Biochimica et Biophysica Acta, 459 (1977) 516–524

BBA 47255

SECONDARY ELECTRON TRANSFER IN REACTION CENTERS OF *RHODOPSEUDOMONAS SPHAEROIDES*

OUT-OF-PHASE PERIODICITY OF TWO FOR THE FORMATION OF UBISEMIQUINONE AND FULLY REDUCED UBIQUINONE

ANDRE VERMEGLIO*

Section of Genetics, Development and Physiology, Cornell University, Ithaca, N.Y. 14853 (U.S.A.)

(Received August 2nd, 1976)

SUMMARY

Electron transfer between purified reaction centers from *Rhodopseudomonas sphaeroides* and exogenous ubiquinone has been studied in the presence of electron donors by measurements of light-induced absorbance changes following a sequence of short actinic light flashes. Each odd flash promotes the formation of a molecule of ubisemiquinone; after each even flash the semiquinone disappears and a molecule of the fully reduced quinone appears.

We interpret these results by means of a model where a specialized molecule of ubiquinone is reduced by the primary electron acceptor in a one-electron transfer reaction after each flash, and is reoxidized by a molecule of the ubiquinone pool in a two-electron transfer reaction every two flashes.

INTRODUCTION

The primary and secondary electron acceptors in bacterial photosynthesis are thought to be molecules of quinone, interacting with an iron atom in the case of the former [1–3]. The evidence comes mainly from analysis of light-induced absorbance changes [4–7] and of extraction-reconstitution experiments with exogenous ubiquinone [8, 9]. However, the mechanism of interaction between the primary and secondary electron acceptors is still not well understood. One puzzling problem is the fact that the primary acceptor is a one-electron carrier as demonstrated by Parson [10], but each molecule of the pool of secondary acceptor is a two-electron carrier. Any model describing the interaction between the primary electron acceptor and the pool of secondary acceptor will have to account for this fact. Parson [10] has proposed that electron chains are coupled through the ubiquinone pool, so that two

* On leave from Service de Biophysique, Département de Biologie, Centre d'Etudes Nucléaires de Saclay BP No. 2, 91190 Gif-sur-Yvette, France.

molecules of ubisemiquinone disproportionate to give one molecule of fully reduced dihydroquinone and one of the oxidized quinone.

The same type of hypothesis was postulated by Stiehl and Witt [11] to solve the same problem in Photosystem II of green plants. However, the works of Bouges-Bocquet [12] and Velthuys and Amesz [13] have shown that in fact there is no cooperation between different electron chains at the level of the plastoquinone pool. Instead, a specialized secondary acceptor, presumably a molecule of plastoquinone, equilibrates with the pool of plastoquinone in a two-electron transfer reaction.

In order to test the same mechanism for photosynthetic bacteria we have studied light-induced absorbance changes related to the acceptor side of purified reaction centers of *Rhodopseudomonas sphaeroides*. To observe these changes, it is necessary to rereduce rapidly the oxidized primary electron donor by means of a secondary electron donor [4]. Under these conditions, and if exogeneous ubiquinone has been added, the difference spectrum induced by a first flash is like that for the formation of ubisemiquinone "in vitro" [14], plus changes due to bacteriopheophytin and bacteriochlorophyll absorption band shifts. On a second flash the absorption spectrum of the ubisemiquinone almost disappears; there remain mainly only absorbance changes due to fully reduced ubiquinone. A periodicity of two with damping properties is observed for the appearance and disappearance of the ubisemiquinone molecule in a sequence of flashes. On the other hand, the fully reduced ubiquinone accumulates upon flash excitation; one molecule every two flashes.

We propose for the electron transfer between primary and secondary electron acceptors a model similar to the one proposed for the acceptor side of Photosystem II of green plants [12, 13]: After a single turnover flash, a specialized molecule of ubiquinone can hold one electron; after a second actinic flash this specialized molecule can reduce a molecule of the ubiquinone pool in a two-electron transfer reaction.

MATERIALS AND METHODS

R. sphaeroides, carotenoidless mutant R26 was grown as described earlier [15]. The method of preparing chromatophores has also been described [16].

Solubilization of reaction centers was achieved by adding lauryldimethyl amine-*N*-oxide (LDAO*), final concentration 1 %, to a suspension of chromatophores ($A = 40$ at 865 nm for 1 cm path length). The suspension was then centrifuged at 150 000 $\times g$ for 90 min. The "crude" reaction centers (supernatant) were dialyzed overnight against 0.1 % LDAO, 0.01 M Tris · HCl, pH 7.5, at 4 °C and applied to a DEAE-cellulose anion-exchange column (Whatman DE-52) equilibrated in 0.1 % LDAO, 0.01 M Tris · HCl, pH 7.5, and eluted with a salt gradient (0.01–0.40 M NaCl) in the presence of 0.1 % LDAO. Alternatively Triton X-100 was used as solubilizing agent (final concentration 2 %) and its concentration in the column eluant was 0.5 %.

Light-induced absorbance changes were measured either with a single beam spectrophotometer or with a split beam spectrophotometer described in ref. 17. For measurement in the ultraviolet range the measuring lamp of the single beam instrument was a deuterium arc lamp (Model D102, G.W. Gates & Co., Inc., Long

* Generously donated by Onyx Chemical Co., Jersey City, New Jersey.

Island, N.Y.). Actinic illumination was provided either by a Xenon flash lamp, operated at 100 J and 0.1 ms half-time, filtered through Corning filters 4-97 (blue) or 7-69 (near infrared), or by a Q-switched ruby laser, Korad model K-15. Adequate filters were put in front of the detector to block scattered exciting light.

Ubiquinone 6, horse heart cytochrome *c*, and sodium ascorbate were purchased from the Sigma Chemical Co., St. Louis, Mo., and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (diaminodurene or DAD) from the Aldrich Chemical Co., Inc., Milwaukee, Wisc.

RESULTS

In order to abstract the absorbance changes due to primary and secondary acceptors from the total absorbance changes, the oxidized primary electron donor must be quickly rereduced by an adequate electron donor [4]. We used the following electron donors: DAD, sodium ascorbate and reduced cytochrome *c*. This choice was dictated by the facts that upon oxidation no changes or very small changes occur in the wavelength range 255–295 nm for the cytochrome *c* and for wavelengths greater than 320 nm for the two other electron donors. Moreover, the oxidized form of the electron donor must not be able to play the role of secondary electron acceptor (which is the case, for example, for *N*-methylphenazonium methosulphate), if one wants to study more intrinsic secondary reactions of reaction centers.

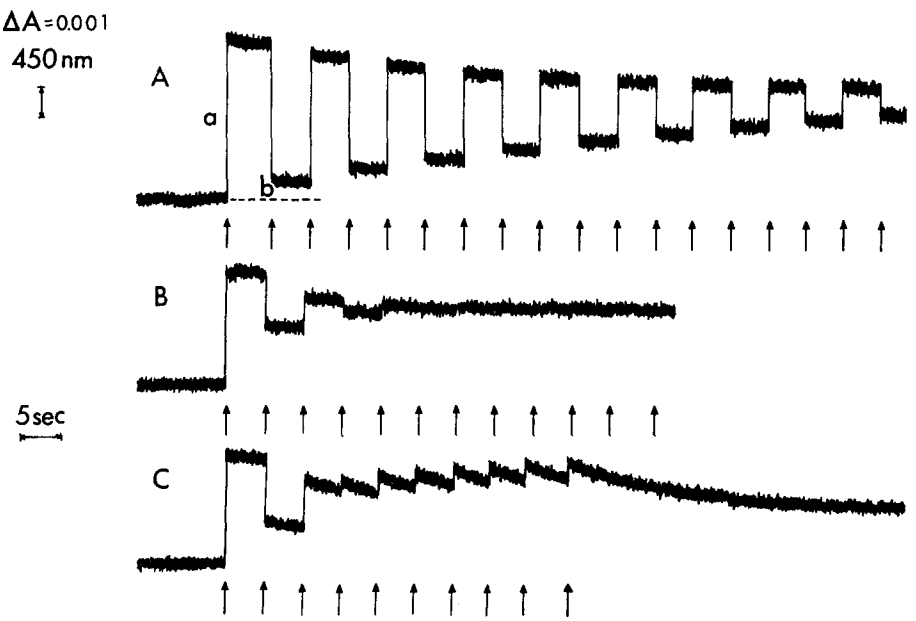


Fig. 1. Light-induced absorbance changes in reaction centers (2 μM, in 0.3 % LDAO, 0.01 M Tris · HCl, pH 7.5) measured at 450 nm. Actinic illumination was provided by Xenon flashes spaced 5 s apart, indicated by the arrows. Electron donor DAD (100 μM) for each trace. Trace A, with 25 μM exogeneous ubiquinone 6. Trace B, same as A plus 1 mM *o*-phenanthroline. Trace C, without ubiquinone or *o*-phenanthroline.

Fig. 1, trace A, shows the behavior of photoinduced absorbance changes at 450 nm in response to a sequence of actinic flashes spaced 5 s apart, in the presence of DAD as electron donor and ubiquinone 6 as secondary electron acceptor. In these conditions the rereduction of the oxidized primary donor P^+ is fast and no changes due to P^+ are observed due to the time constant of the apparatus (20 ms). The absorbance changes are therefore related only to photoreduced products. Clearly a periodicity of two is observed, each odd flash inducing an absorbance increase and each even flash an absorbance decrease. Oscillations can be seen up to 20 flashes.

The oscillatory pattern is partly inhibited in the presence of 1 mM *o*-phenanthroline (Fig. 1, trace B) which is known to block electron transfer between primary and secondary acceptors. To completely inhibit absorbance changes on the second and successive flashes one must add up to 4 mM *o*-phenanthroline (data not shown).

If no external ubiquinone is added (Fig. 1, trace C) oscillation is observed only on the first two flashes, the subsequent ones inducing an absorbance increase that relaxes slowly in the dark.

Reaction centers purified by ammonium sulfate fractionation gave either no oscillatory pattern or oscillations with high damping, even with excess ubiquinone. If no ubiquinone was added, we did not observe a single oscillation with $(NH_4)_2SO_4$-purified reaction centers. Instead the behavior for flashes 1, 2, 3, . . . was like that in Fig. 1 (C) for flashes 3, 4, 5, . . . This explains why Wraight et al. [18] did not observe oscillations for the 431 nm absorbance changes in the presence of reduced cytochrome *c* and excess ubiquinone.

In the presence of sodium ascorbate (10 mM) as electron donor, the reduction of P^+ was much slower ($t_{\frac{1}{2}} = 160$ ms) but still the absorbance changes at 450 nm showed a periodicity of two in a series of actinic flashes (Fig. 2). Under these conditions, absorbance changes due to P^+ were easily observable because of the slow rate of rereduction. These are the rapid transient changes in Fig. 2. We found no periodicity for the photooxidation of P^+, observed at 605 or 870 nm in a sequence of saturating flashes: each flash photooxidized the same amount ($\pm 10\,\%$) of primary electron donor (data not shown).

The amplitude of the absorbance decrease induced by the second flash depended on the time elapsed after the first flash (Fig. 3). A decrease still occurred if the time interval between the flashes was as long as 3 min; more than 15 min between

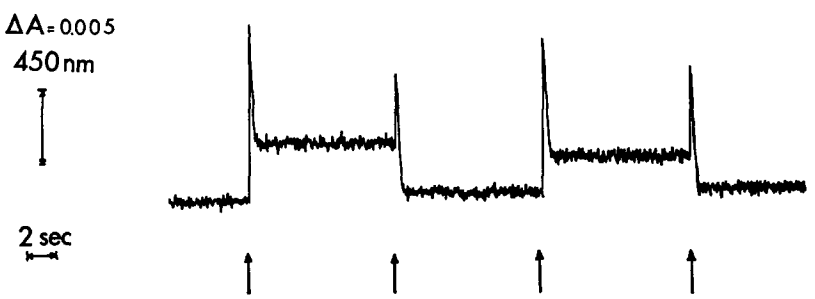


Fig. 2. Light-induced absorbance changes observed at 450 nm. Reaction center concentration 2 μM in 0.3 % LDAO, 0.01 M Tris · HCl, pH 7.5. Xenon flashes spaced by 10 s. Electron donor sodium ascorbate (10 mM); ubiquinone (25 μM) as secondary electron acceptor.

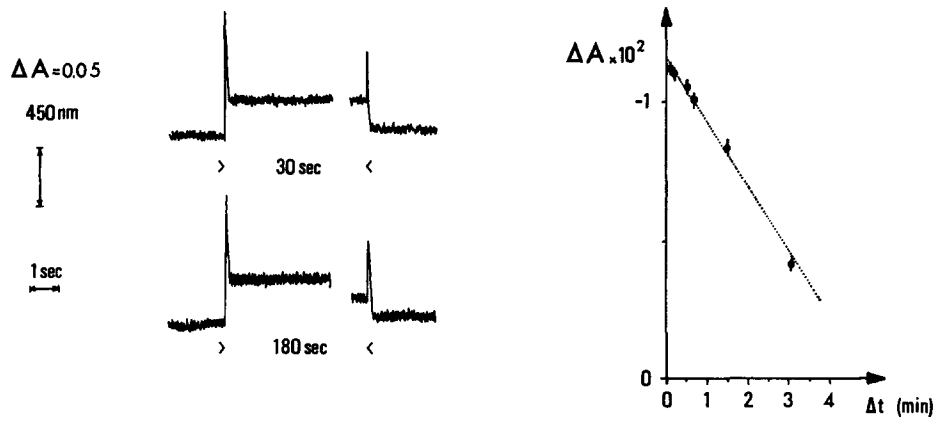


Fig. 3. Left part: light-induced absorbance changes observed at 450 nm. Same conditions as Fig. 2 except that the two flashes were spaced by 30 and 180 s. Right part: plot of the light-induced absorbance changes occurring on the second flash (measured from experiments similar to those reported in the right part) vs. the time elapsed between the flashes (Δt).

the two flashes was required to obtain on the second flash the same absorbance increase as occurred on the first one.

For all wavelengths tested from 310 nm to the near infrared the light-induced absorbance changes exhibited an oscillatory pattern, but no oscillations were observable at wavelengths below 300 nm. Fig. 4 (trace A) shows the light-induced changes occurring at 282 nm in the presence of reduced cytochrome *c* (25 μM) and ubiquinone

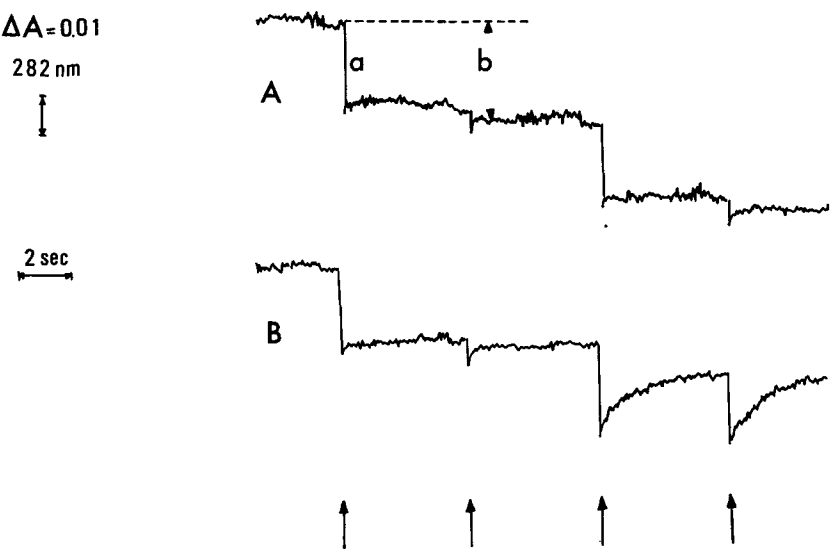


Fig. 4. Light-induced absorbance changes observed at 282 nm. Excitation: laser flashes spaced 5 s apart as indicated by the arrows. Reaction center concentration 2 μM in 0.3 % LDAO, 0.1 M Tris · HCl, pH 7.5, in the presence of 25 μM ubiquinone. The electron donor was reduced cytochrome *c*: 25 μM for trace A and 5 μM for trace B.

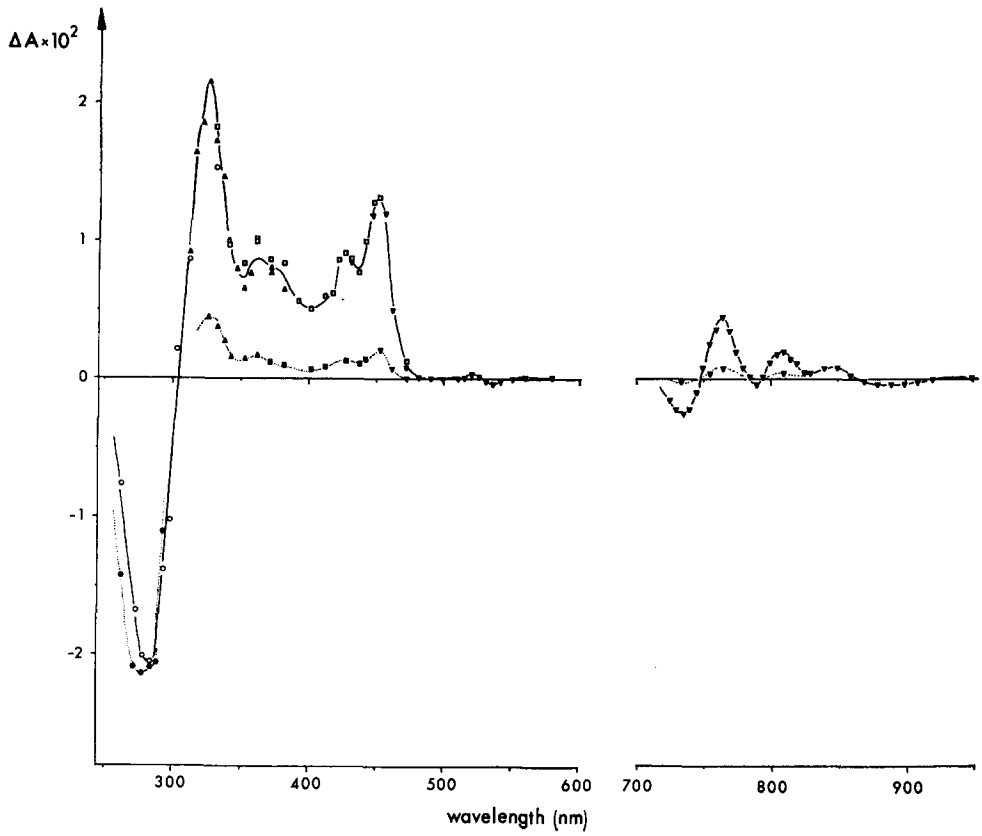


Fig. 5. Difference spectra of light-induced absorbance changes: open symbols, full line, changes induced by the first flash; closed symbols, dotted line, changes remaining after the second flash. ○, ●, electron donor: reduced cytochrome *c* (25 μM); excitation by laser flashes; absorbance changes measured with the single beam spectrophotometer equipped with a deuterium arc lamp. △, ▲, electron donor: DAD (100 μM); excitation by laser flashes; absorbance changes measured with the single beam spectrophotometer equipped with a deuterium arc lamp. □, ■, electron donor: DAD (100 μM); excitation by laser flashes; absorbance changes measured with the split beam spectrophotometer equipped with a tungsten lamp. ▽, ▼, electron donor: sodium ascorbate (10 mM); excitation by Xenon flashes; absorbance changes measured with the split beam spectrophotometer.

(25 μM). The absorbance change due to photooxidized cytochrome *c* has been evaluated to be less than 15 % of the total absorbance change on the first flash, by comparing the extent of cytochrome oxidation at 420 nm. Odd flashes (1 and 3) induced a large absorbance decrease at 282 nm but even flashes (2 and 4) only a small one. In fact, depending on the measuring wavelength between 255 and 295 nm, the second and fourth flashes could induce a small decrease or increase in absorbance as expected from the total difference spectrum of the light-induced changes (see later; Fig. 5).

In the presence of a smaller amount of reduced cytochrome *c* (5 μM) (Fig. 4, trace B) the same behavior was observed for the two first flashes, but subsequent flashes induced absorbance decreases of equal amplitudes relaxing in a few seconds. At wavelengths characteristic of absorbance changes related to P^+ the same kinetics

were observed (data not shown). After consumption of reduced cytochrome *c* in the medium, a back reaction between P^+ and photoreduced product was therefore apparent. In the past, changes around 280 nm have been attributed either to reduction of ubiquinone or photooxidation of *P*-870 [19], resulting in a controversial picture. From the results depicted in Fig. 4, we propose that the decrease in absorbance around 280 nm is due mainly to formation of either ubisemiquinone or fully reduced quinone. We conclude this because the amplitudes of the changes are nearly equal in cases where we expect only ubiquinone participation (first flash, Fig. 4, trace B) or when we expect participation of both ubisemiquinone and oxidized *P*-870 (third flash, Fig. 4, trace B).

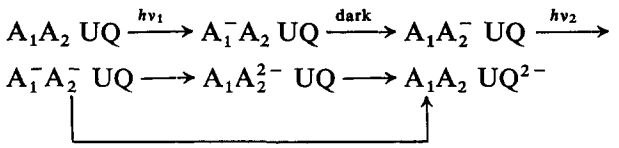
The light-induced difference spectra for the changes occurring on the first flash (indexed as "a" in Fig. 1, trace A, and Fig. 4, trace A) and for the changes remaining after two flashes (indexed as "b" in Fig. 1, trace A, and Fig. 4, trace A) are shown in Fig. 5 by the full and dotted lines, respectively. Each kind of symbol (circles, triangles, etc.) refers to different experimental conditions (flash excitation, electron donor, etc.) as explained in the legend. For each wavelength a new sample was used, or else a sample which had received a small even number of flashes was dark-adapted for at least 20 min before use.

The difference spectrum induced on the first flash (Fig. 5, full line) is almost identical to the difference spectrum between anionic ubisemiquinone and ubiquinone in methanolic solution as reported by Bensasson and Land [14]. However, additional changes can be seen in the Q_x region of bacteriopheophytin absorption and in the near infrared part of the spectrum. The difference remaining after two flashes (Fig. 5, dotted line) exhibits the same shape as the difference between fully reduced and oxidized ubiquinone in solution, apart from 10 to 20 % of absorbance changes attributable to anionic ubisemiquinone, and bacteriopheophytin and bacteriochlorophyll absorption band shifts. If one plots the absorbance changes induced by any odd number of flashes, or the absorbance changes remaining after any even number of flashes, one will obtain difference spectra similar to those shown in Fig. 5 by the full and dotted lines, respectively, except for some mixing of the two spectra due to damping.

DISCUSSION

We have asked whether if, after a single turnover, two electron chains cooperate to fully reduce a molecule of ubiquinone, or if each reaction center needs to accumulate two negative charges before being able to reduce a molecule of the ubiquinone pool. Our results show that the second hypothesis is correct. The close similarity between the difference spectra depicted in Fig. 5 and those obtained in vitro [14] suggests the formation of ubisemiquinone and fully reduced ubiquinone, respectively, after odd and even numbers of flashes. This result can be described by a model in which the first electron is held in the reaction center, and a ubiquinone molecule of the pool is fully reduced in a two-electron transfer reaction after a second charge separation. Since the primary electron acceptor can hold only one electron at a time [10], allowing only a single charge separation, we must postulate that the reaction center contains an additional acceptor distinct from the primary one. This postulate agrees with the finding of Okamura et al. [3] that each reaction center contains two

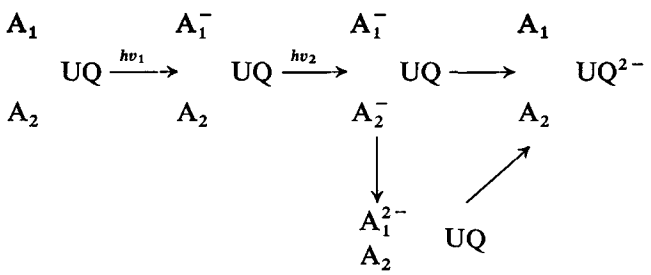
molecules of ubiquinone, one firmly bound and essential for photochemical activity ("primary") and the second more readily extracted. We propose a "series" scheme as follows:



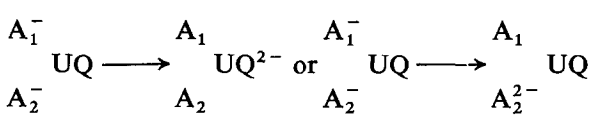
where A_1 and A_2 are the two molecules of ubiquinone in one reaction center and UQ represents a pool of exogenous ubiquinone.

This model explains all of our findings; especially the necessity of adding external ubiquinone to observe repeated oscillations, the occurrence of a single oscillation on the first two flashes if external ubiquinone has not been added and the action of *o*-phenanthroline in abolishing oscillations and preventing electron transfer beyond A_1. This blocking action is probably related to the fact that *o*-phenanthroline can cause selective release of the second ubiquinone (A_2 in our model) from the reaction center [3]. The absence of oscillations in reaction centers purified by ammonium sulfate fractionation is explained if in such preparations the link between A_1 and A_2 has been weakened, or A_2 has been released.

We can also consider a "parallel" model:



This model requires certain ad hoc statements which seem less economical than their counterparts in the series model. First, in the parallel model the limiting step for recovery of reactivity after a flash should be the time of rereduction of the primary electron donor P^+. This is inconsistent with the finding of Parson [10], who reported that in *Chromatium* chromatophores the half-time for the recovery of photochemical activity after a flash is much longer (60 μs) than the half-time of rereduction of P^+ (2 μs). In the parallel scheme we must postulate that after a single flash, some kind of rearrangement must occur before A_2 can become operative as primary acceptor. Second, the half-time for recovery is the same after one or two flashes [10, 20]. We must therefore assert that the half-time of the rearrangement after one flash is the same as the half-time of the reaction



after the second flash. In the series model we need only say that the rate of electron transfer from A_1^- to A_2 is the same as that from A_1^- to A_2^-.

The absorbance changes in the near infrared (Fig. 5) are not identical in shape to those reported earlier [4] as associated with reduction of the primary acceptor. The detailed nature of these pigment band shifts appears to depend on whether the electron is on A_1 or A_2 or neither. Experiments are in progress to exploit this situation to monitor the kinetics of electron transfer between A_1, A_2 and exogenous ubiquinone.

ACKNOWLEDGEMENTS

I wish to express my gratitude to Dr. Roderick K. Clayton for his generous and continuous encouragement and support during this investigation and the preparation of the manuscript.

This work was supported by Contract No. AT(11-1)-3162 with the U.S. Energy Research and Development Administration and by Grant No. PCM 76-10556 from the National Science Foundation.

REFERENCES

1 Feher, G., Okamura, M. Y. and McElroy, J. D. (1972) Biochim. Biophys. Acta 267, 222–226
2 Bolton, J. R. and Cost, K. (1973) Photochem. Photobiol. 18, 417–421
3 Okamura, M. Y., Isaacson, R. H. and Feher, G. (1975) Proc. Natl. Acad. Sci. U.S. 72, 3491–3495
4 Clayton, R. K. and Straley, S. C. (1972) Biophys. J. 12, 1221–1234
5 Slooten, L. (1972) Biochim. Biophys. Acta 275, 208–218
6 Bales, H. and Vernon, L. P. (1963) in Bacterial Photosynthesis (Gest, H., San Pietro, A. and Vernon, L. P., eds.), pp. 269–274, Antioch Press, Yellow Springs
7 Takamiya, K. and Takamiya, A. (1970) Biochim. Biophys. Acta 205, 77–85
8 Halsey, Y. D. and Parson, W. (1974) Biochim. Biophys. Acta 347, 404–416
9 Cogdell, R. J., Brune, D. C. and Clayton, R. K. (1974) FEBS Lett. 45, 344–347
10 Parson, W. (1969) Biochim. Biophys. Acta 189, 384–396
11 Stiehl, H. H. and Witt, H. T. (1969) Z. Naturforsch. 24b, 250–256
12 Bouges-Bocquet, B. (1973) Biochim. Biophys. Acta 314, 250–256
13 Velthuys, B. R. and Amesz, J. (1974) Biochim. Biophys. Acta 333, 85–94
14 Bensasson, R. and Land, E. J. (1973) Biochim. Biophys. Acta 325, 175–181
15 Clayton, R. K. (1960) Biochim. Biophys. Acta 37, 503–512
16 Clayton, R. K. and Clayton, B. J. (1972) Biochim. Biophys. Acta 283, 492–504
17 Arnold, W. and Clayton, R. K. (1960) Proc. Natl. Acad. Sci. U.S. 46, 769–776
18 Wraight, C. A., Cogdell, R. J. and Clayton, R. K. (1975) Biochim. Biophys. Acta 396, 242–249
19 Loach, P. A., Bambara, R. A. and Ryan, F. J. (1971) Photochem. Photobiol. 13, 247–257
20 Case, G. D. and Parson, W. (1971) Biochim. Biophys. Acta 253, 187–202