10 mM Tris (pH 8.0), 10 μM EDTA. Iron content 0.86 Fe/reaction center. •, iron-depleted, 2.4 μM reaction centers, 0.28 Fe/reaction center. The data were normalized at the highest light intensity. Before normalization the absorbance changes for the two samples were within 10% of each other. (b) Theoretical light saturation curve. These curves are plots of the cumulative one-hit Poisson distribution with provision for quantum yield less than one. The equation plotted is $y = 1\text{-}e^{-\Phi I}$, where Φ is the quantum yield and I is the number of photons absorbed per reaction center. Left curve, $\Phi = 1.0$; right curve, $\Phi = 0.1$.

ized laser flash, because similar saturation curves were obtained when a quarter wave-plate was added to make the laser light circularly polarized.

Previous work has shown that the photooxidation of *P*-870 involves the transient reduction of an initial electron acceptor (I), which appears to be bacteriopheopytin. The *P*-870$^+$ I$^-$ radical pair then transfers an electron to

another acceptor, presumably Q_A, in about 250 ps [34,35]. Picosecond experiments were performed in collaboration with D. Holten and M. Windsor of Washington State University to determine whether removing iron altered the seond of these steps. The $1/e$ time for the oxidation of I^-, monitored by the decay of the absorption band due to I^- at 680 nm, was found to be 250 ± 50 ps in both control and iron-depleted reaction centers. The initial formation of $P\text{-}870^+$ I^- was complete in less than 20 ps in both cases. These results, coupled with the results in Fig. 1, indicate that iron removal does not measurably affect either the speed or the efficiency of electron transfer between *P*-870 and Q_A.

Secondary reactions

The second quinone acceptor (Q_B) is bound to the reaction center more loosely than Q_A and is largely removed during the isolation procedure of Clayton and Wang [27]. In such reaction centers, most of the back reaction between the reduced quinone acceptor and $P\text{-}870^+$ occurs in a single step, with a half-time of ≈80 ms at room temperature (Fig. 2a and ref. 36). By decreasing the detergent concentration during the purification procedure, reaction centers with nearly two ubiquinones per reaction center can be prepared [11]. In these the back reaction has a pronounced slow phase, with a half-time of 0.8—1 s (Fig. 2b). Reaction centers isolated with Q/reaction center ratios intermediate between these two extremes show biphasic kinetics, and the amount of slow phase is linearly related to the amount of quinone above one per reaction center (Fig. 2d). Addition of Q to reaction centers with only one

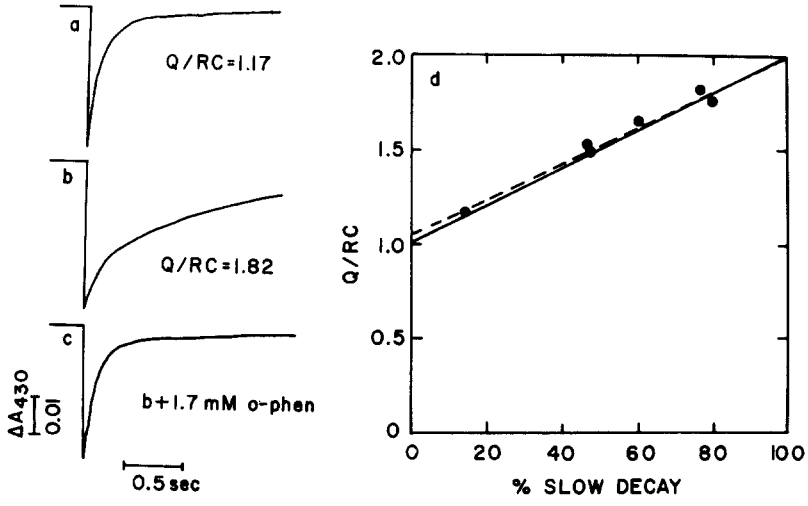

Fig. 2. *P*-870$^+$ decay kinetics in reaction centers with varying quinone contents. Parts a, b and c show the optical absorbance changes at 430 nm, when reaction center suspensions are excited with single ruby laser flashes. A downward deflection is an absorbance increase. No reductants for *P*-870$^+$ were added; the decay is taken to reflect back reactions with Q_A^- and Q_B^-. a, Q/reaction center = 1.17; b, Q/reaction center = 1.82; c, sample b after addition of 1.7 mM *o*-phenanthroline; d, plot of Q/reaction center vs. the percentage of the decay that occurs in the slower of the two phases, with a half-time of 0.8—1.0 s. The solid line in d is what is expected if the second Q is necessary for secondary electron transfer activity; the dashed line is a least-squares fit to the data points. Buffer solution was 0.025% lauryl dimethylamine oxide, 10 mM Tris (pH 8.0), 10 μM EDTA.

Q results in a conversion to predominantly slow decay kinetics (Fig. 3a and 3b; ref. 36); it has little effect on the kinetics in reaction centers that are isolated so as to contain two ubiquinones per reaction center. The simplest explanation for these observation is that the more rapid decay kinetics reflect back electron transfer to $P\text{-}870^+$ from Q_A^-, and the slow kinetics reflect a back reaction from Q_B^- [36]. In support of this interpretation, addition of *o*-phenanthroline to reaction centers with slow decay kinetics causes the decay to assume the 80 ms half-time characteristic of reaction centers with only one quinone (Fig. 2c and 3c, and ref. 23). This is consistent with other evidence [16,22] that *o*-phenanthroline blocks the secondary reaction between Q_A^- and Q_B. The amount of decay that occurs with 1-s kinetics is therefore a quantitative measure of the extent to which the secondary reaction has occurred. By monitoring the bacteriopheophytin band shift at 750 nm [18], we measured a half-time of 110 ± 20 μs for the secondary reaction in reaction centers containing two quinones (data not shown).

In Fig. 3d, e and f, the $P\text{-}870^+$ decay kinetics are used to determine the extent of the secondary reaction in iron-depleted reaction centers. Iron-depleted reaction centers have predominantly fast decay kinetics (Fig. 3d). The kinetics are not affected by the addition of Q (Fig. 3d, e), nor by *o*-phenanthroline (Fig. 3f). They exhibit little evidence of secondary reaction regardless of the amount of Q added.

Another means of monitoring the extent of secondary reaction is to use two closely-spaced flashes [15—17]. If an efficient electron donor such as reduced cytochrome *c* is added to reduce $P\text{-}870^+$ quickly, the amount of *P*-870 oxida-

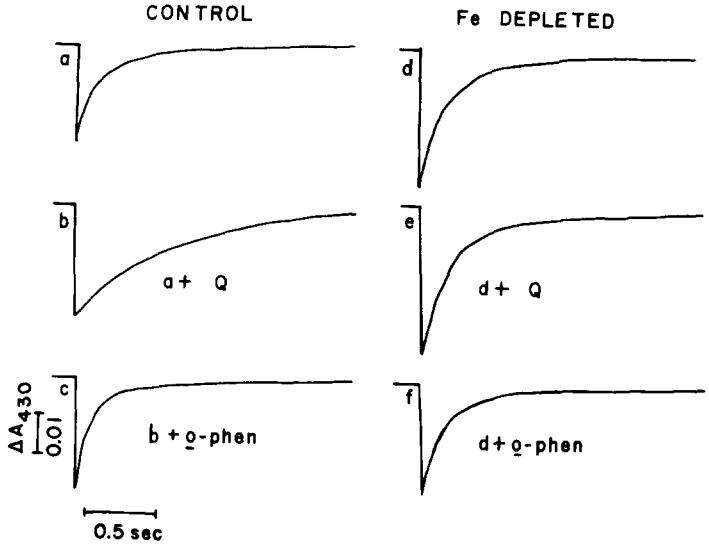

Fig. 3. $P\text{-}870^+$ decay kinetics in control and iron-depleted reaction centers. Curves a, b and c were obtained using 1.6 μM control reaction centers with 0.86 Fe/reaction center. Curves d, e and f were obtained with 1.9 μM iron-depleted reaction centers containing 0.28 Fe/reaction center. a and d, no additions, b and e, plus 31 μM Q; c and f, plus 31 μM Q and 0.8 mM *o*-phenanthroline. Further addition of either Q or *o*-phenanthroline had no additional effect. Buffer solution was 0.05% Triton X-100, 10 mM Tris (pH 8.0), 10 μM EDTA; other conditions as in Fig. 2.

tion observed on the second flash reflects the amount of Q_A^- that has become reoxidized during the interval between the flashes. Fig. 4 shows the results of such an experiment on untreated and Fe-depleted reaction centers. With no additions, the decay of $P\text{-}870^+$ involves the back reactions discussed above, and is slow on the time scale of the experiment (Fig. 4a, e). Addition of cytochrome *c* speeds the decay on the first flash, but little photooxidation occurs on the second flash (Fig. 4b, f). Addition of Q restores activity on the second flash in the control, but not in iron-depleted reaction centers (Fig. 4c and g). Addition of *o*-phenanthroline decreases the activity on the second flash in the control, as expected if it blocks the reoxidation of Q_A^-, but has little effect on the iron-depleted sample (Fig. 4d and h). These experiments confirm that the iron-depleted reaction centers are deficient in the secondary reaction between Q_A and Q_B. In addition, they show that Q_A acts as only a single electron acceptor, even if the iron is removed.

For the experiment of Fig. 5, the concentration of $LiClO_4$ and *o*-phenanthroline were varied in order to remove varying amounts of iron, and the secondary reaction activity was plotted as a function of iron content. The secondary activity was determined as the fraction of $P\text{-}870^+$ decay with slow back reaction kinetics (cf. Fig. 2). The solid line in Fig. 5 is what is expected if iron content is linearly related to secondary electron transfer activity; the

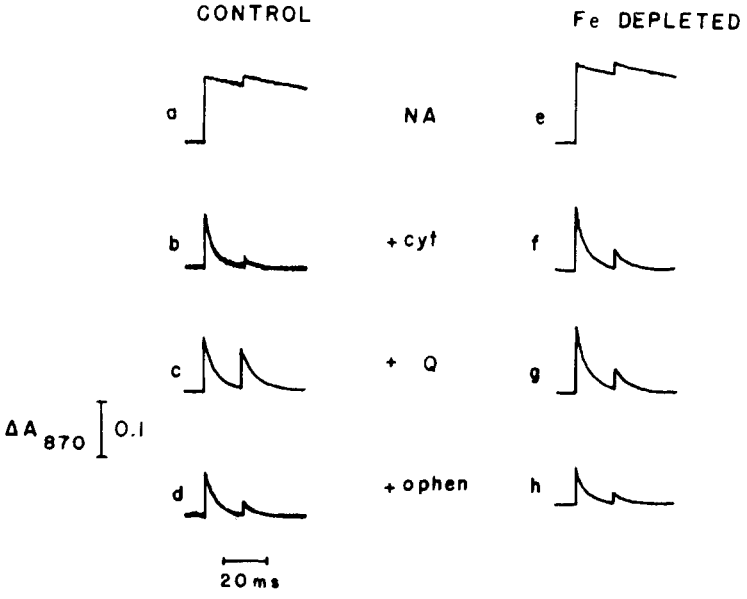

Fig. 4. Double-flash experiment on control and iron-depleted reaction centers. Curves a, b, c, and d were obtained using 1.1 μM control reaction centers with 0.86 Fe/reaction center. Curves e, f, g, and h were obtained using 1.2 μM iron-depleted reaction centers with 0.28 Fe/reaction center. Reaction buffer: 0.05% Triton X-100, 10 mM Tris (pH 8), 10 μM EDTA, 5 μM *N*-methylphenazinium methosulfate. The redox potential of the sample was adjusted to +200 mV (NHE) prior to each measurement, by addition of $Na_2S_2O_4$ or $K_3Fe(CN)_6$. Curves a and e, no additions; curves b and f, plus 5.6 μM horse cytochrome *c*; curves c and g, plus 5.7 μM cytochrome *c* and 15 μM Q; curves d and h, plus 5.7 μM cytochrome *c*, 15 μM Q, and 0.8 mM *o*-phenanthroline. The traces show flash-induced absorbance changes at 870 nm. Decreasing the measuring light intensity to 8% of its original value did not affect the results.

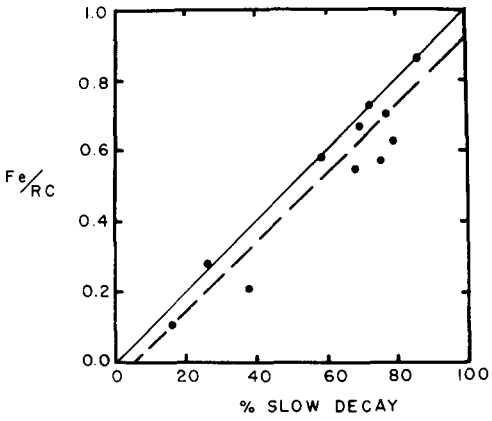

Fig. 5. Correlation between iron content and secondary activity in $LiClO_4$-treated reaction centers. Secondary reaction activity measured by the % of the $P\text{-}870^+$ decay (measured at 430 nm) that had a half-time of 0.8—1.0 s. The half-time of the more rapid decay phase was 85 ± 20 ms in all samples. Ubiquinone was added until the decay kinetics were constant (the amount of slow phase was maximal). The solid line is the expected behavior for 1 : 1 correlation between iron content and secondary reaction activity. The dashed line is a least-squares fit to the data.

dashed line is a least-squares fit to the data points. The data indicate a good correlation between iron content and secondary reaction activity. This figure includes data obtained with several preparations of reaction centers, with Q contents ranging from close to 1 to close to 2. Excess ubiquinone was added to maximize the amount of slow decay in all cases.

To investigate whether the loss of secondary activity is indeed due to loss of iron, or to a side effect of the treatment with $LiClO_4$ and *o*-phenanthroline, we sought an alternative procedure for removing the iron. Fig. 6 (left) shows the results of treating reaction centers with varying amounts of SDS. Incubation with SDS causes a decrease in both the iron content (circles) and the secondary reaction activity (squares), as measured by the $P\text{-}870^+$ decay kinetics. The slow decay phase was somewhat slower ($t_{1/2} \approx 1$—1.5 s) in SDS-treated reaction centers than in control or $LiClO_4$-treated reaction centers. Samples treated with SDS also lose photochemical activity, as measured by the amount of *P*-870 oxidation induced by a single flash (Fig. 6 right, triangles). In parallel with the loss of activity, there is an increase in the amount of the triplet state of *P*-870 (also called P^R; see ref. 31) that is generated by a flash (Fig. 6 right, crosses). Addition of Q to these reaction centers does not restore *P*-870 photooxidation, nor decrease triplet formation. Reaction centers that have been depleted of Q by the gentler method of Okamura et al. [11] also exhibit triplet formation, but addition of Q fully restores their primary and secondary activity and abolishes the formation of triplets (data not shown).

Fig. 7 shows a plot of iron content vs. secondary reaction activity in reaction centers that were treated with SDS. The correlation is considerably poorer than that obtained with $LiClO_4$-treated reaction centers. The secondary electron transfer activity generally is higher than one would expect, judging from the Fe content. However, the secondary reaction assay monitors only reaction centers that are photochemically active, while both active and inactive reaction centers are included in the measurements of Fe content. If the iron content of inactive

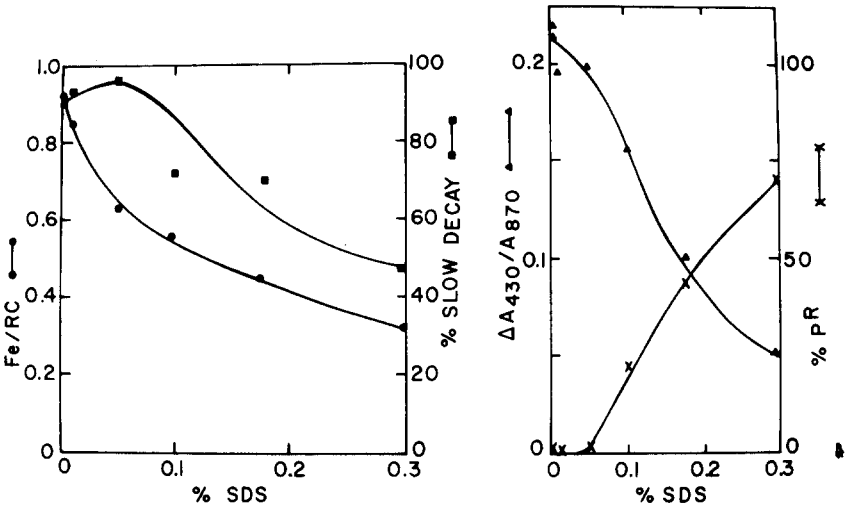

Fig. 6. SDS incubation as a method to remove iron. An 18-h incubation of 1-ml aliquots of 28 μM reaction centers in 0.025% lauryl dimethylamine oxide, 10 mM Tris (pH 8.0), 1.0 mM EDTA and the indicated SDS concentrations (w/v) was followed by dialysis vs. 0.025% lauryl dimethylamine oxide, 10 mM Tris (pH 8.0), 10 μM EDTA for 2 days. Left panel: ●———●, Fe/reaction center; ■———■, % of the P-870$^+$ with slow decay kinetics. Right panel: ▲———▲, primary photochemical activity, measured as total light-induced absorbance increase at 430 nm divided by the 870 nm absorbance of the sample; X———X, % of the excited reaction centers that go to the triplet state P^R, rather than to P-870$^+$, measured as the fraction of the flash-induced absorbance increase at 510 nm that decays with $t_{1/2}$ = 25 μs. At 510 nm the extinction coefficients of P-870$^+$ and P^R are approximately equal [31]. The ratio $\Delta A_{430}/A_{870}$ is used to normalize samples with differing reaction center concentrations.

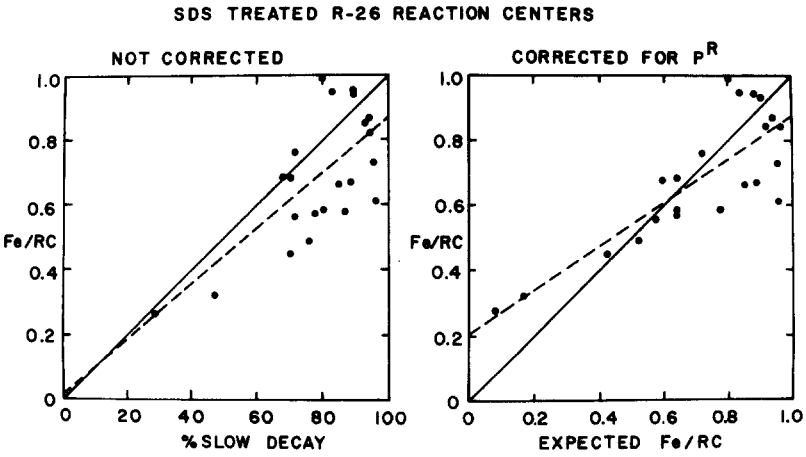

Fig. 7. Correlation between the iron content and secondary reaction activity in SDS-treated reaction centers. SDS concentrations of 0—0.25% (w/v) and incubation times of 18 h to 4 days were used to deplete reaction centers partially of iron. Other conditions as in Fig. 6. Secondary activity was measured as in Fig. 2. Left panel: not corrected for loss of primary photochemical activity; right panel: corrected for loss of primary activity. Expected Fe/reaction center was calculated using the formula: Expected Fe/reaction center = [P-870$^+$/(P-870$^+$ + P^R)] X (Fraction of P-870$^+$ with slow decay). This equation assumes that reaction centers that exhibit P^R rather than P-870$^+$ have no iron. The relative amounts of P^R and P-870$^+$ formed on flash excitation were measured as in Fig. 6. All measurements were done in the presence of saturating Q.

reaction centers is different from that of active reaction centers, the plot of Fe/reaction center vs. secondary activity will be distorted. One possibility is that inactive reaction centers that cannot be reconstituted with added Q are missing both iron and Q, since reaction centers lacking only Q are readily reconstituted. In support of this assumption, the decay kinetics of the triplet state seen in the SDS-treated reaction centers are slower ($t_{1/2}$ = 25 μs) than those ($t_{1/2}$ = 10 μs) in control reaction centers in which photochemistry is blocked by the chemical reduction of Q_A. Fe-depleted reaction centers prepared by $LiClO_4$ treatment show similar slow decay kinetics for the triplet state after reduction of Q_A [26]. Fig. 7b shows a plot of the data after 'correction' in accordance with this assumption. The correlation in the middle range of Fe content is improved, but the samples with low iron content now appear to have somewhat less secondary activity than expected. In addition a number of samples with high iron content still have greater activity than expected. This poor correlation is most apparent when relatively low concentrations of SDS (0.05—0.07%) are used to treat the reaction centers (Fig. 6). At these low concentrations, SDS does not significantly decrease primary photochemical activity, or cause triplet formation. The iron content of the reaction center sample decreases upon treatment with low concentrations of SDS, but the secondary reaction activity is unchanged or actually increases slightly. This unexpected effect of low SDS concentrations was observed in three separate experiments of this type. Similar anomalous behavior was observed when reaction centers were treated with 0.07% SDS for varying periods of time (data not shown). The secondary activity increased slightly with a one-day incubation, while the iron content decreased slightly. Longer incubation resulted in a further loss of iron, accompanied by decreased secondary activity, and eventual loss of some of the primary activity after four days incubation.

Addition of *o*-phenanthroline to $LiClO_4$ or SDS-treated reaction centers always converted 90% or greater of the *P*-870$^+$ decay to 80—100 ms kinetics, just as it did with untreated reaction centers.

Reaction centers contain small amounts of Mn, which presumably substitutes for Fe in some reaction centers [1,3]. Mn analyses were performed on a few samples; they were found to contain 0.06 Mn/reaction center. It was not determined quantitatively if the conditions that bring about Fe depletion also remove Mn, but the few experiments that were performed suggested that Mn was somewhat more resistant to removal than Fe.

The mechanism of the back reaction between Q_B^- and P-870$^+$

The kinetics of the back reaction between *P*-870$^+$ and Q^- have been used to assay the extent of secondary reaction that occurs after flash excitation. However, it is not clear whether the 1 s back reaction represents a direct back reaction between Q_B^- and *P*-870$^+$, or the thermal repopulation of Q_A^- followed by a back reaction between Q_A^- and *P*-870$^+$. The former mechanism predicts that addition of *o*-phenanthroline to reaction centers in the *P*-870$^+$ Q_B^- state would have no effect on the back reaction kinetics. The latter mechanism predicts that the back reaction will slow down, since the rate-limiting step between Q_B^- and Q_A would be slowed considerably by *o*-phenanthroline. To investigate this point, the stopped-flow experiment shown in Fig. 8 was performed. Reaction

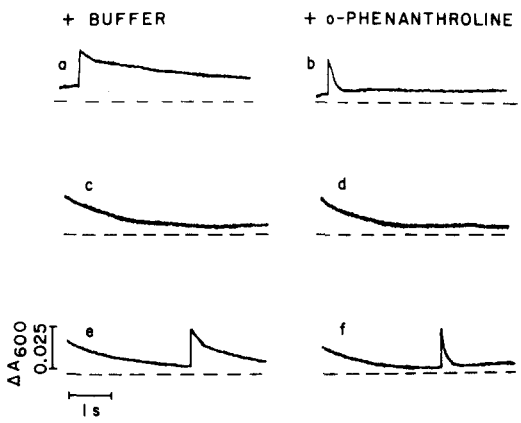

Fig. 8. Stopped-flow back reaction kinetics. Experiment performed as described in Materials and Methods and text. Curves a, c, e; 10 μM reaction centers were mixed with 0.025% lauryl dimethylamine oxide, 10 mM Tris (pH 8.0), 10 μM EDTA, and 4% ethanol. Curves b, d, and f; 10 μM reaction centers were mixed with the same buffer solutions including 4 mM *o*-phenanthroline. In curves a and b dark-adapted samples were mixed and then given a broad-band infrared flash. In curves c and d, the samples were preilluminated with white light and then mixed. In curves e and f the samples were preilluminated, mixed and the flashed. The traces show the optical absorbance at 600 nm; an upward deflection is an absorbance decrease. Optical path length = 2.2 cm.

centers containing nearly two quinones and one iron per reaction center were mixed with buffer alone or with buffer containing 4 mM *o*-phenanthroline, and the back reaction kinetics were observed.

Fig. 8a and b are control experiments designed to determine if there is any significant delay before *o*-phenanthroline is able to exert its blocking effect on the secondary reaction. The solutions were mixed and a short time later a flash was given. Fig. 8a shows that control reaction centers mixed with buffer alone decay predominantly by the 1 s pathway, as expected. Fig. 8b shows that reaction centers mixed with *o*-phenanthroline and then excited with a flash show the 100 ms decay half-time characteristic of blocked systems. This establishes that *o*-phenanthroline is able to block the secondary reaction within a few hundred milliseconds after mixing *.

Fig. 8c and d shows that decay kinetics of samples preilluminated with continuous light prior to mixing with buffer (Fig. 8c) or with buffer plus *o*-phenanthroline (Fig. 8d). In this case, Q_B is reduced before the addition of the *o*-phenanthroline. The decay occurs via the 1 s path in both Fig. 8c and d; *o*-phenanthroline has no effect. Fig. 8e and f show decay kinetics of samples which were preilluminated, mixed and then given a flash. The decay in reaction centers mixed with buffer alone (Fig. 8e) is predominantly slow both after the preillumination and after flashes that follow the mixing. Reaction centers preilluminated and then mixed with *o*-phenanthroline exhibit slow decay kinetics following the preillumination, but flashes given after mixing

* These controls, and the similar ones in Fig. 8e and f, show that *o*-phenanthroline reacts rapidly with reaction centers that are in the state *P*-870 Q_AQ_B. An ambiguity in the experiments is that the controls cannot show rigorously that *o*-phenanthroline reacts rapidly if the reaction centers are in the state *P*-870$^+$ $Q_A\bar{Q}_B$.

induce rapid decay, as they do in Fig. 8b. These experiments show that reaction centers in state $P\text{-}870^+$ Q_B^- decay with a rate constant of approx. 1 s^{-1}, regardless of the presence of *o*-phenanthroline, thus supporting the direct back reaction between $P\text{-}870^+$ and Q_B^- as the decay mechanism. The back reaction via Q_A^- appears to be slow, relative to the 1 s^{-1} rate of the reaction between Q_B^- and $P\text{-}870^+$.

Discussion

The data presented in this paper indicate that the primary photochemical reaction proceeds unperturbed in reaction centers that have been depleted of iron. This agrees with earlier findings that preparations treated to remove iron retained photochemical activity [6,7,37]. With the exception of the work of Loach and Hall [6], earlier studies have not included quantum yield determinations and so have been inconclusive as to whether iron plays any role in the primary reaction. Loach and Hall found that the quantum yield of $g = 2$ radical formation in Fe-depleted subchromatophore particles was higher than in chromatophores. In chromatophores, the $g = 2$ ESR signal is thought to be due almost exclusively to $P\text{-}870^+$, whereas in iron-depleted preparations it also arises from Q_A^-. By assuming that the quantum yield of $P\text{-}870^+$ formation was the same as it is in chromatophores, Loach and Hall calculated a relative quantum yield of 60% for Q_A^- in the subchromatophore particles. This was close to the percentage of the reaction centers that had been depleted of Fe in the particles. Our experiments (Fig. 1) provide a somewhat more direct demonstration that the quantum yield in iron-depleted reaction centers is the same as it is in control reaction centers. The picosecond experiments indicate that the rate of electron transfer between I^- and Q_A is undisturbed in iron-depleted reaction centers. In summary, iron seems to play no significant role in the primary reaction.

The data obtained using $LiClO_4$ incubation as a means to deplete reaction centers of iron indicate a 1 : 1 relationship between iron content and the extent of the secondary reaction between Q_A^- and Q_B. The experiments in which SDS was used to deplete reaction centers of iron show qualitatively the same results, but quantitatively the correlation between iron content and secondary reaction activity is not as good. The SDS experiments are complicated by the tendency of the reaction centers to lose primary activity upon treatment with higher concentrations of SDS. However, the correlation between Fe content and secondary activity is poor even at SDS concentrations below those that cause a decrease in primary activity. Consistently more secondary activity is observed than is expected on the basis of the iron content.

SDS treatment was used by Feher et al. [7] to deplete reaction centers of iron. They reported that SDS caused some loss of primary activity at room temperature, and a severe loss of activity at low temperature. The effects of SDS thus appear to be complex, and they could be attended by structural reorganization in the reaction center. SDS-treated reaction centers that have been extensively dialyzed to remove SDS bind irreversibly to DEAE cellulose, implying that there is still a considerable amount of SDS attached to the reaction centers. For these reasons, we place more weight on the experiments

employing $LiClO_4$, and favor the interpretation that Fe is required for electron transfer from Q_A^- to Q_B.

Recent ESR experiments have shown that either Q_A^- or Q_B^- can couple magnetically to the Fe of the reaction center, so that the ESR spectra of the two radicals are similar, though not quite identical [20,24,25]. This suggests that iron is nearly equidistant from the two quinones, and in a position to mediate electron transfer between them. No experiments reported so far, however, indicate whether the iron becomes transiently reduced or oxidized during the secondary reaction. Iron could serve a purely structural role, correctly positioning the quinones with respect to each other. If *o*-phenanthroline binds to the iron, it might simply displace Q_B. Reaction centers treated with *o*-phenanthroline exhibit slightly different ESR spectra from untreated reaction centers [2], and treatment with *o*-phenanthroline facilitates the removal of quinones from the reaction center by detergent [11]. Both untreated and chemically reduced reaction centers show Mössbauer spectra [38] and magnetic susceptibility [39] characteristic of ferrous iron. However, transient oxidation or reduction of the iron would not be observed in these measurements.

The double-flash experiments indicate that the first quinone acceptor normally is able to be reduced only as far as the semiquinone, in both control and iron-depleted reaction centers. Although Q_A^- apparently can be reduced further to $Q_A^=$ (or the dihydroquinone Q_AH_2) under certain conditions, this is evidently a very slow process [40]. The nature of the environment that confers this unusual property to Q_A is unclear, except that iron appears not to be the major factor. It has been reported recently that treatment of reaction centers with SDS or chaotropic agents converts redox titrations of Q_A from one-electron to two-electron curves [41]. This apparent discrepancy remains to be resolved, but the redox titrations also could reflect processes that are too slow to be important during the photochemical electron transfer reactions.

Fig. 2 shows that reaction centers that contain two quinone molecules exhibit slow back reaction kinetics. Previous studies have shown that addition of excess Q to reaction centers produces slow decay kinetics, but it was not established until now whether endogenous Q_B elicited the same behaviour, or if the slow back reaction occurred from a tertiary acceptor. The 10-fold difference in back reaction rate from Q_A^- and Q_B^- is easily explained using recent theories of electron transfer [42—45] if the distance between *P* and Q_B is slightly larger than the distance between Q_A and *P*, as might be expected in a series scheme. We favor the view that the reaction center contains a series arrangement in which one Q always serves as primary acceptor and another serves as secondary acceptor. A parallel scheme in which *P*-870 can reduce either Q_A or Q_B directly would require the ad hoc assumption that a conformational change occurs after the first Q is reduced, and that during this conformational change the system is refractory to another electron transfer. The conformational change would have to displace Q^- from *P* such that the back reaction rate is slowed by a factor of ten. In addition, a parallel scheme requires that the conformational change not occur in reaction centers which contain only one Q, those which have lost Fe, and those treated with *o*-phenanthroline. The series scheme very naturally explains all the data obtained to date.

In reaction centers that contain Fe and both Q_A and Q_B, the decay of *P*-870$^+$

after flash excitation occurs by a relatively slow process, with a halftime of about 1 s. The stopped flow experiments shown in Fig. 8 support the interpretation that the 1 s decay is due to a direct back reaction between Q_B^- and $P\text{-}870^+$. This allows one to calculate a limit for the equilibrium constant for electron transfer between Q_A and Q_B. Since Q_A^- decays by a back reaction with a rate constant of about $10\ s^{-1}$, the apparent rate constant for the decay of an equilibrium mixture of $Q_A^-Q_B$ and $Q_AQ_B^-$ via Q_A would be $10\ s^{-1} \times (Q_A^-Q_B)/[Q_A^-Q_B) + (Q_AQ_B^-)]$. An upper limit of $0.2\ s^{-1}$ for this (20% of the overall rate constant of about $1\ s^{-1}$) means that $K_{eq} = (Q_AQ_B^-)/(Q_A^-Q_B) \gtrsim 50$. The apparent midpoint redox potential of Q_B must be $\gtrsim 100$ mV more positive than that of Q_A. Redox titrations of *C. vinosum* chromatophores [32] have given apparent midpoint redox potential values consistent with this conclusion. For reasons that are not clear, however, the titrations depend on whether *N*-methylphenazinium methosulfate is used as a mediator.

Acknowledgements

We thank D. Holten and M. Windsor of Washington State University for the picosecond absorbance measurements, M. Okamura, D. Ort and C. Schenck for helpful suggestions and discussions, D. Johnson and G. Christian of the Univeristy of Washington Chemistry Department for assistance with the atomic absorption analyses, and S. Felton of the Fisheries Department for permitting us to do the stopped-flow experiments in his laboratory. This work was supported by grant number PCM 77 13290 from the National Science Foundation and an NSF National Needs postdoctoral fellowship to R.E.B.

References

1 Feher, G. (1971) Photochem. Photobiol. 14, 373—387
2 Feher, G. and Okamura, M.Y. (1978) in The Photosynthetic Bacteria (Clayton, R.K. and Sistrom, W.R., eds.), Plenum, New York
3 Feher, G., Isaacson, R.A., McElroy, J.D., Ackerson, L.C. and Okamura, M.Y. (1974) Biochim. Biophys. Acta 368, 135—139
4 McElroy, J.D., Feher, G. and Mauzerall, D. (1970) Biophys. J. 10, 204a
5 Leigh, Jr., J.S. and Dutton, P.L. (1973) Ann. N.Y. Acad. Sci. 222, 838—845
6 Loach, P.A. and Hall, R.L. (1972) Proc. Natl. Acad. Sci. U.S. 69, 786—790
7 Feher, G., Okamura, M.Y. and McElroy, J.D. (1972) Biochim. Biophys. Acta 267, 222—226
8 Clayton, R.K. and Straley, S.C. (1972) Biophys. J. 12, 1221—1234
9 Slooten, L. (1972) Biochim. Biophys. Acta 275, 208—218
10 Cogdell, R.J., Brune, D.C. and Clayton, R.K. (1974) FEBS Lett. 45, 344—347
11 Okamura, M.Y., Isaacson, R.A. and Feher, G. (1975) Proc. Natl. Acad. Sci. U.S. 72, 3491—3495
12 Morrison, L., Runquist, J. and Loach, P. (1976) Photochem. Photobiol. 25, 73—84
13 Romijn, J.C. and Amesz, J. (1977) Biochim. Biophys. Acta 461, 327—338
14 Feher, G. and Okamura, M.Y. (1976) Brookhaven Symp. Biol. 28, 183—194
15 Parson, W.W. (1969) Biochim. Biophys. Acta 189, 384—396
16 Halsey, Y.D. and Parson, W.W. (1974) Biochim. Biophys. Acta 347, 404—416
17 Chamorovsky, S.K., Remennikov, S.M., Kononenko, A.A., Vendiktov, P.S. and Rubin, A.B. (1976) Biochim. Biophys. Acta 430, 62—70
18 Vermeglio, A. and Clayton, R.K. (1977) Biochim. Biophys. Acta 461, 159—165
19 Vermeglio, A. (1977) Biochim. Biophys. Acta 459, 516—524
20 Wraight, C.A. (1977) Biochim. Biophys. Acta 459, 525—531
21 Barouch, Y. and Clayton, R.K. (1977) Biochim. Biophys. Acta 462, 785—788
22 Parson, W.W. and Case, G.D. (1970) Biochim. Biophys. Acta 205, 232—245
23 Clayton, R.K., Szuts, E.Z. and Fleming, H. (1972) Biophys. J. 12, 64—79

24 Wraight, C. (1978) Biophys. J. 21, 8a
25 Okamura, M.Y., Isaacson, R.A. and Feher, G. (1978) Biophys. J. 21, 8a
26 Blankenship, R. and Parson, W. (1977) Abst. 4th Inter. Cong. on Photosynthesis, Reading, U.K., Sept. 4—9, 1977, (Hall, D.O.). p. 37
27 Clayton, R.K. and Wang, R.T. (1971) Methods Enzymol. 23, 696—704
28 Straley, S.C., Parson, W.W., Mauzerall, D.C. and Clayton, R.K. (1973) Biochim. Biophys. Acta 305, 597—609
29 Takamiya, K. and Takamiya, A. (1969) Plant Cell Phys. 10, 363—373
30 Pumphrey, A.M. and Redfearn, E.R. (1960) Biochem. J. 76, 61—64
31 Parson, W.W., Clayton, R.K. and Cogdell, R.J. (1975) Biochim. Biophys. Acta 387, 265—278
32 Case, G.D. and Parson, W.W. (1971) Biochim. Biophys. Acta 253, 187—202
33 Van Zee, J. (1976) DECUS Proc. 2, 405—412
34 Rockley, M.G., Windsor, M.W., Cogdell, R.J. and Parson, W.W. (1975) Proc. Natl. Acad. Sci. U.S. 72, 2251—2255
35 Kaufmann, K.J., Dutton, P.L., Netzel, T.L., Leigh, J.S. and Rentzepis, P.M. (1975) Science 188, 1301—1304
36 Clayton, R.K. and Yau, H.F. (1972) Biophys. J. 12, 867—881
37 Bolton, J.R. and Cost, K. (1973) Photochem. Photobiol. 18, 417—421
38 Debrunner, P.G., Schulz, C.E., Feher, G. and Okamura, M.Y. (1975) Biophys. J. 15, 226a
39 Butler, W.F., Johnston, D.C., Okamura, M.Y., Shore, H.B. and Feher, G. (1978) Biophys. J. 21, 8a
40 Okamura, M.Y., Isaacson, R.A. and Feher, G. (1977) Biophys. J. 17, 149a
41 Tiede, D.M., Prince, R.C. and Dutton, P.L. (1978) Biophys. J. 21, 196a
42 Hopfield, J.J. (1974) Proc. Natl. Acad. Sci. U.S. 71, 3640—3644
43 Jortner, J. (1976) J. Chem. Phys. 64, 4860—4867
44 Hopfield, J.J. (1977) Biophys. J. 18, 311—321
45 Blankenship, R.E. and Parson, W.W. (1979) in Topics in Photosynthesis (Barber, J., ed.), Vol. 3, Elsevier, Amsterdam, in the press