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SOME EXPERIMENTS ON THE PRIMARY ELECTRON ACCEPTOR IN REACTION CENTRES FROM *RHODOPSEUDOMONAS SPHAEROIDES*

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SUMMARY

The bacterial reaction center absorbance change at 450 nm (*A*-450) assigned to an anionic semiquinone, has been suggested as a candidate for the reduced form of the primary electron acceptor in bacterial photosynthesis. In reaction centers of *Rhodopseudomonas sphaeroides* we have found kinetic discrepancies between the decay of *A*-450 and the recovery of photochemical competence. In addition, no proton uptake is measurable on the first turnover, although subsequent ones elicit one proton bound per electron. These results are taken to indicate that the acceptor reaction after a long dark period may be different for the first turnover than for subsequent ones. It is suggested that *A*-450 is still a likely candidate for the acceptor function but that in reaction centers, additional quinone may act as an adventitious primary acceptor when the "true" primary acceptor is reduced. Alternatively, the primary acceptor may act in a "ping-pong" fashion with respect to subsequent photoelectrons.

INTRODUCTION

In a simple model of the early electron transfer events in bacterial photosynthesis, following a saturating, single-turnover flash, the primary donor (P) is oxidized and the primary acceptor (A) is reduced:

$$PA \rightarrow P^+A^-$$

The two species P^+ and A^- are thus produced simultaneously although their subsequent fates are largely independent. The nature of P has been firmly established as a spe-

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cialized bacteriochlorophyll molecule or group of molecules but the nature of A is still obscure. Detection of a characteristic spectrum has been hindered by the large attendant absorbance changes due to P/P^+. However, using reaction centre preparations in the presence of a weak reductant such as ascorbate it has been possible to detect a light-induced spectrum after re-reduction of P^+ [1, 2] which was tentatively assigned to A/A^-. The spectrum so obtained correlates quite closely with that of the ubisemiquinone anion radical, UQ^-, [3, 4] and has been ascribed to this species; however, since there are still some uncertainties as to its nature we will refer to it as *A*-450*, after the peak absorption wavelength.

The need to re-reduce P^+ in order to observe *A*-450 essentially limits its detection to room temperature and it is thus difficult to assess its candidacy as primary acceptor with that of the second major contender, an iron-sulphur EPR signal ($g = 1.82$) observable only at liquid helium temperatures [5, 6]. This signal, apparent in a variety of species and preparations of photosynthetic bacteria, fulfills all of the kinetic and thermodynamic requirements of the primary acceptor at low temperature but, of course, is of indeterminate relevance at room temperature since it is not observable.

In order to test further the possible role of *A*-450 as primary acceptor we have undertaken a more rigorous kinetic testing of this component in reaction centres using cytochrome *c* to re-reduce P^+ in a rapid, second-order reaction [7]. Following a saturating, single-turnover flash, the reaction centres are left in the state PA^- and the ability of a second saturating flash to oxidize another molecule of cytochrome *c* (via P^+) should follow the reoxidation of A^- by secondary acceptors. We report here on comparisons of the decays of flash-induced *A*-450 and fluorescence with the recovery of photochemical activity as indicated by cytochrome *c* oxidation by a second flash.

METHODS

Reaction centres were prepared by lauryl dimethylamine N-oxide treatment of chromatophores from *Rhodopseudomonas sphaeroides* R26 and purified as previously described [9].

In order to observe the flash-induced *A*-450 absorbance change, the contribution due to P^+ was quenched by electron transfer from mammalian cytochrome *c* in large excess. Cytochrome *c* has been shown to donate to P^+ by a second-order reaction [7], permitting the attainment of half-reaction times of 40–60 μs with reasonable concentrations of reaction centres (2–3 μM) and cytochrome (50–100 μM). *A*-450 was monitored on a split-beam differential spectrophotometer [10] using the cytochrome *c* isosbestic wavelength of about 431 nm. This wavelength setting was carefully determined beforehand by reducing oxidized cytochrome with minimal amounts of dithionite.

Cytochrome *c* oxidation, rapid proton uptake and rapid *A*-450 measurements were made on a kinetic single-beam spectrophotometer of conventional design with a response capability of 10 μs. Proton uptake was monitored with the pH indicator

* This component was previously referred to as *P*-450 but confusion has arisen with the microsomal complex (also *P*-450). We, therefore, propose to distinguish it by the label *A*-450 indicating merely an absorbance change at 450 nm.

phenol red as previously described [11]. Fluorescence was measured in a simple fluorimeter measuring at right angles to the actinic light. Flash excitation was provided by one of three sources; a Q-switched ruby laser (Korad model TRG 104 A, Santa Monica, Calif. 90406), a xenon flash lamp (100 J, half width $\approx$ 0.1 ms) and a xenon camera flash (half width $\approx$ 0.4 ms).

Ubiquinone$_6$ and horse heart cytochrome *c* were purchased from the Sigma Chemical Co., St. Louis, Mo.

RESULTS AND DISCUSSION

1. Some characteristics of the flash-induced A-450 change

(*a*) *Extinction coefficient.* Previous estimates of the extinction coefficient of *A*-450 have indicated values between 2 and 4 $mM^{-1}cm^{-1}$ [1, 2] although Slooten's value [2] was obtained by comparison with the rather low value of ΔA at 600 nm for reaction centres of 16 $mM^{-1}cm^{-1}$. A recent revision of this to 26 $mM^{-1}cm^{-1}$ [7] would give ΔA for *A*-450 of about 6 $mM^{-1}cm^{-1}$ which is in agreement with that expected for $Q^{-}{}_{10}$ [4]. Since some oxidized product of ascorbate, used in all these earlier determinations of ΔA of *A*-450, causes irreversible destruction of reaction centres (Wraight C.A. and Clayton, R. K., unpublished observations) this parameter was redetermined by comparison of the extent of the *A*-450 change (at 431 nm) with the extent of cytochrome *c* oxidation ($\Delta A_{550nm} = 20.4$ [12]). An average of six determinations gave a value at 431 nm of 6.5 $\pm$ 0.5 $mM^{-1}cm^{-1}$; this indicates a value at the absorbance peak at 450 nm of about 8–9 $mM^{-1}cm^{-1}$, somewhat larger than the value for $Q^{-}{}_{10}$ in methanolic solution.

(*b*) *Kinetics.* The kinetics of the flash-induced *A*-450 change are shown in Fig. 1. Determination of the rise time was limited by the rate of removal of the interfering

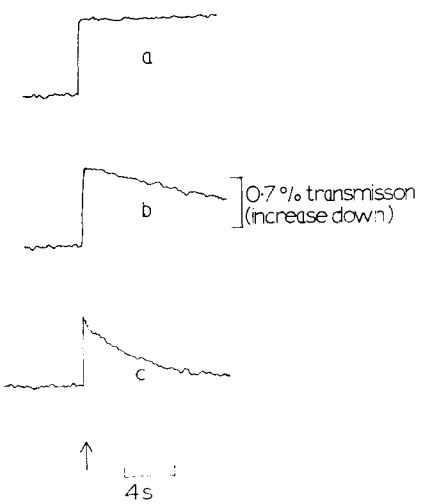

Fig. 1. Kinetics of the laser-induced *A*-450 change. *A*-450 changes were monitored at $\approx$ 431 nm. The sample contained 10 mM Tris-HCl pH 7.5, 0.3 % lauryl dimethylamine N-oxide, 2 μM reaction centers and 25 μM reduced cytochrome *c*. (a) No further additions, (b) + 33 μM 1,4-naphthoquinone, (c) + 33 μM 1,4-naphthoquinone and 2 mM *o*-phenanthroline. ↑, Laser fires.

P^+ absorbance change and was certainly faster than the re-reduction of P^+ by cytochrome *c* (< 60 μs, see Methods). The recovery of the *A*-450 signal was slow. The extent of the flash-induced absorbance change was unaffected by additions of lauryl dimethylamine N-oxide (up to 2 %), *o*-phenanthroline or Q. However, the rate of decay was somewhat slowed by addition of Q and accelerated by 1,4-naphthoquinone. Rather unexpectedly, this acceleration was enhanced by addition of *o*-phenanthroline (Fig. 1) for which we can offer no explanation, at present.

2. The decay of flash-induced A-450 and the recovery of photochemistry in a second flash

The decay of flash-induced *A*-450 was measured as the time course for recovery of the change elicited by a second flash administered at different times after the first. In parallel experiments the recovery of photochemistry in a second flash was determined by the ability to photooxidize cytochrome *c* (Table I).

With a long xenon flash (0.1 – 0.4 ms), reaction centres suspended in low concentrations of lauryl dimethylamine N-oxide (⩽ 0.3 %) were capable of oxidizing 2–3 mol of cytochrome *c* per mol of *P*-870. Higher levels of lauryl dimenthylamine N-oxide progressively decreased this to a limiting value of 1 mol cytochrome *c*/mol *P*-870 indicating that high lauryl dimethylamine N-oxide (e.g. 2 %) effectively decoupled any endogenous secondary acceptors in the reaction centre preparations. In the absence of an effective secondary electron acceptor pool, the recovery of the flash-induced *A*-450 change and cytochrome oxidation exhibited similar half times of 1–2 min.

In the presence of high concentrations of lauryl dimethylamine N-oxide, added Q can act as an effective secondary acceptor. This strongly supports an earlier suggestion that high lauryl dimethylamine N-oxide acts by diluting out the endogenous acceptor pool [13]. Under these conditions, recovery of the *A*-450 change was somewhat slowed ($t_{\frac{1}{2}}$ = 2–4 min), whereas recovery of photochemical competence (cytochrome oxidation) was greatly accelerated and was complete within the time required to recharge the flash system (about 10 s).

TABLE I

A COMPARISON OF THE RECOVERY KINETICS OF THE *A*-450 CHANGE AND PHOTOCHEMISTRY

Excitation was provided by a xenon flash. The sample contained 10 mM Tris-HCl pH 7.5, 0.3 % lauryl dimethylamine N-oxide (LDAO), 24 μM reduced cytochrome *c* and 1.5 μM reaction centers.

Change	Additions	$t_{1/2}$ decay(s)
A-450	2 % LDAO	90
	20 μM Q +2 % LDAO	240
	20 μM Q +2 % LDAO +2 mM *o*-phenanthroline	90
Cytochrome *c* oxidation (photochemistry)	2 % LDAO	90
	20 μM Q +2 % LDAO	< 10
	20 μM Q +2 % LDAO +2 mM *o*-phenanthroline	< 10

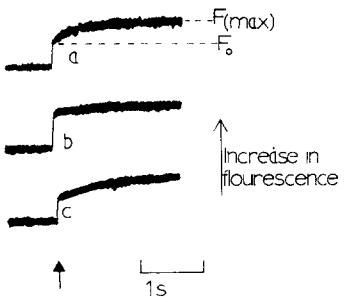

Fig. 2. Light-induced fluorescence changes of reaction centers with *A*-450 reduced or oxidized prior to the measurement. The sample contained 10 mM Tris-HCl pH 7.5, 0.3 % lauryl dimethylamine N-oxide, 24 μM reduced cytochrome *c* and 4.2 μM reaction centers. ↑, continuous light on (intensity $\approx$ 1 mW · cm^{-2}). (a) Illumination after 15 min dark incubation, (b) illumination 5 s after a Q-switched laser flash, (c) conditions as (b) but with 40 μM Q present.

3. Kinetics of fluorescence recovery

It was clear from the results of the previous section that recovery of *A*-450 did not necessarily parallel that of photochemistry. In view of recent results questioning the validity of fluorescence measurements as an indicator of photochemistry [14, 15], correlation between fluorescence and either *A*-450 or photochemistry was sought. The variable fluorescence of reaction centres was measured in the presence of cytochrome *c* before and shortly after a saturating, single-turnover flash with and without added ubiquinone (Fig. 2). In the absence of Q, the flash preillumination raised the initial fluorescence level to the maximum value (F_{max}); thus no decay of the fluorescence occurred in the 2–3 s dark period between flash and measurement (Fig. 2B). However, in the presence of ubiquinone the initial fluorescence level had decayed more or less to the minimum value (F_0) in this time (Fig. 2C); thus the variable fluorescence paralleled the photochemical activity rather than the redox state of *A*-450.

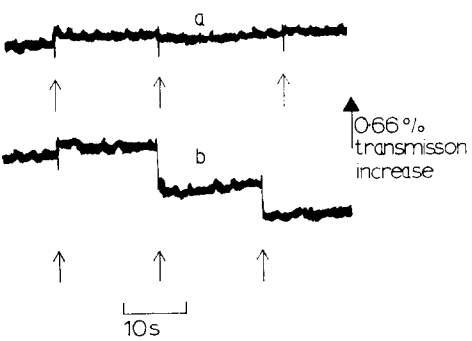

Fig. 3. The laser-induced rapid proton uptake by reaction centers. The absorbance changes of the pH indicator phenol red were monitored at 557 nm. Arrows indicate laser flash. The cuvette contained 100 mM KCl, 50 μM phenol red pH 7.5, 4.2 μM reaction centers and 24 μM reduced cytochrome. 10 μM 1,4-naphthoquinone were added to allow repeated turnover of the system (naphthoquinone rather than Q was used because Q needs lauryl dimethylamine N-oxide to interact with the reaction centers and lauryl dimethylamine N-oxide buffers, making the measurement of small pH changes difficult). (a) With 20 mM Tris-HCl, (b) in the absence of added buffer; the samples were in the dark for 15 min prior to the first flash.

4. Proton-binding in reaction centres and the distinction between the first and subsequent turnovers

These results suggested the possibility that a "true primary acceptor" functions on the first flash while external Q might substitute on the second flash. Previous work had shown that reaction centres supplemented with reduced cytochrome *c* exhibit a flash-induced proton uptake stoichiometric with Q reduction [16]. However, these measurements involved an initial flash in the absence of indicator to check the isosbestic wavelength. There was, therefore, a possibility that the first turnover may have a different H^+-binding character than subsequent ones. Fig. 3 shows that, indeed, the first flash was not accompanied by proton-binding, although subsequent ones were. This is a further indication of a distinction between first and second flash events and also lends support to the identity of *A*-450 as an anionic (unprotonated) ubisemiquinone [1, 2]. It is noteworthy that the midpoint potential of the primary acceptor of reaction centres is pH-independent [17] although that of chromatophores shows a 60 mV/pH unit dependence [18].

If exogenous Q is able to substitute for the primary acceptor function it might be hoped that it would appear as a change in the quantum yield, although this is certainly not a necessary consequence. The relative quantum yield of cytochrome *c* photo-oxidation in the presence of added Q was measured before and immediately after a single-turnover flash. No difference could be discerned within the precision of the relative determinations ($\pm$ 5 %).

CONCLUSIONS

The present results show that the photochemical activity of isolated reaction centres can recover well in advance of the decay of *A*-450. This, however, cannot be taken to rule out *A*-450 as the primary electron acceptor since it is also strongly indicated that the nature of the acceptor reaction in the first turnover after a long dark period is different from that of subsequent ones. Conceivably, ubisemiquinone could be formed on the first flash and the fully reduced form on the second; this would facilitate communication between a one-electron primary acceptor and a two-electron secondary acceptor pool. Observation of absorbance changes at 270 nm was not helpful since the differential extinction coefficients of the semiquinone and fully reduced forms are similar. It is clear from the *A*-450 spectral region, however, that this component does not alter on the second flash.

We have considered two relatively simple explanations of our results. Firstly, that a specialized primary acceptor, including the *A*-450 component, is active on the first flash but that additional, unspecialized quinone can substitute when the "true" acceptor is reduced. It remains to be seen if this adventitious acceptor function is exhibited by the endogenous quinone pool in vivo. It has been established that intact chromatophores do indeed bind protons on the first flash, but this H^+-binding is correlated with rapid transfer of the photoelectron to a secondary acceptor. The addition of *o*-phenanthroline to chromatophores diminishes the H^+-binding to a somewhat variable extent but does not seem to abolish it [11].

A second interpretation would be that the primary electron acceptor acts in a "ping-pong" fashion, the first photoelectron moving onto a secondary acceptor as the second photoelectron appears. If this were the case, a rapid transient change in the

A-450 component might be expected on the second and subsequent flashes; however, the kinetic limitation on the measurement of the *A*-450 rise-time (see Methods) precludes a direct test of this possibility.

Since a single turnover flash can generate both the *A*-450 optical and $g = 1.82$ EPR signals, it seems convenient to view the primary electron acceptor as an iron · Q complex [19, 20], and a rationalization of the low temparature EPR signal has been offered in this context (Feher, G. personal communication). Recent experimental results from Feher's laboratory and by Debrunner [21] have cast serious doubt on the necessity for iron in the primary acceptor complex, although its involvement when present is still probable.

In collaboration with Prince and Dutton we have looked at the room temperature decay characteristics of the $g = 1.82$ EPR signal by flash illumination followed by rapid freezing in liquid nitrogen. However, comparison with the *A*-450 data is complicated by the very much higher concentrations of reaction centers required for EPR measurements and no conclusive results have been obtained so far.

The work reported here highlights two significant differences between isolated reaction centers and those in situ in the chromatophore; the pH dependence of the midpoint potential of the primary acceptor and the life-time of the reduced primary acceptor after a single flash (in chromatophores the electron can be seen to move through the entire electron transport chain in a few milliseconds [22]). Rather than resort to unconstructive criticism of the isolated reaction center as a model system for the primary processes of photosynthesis, we would like to suggest that the proton-binding capacity of the in situ primary acceptor may be associated with some other membrane component sensitive to the redox state of the primary acceptor. In addition, the secondary electron transfer step may also be sensitive to the environment presented by the intact system. These proposals suggest several critical and challenging experiments in reconstituting the in vivo behavior of the reaction center preparation.

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REFERENCES

1 Clayton, R. K. and Straley, S. C. (1972) Biophys. J. 12, 1221–1234
2 Slooten, L. (1972) Biochim. Biophys. Acta 275, 208–218
3 Land, E. J., Simic, M. and Swallow, A. J. (1971) Biochim. Biophys. Acta 226, 239–240
4 Bensasson, R. and Land, E. J. (1973) Biochim. Biophys. Acta 325, 175–181
5 Leigh, J. S. and Dutton, P. L. (1972) Biochem. Biophys Res. Commun. 46, 416–421
6 Feher, G. (1971) Photochem. Photobiol. 14, 383–387
7 Prince, R. C., Cogdell, R. J. and Crofts, A. R. (1974) Biochim. Biophys. Acta 347, 1–13
8 Parson, W. W. (1969) Biochim. Biophys. Acta 189, 386–396
9 Clayton, R. K. and Wang, R. T. (1971) in Methods in Enzymology (Colowick, S. P. and Kaplan, N., eds, San Pietro, A., Vol. ed.), Vol. 23, pp. 696–704, Academic Press, New York
10 Clayton, R. K. (1966) Photochem. Photobiol. 5, 679–688
11 Cogdell, R. J., Jackson, J. B. and Crofts, A. R. (1973) J. Bioenerget. 4, 412–429

12 Straley, S. C., Parson, W. W., Mauzerall, D. C. and Clayton, R. K. (1973) Biochim. Biophys. Acta 305, 597–607
13 Clayton, R. K., Fleming, H. and Szuts, E. Z. (1972) Biophys. J. 12, 46–63
14 Wraight, C. A. and Clayton, R. K. (1973) Biochim. Biophys. Acta 333, 246–260
15 Wraight, C. A., Leigh, J. S., Dutton, P. L. and Clayton, R. K. (1974) Biochim. Biophys. Acta 333, 401–408
16 Cogdell, R. J., Prince, R. C. and Crofts, A. R. (1973) FEBS Lett. 35, 204–208
17 Dutton, P. L., Leigh, J. S. and Wraight, C. A. (1973) FEBS Lett. 36, 169–173
18 Jackson, J. B., Cogdell, R. J. and Crofts, A. R. (1973) Biochim. Biophys. Acta 292, 218–225
19 Feher, G., Okamura, M. Y. and McElroy, J. D. (1972) Biochim. Biophys. Acta 267, 222–226
20 Bolton, J. R. and Cost, K. (1973) Photochem. Photobiol. 18, 417–422
21 Feher, G., Isaacson, R. A., McElroy, J. D., Ackerson, L. C. and Okamura, M. Y. (1974) Biochim. Biophys. Acta 368, 135–139
22 Dutton, P. L., Petty, K. M., Prince, R. C. and Cogdell, R. J. (1975) Biochim. Biophys. Acta in press