Biochimica et Biophysica Acta, 387 (1975) 536–556

BBA 46926

CYTOCHROME c_2 AND REACTION CENTER OF *RHODOSPEUDOMONAS SPHEROIDES* Ga. MEMBRANES. EXTINCTION COEFFICIENTS, CONTENT, HALF-REDUCTION POTENTIALS, KINETICS AND ELECTRIC FIELD ALTERATIONS

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(Received October 28th, 1974)
(Revised manuscript received January 27th, 1975)

SUMMARY

1. The reduced minus oxidized extinction coefficients ($\Delta\varepsilon^{red\text{-}ox}$) of reaction center P605 when in the chromatophore is about 20 % smaller than in the detergent-isolated state. Presumably the coupling of the reaction center protein to the antenna bacteriochlorophylls and carotenoids causes this hypochromism. The chromatophore values for P605 are 19.5 $mM^{-1} \cdot cm^{-1}$ with the spectrophotometer on single beam mode at 605 nm, and 29.8 $mM^{-1} \cdot cm^{-1}$ on dual wavelength mode set at 605–540 nm. Cytochrome c_2, which is not affected by detergent, has a $\Delta\varepsilon^{red\text{-}ox}$ value at 550–540 nm of 19.0 $mM^{-1} \cdot cm^{-1}$.

2. The total bacteriochlorophyll to reaction center bacteriochlorophyll protein (P) ratio is about 100 : 1. The cytochrome c_2: reaction center protein ratio approaches 2. In current French press chromatophore preparations, about 70 % of the reaction centers are each associated on a rapid kinetic basis with two cytochrome c_2 molecules (intact P-c_2 units). The remaining reaction center proteins are not associated with cytochrome c_2 on a kinetically viable basis and may be the result of damage incurred during mechanical rupture of the cells.

3. The half-reduction potential of cytochrome c_2 in the isolated state is 345 mV. In the chromatophore, two electrochemical species of cytochrome c_2 are recognized. The majority has a value of approx. 295 mV and is identifiable with cytochrome c_2 in a reaction center protein-associated state (kinetically active, intact P-c_2 units); the remainder has an approx. 350 mV half-reduction potential and is probably cytochrome c_2 in the "free" or reaction center-dissociated state (possibly from damaged P-c_2 units). It appears that there is no exchange of cytochrome c_2 between the reaction center-associated and the reaction center-dissociated state.

4. The half-reduction potential of cytochrome c_2 is pH independent (from pH 5 to 9) whether measured in the free state or when associated with the chromatophore membrane. This shows that a proton is not involved in the oxidation and reduction of cytochrome c_2 in the physiological pH range.

5. The kinetics of the intact reaction center, P, and cytochrome c_2 units in chromatophores and whole cells of *Rhodopseudomonas spheroides* are described. The

two cytochrome c_2 molecules which are associated with one P exhibit similar oxidation kinetics; both are biphasic. The fast phase is estimated to be 20–40 μs in half time. The second slower phase is variable depending on the ionic strength of the medium used for the preparation of the chromatophores; it varies from 0.3 to 8 ms.

6. An equilibrium for cytochrome c_2 and the reaction center and/or the membrane is suggested. The two states of the equilibrium are described by a population of cytochrome c_2 functionally "close" to the P^+, and a population functionally distant from the P^+, which might be physically off the binding site, or orientated unfavorably to the P^+. The former population is identified by the 20–40 μs oxidation rate; the latter variable and somewhat slower oxidation (0.3–8 ms) is that whose rate is governed by the diffusional processes of the equilibrium which brings the cytochrome to the close position.

7. Carotenoid bandshifts are kinetically compatible (a) with the P oxidation which is too fast to measure, and (b) with the two phases of cytochrome c_2 oxidation. These are interpreted as arising from local electric field alterations occurring during the electron transfer events in the reaction center and cytochrome c_2.

INTRODUCTION

This report is a basic investigation into quantitative aspects of the cytochrome and reaction center content of photosynthetically grown *Rhodopseudomonas spheroides*. The information is needed before further progress can be made into problems of electron and proton transfer mechanisms and energy conservation in *Rps. spheroides*. The report focuses on the determination of functional in situ reduced minus oxidized extinction coefficients of the cytochrome c_2* and reaction center bacteriochlorophyll protein** in the chromatophore, and also on the kinetic relationships of these two components. These determinations are intended to permit a more accurate electron counting in multiple single-turnover kinetic analyses (cf. refs 1 and 2), and also a better assessment of the total and kinetically viable amounts of cytochromes present in the chromatophore per reaction center protein; damage of electron transfer steps has been acknowledged as being a real possibility for years [3, 5]. Finally, we have determined the pH dependence of the half-reduction potentials of the cytochrome c_2 to find out if it can function as a proton carrier in addition to its well-known electron carrying capabilities.

* We have previously [1, 2] called the *c*-type cytochrome of *Rps. spheroides* cytochrome c_{295} (the subscript indicating the measured half-reduction potential) because we were not sure that this was the chromatophore-associated form of the familiar buffer-soluble cytochrome c_2. Recent evidence presented here and elsewhere [6] indicates that this is the case and so we shall drop the previously used cytochrome c_{295} nomenclature unless it is used parenthetically in combination with the c_2 designation to indicate the c_2 half-reduction potential when in the membrane-associated state. This serves to identify the membrane-associated cytochrome c_2 as a distinct electrochemical species from that in the isolated state where its half-reduction potential is 60 mV more positive (work presented here; see also refs 6 and 8). Current evidence [2, 6] suggests cytochrome c_2 resides on the inner side of the chromatophore membrane.

** The reaction center is often abbreviated to P, followed by the wavelength of the absorption band if germain to the discussion; e.g. P870, P605, etc. From its hydrophobic nature, the reaction center protein is considered to reside within the hydrophobic core of the membrane matrix (see refs 2, 6 and 8).

MATERIALS AND METHODS

Preparative procedures

Rps spheroides strain Ga. (green mutant) was grown anaerobically in the light in 1 l bottles containing the mineral medium which has been previously described [6], but with succinate as sole carbon source. The bottles were approx. 3 inches away from a row of 100 W tungsten lamps spaced 6 inches apart. To ensure that all cells were as active as possible, the bacteria were transferred in liquid culture serially every day (10 % inoculum) for at least 3 days before the batch used for experiments; the growth time for the experimental batch was about 20 h. Growth temperature was 30 ± 2 °C. Chromatophores were prepared immediately after harvesting and cell washing, and were used for up to a week after preparation, during which time they were stored in ice but without freezing. Chromatophores were prepared by the alumina grinding method as described by Baltscheffsky [9] or by the French press method, passing the cells through the press once at a pressure of 20 000 lb/inch^2. The chromatophores were isolated by the usual differential centrifugation procedures and thoroughly washed twice. After the final suspension of the chromatophores to a concentration of 1–3 mM bacteriochlorophyll, they were centrifuged twice at $25\,000 \times g$ to remove any aggregated fragments. This yielded an optically clear preparation which was of fairly uniform composition as indicated by sucrose density gradient centrifugation. The buffer medium used throughout preparation was normally 100 mM KCl/20 mM morpholinopropane sulfonic acid, pH 7.2. In certain preparations in which a low ionic strength medium was used, the buffer composition was 100 mM sucrose/20 mM Tris/morpholinopropane sulfonic acid.

Redox titrations

Determination of the half-reduction potentials of cytochrome c_2 were done as previously described [1, 10]. The following buffers were used over the pH 6–9 range examined, as follows: pH 5.5–6.5, 50 mM morpholinoethane sulfonate; pH 6.5–7.5, 50 mM morpholinopropane sulfonate; pH 7.5–9.0, 50 mM Tris · HCl or glycyl glycine. The following redox mediators ("redox buffers") were present for the cytochrome c_2 titrations: $> 100\ \mu$M potassium ferro/ferricyanide and 50 μM diaminodurol.

Kinetic studies

Redox potentiometry in combination with spectrometry and flash activation was as previously described [1, 10]. For activation of chromatophores, the xenon flash lamp was used; it had a pulse of 4–6 μs full width at half height duration and was 95 % complete by 10 μs; it was sufficient to 85–95 % saturate the chromatophore reactions studied. The precise saturation level was determined for each preparation and chromatophore concentration used to facilitate calculations. Other flash activation was provided by a 20-ns Q-switched laser pulse. The laser was bright enough to produce nearly 100 % conversion of $P \rightarrow P^+$ with or without the aid of the antenna bacteriochlorophyll network.

Estimation of the reduced minus oxidized extinction coefficients ($\Delta\varepsilon^{red\text{-}ox}$) of reaction center at 605 nm (P605) and the α-band of cytochrome c_2

(a) *P605*. P oxidation-reduction was measured spectrophotometrically using

a fast dual-wavelength instrument set at 605 nm. The changes were assayed either on a single beam mode at 605 nm or on a double beam mode at 605 minus 540 nm. The absorbance changes occurring during oxidation-reduction were recorded following a saturating 20-ns Q-switched ruby laser pulse. Chromatophores were suspended in the anaerobic redox cuvette previously described [10] in 100 mM KCl, 20 mM morpholinopropane sulphonate, pH 7.2, at a redox potential (E_h) of 380 mV. The redox mediator was 100 μM ferri/ferrocyanide. This high redox potential was chosen because it is sufficiently electropositive to render the cytochrome c_2 more than 90 % oxidized (E_m+295 mV, $n = 1$; see ref. 1 and Fig. 2a). Thus, at this potential, flash-oxidized reaction center, designated P^+, is not rapidly re-reduced by electron donation from cytochrome c_2, and so the absorption change measurement of the P^+ formation is somewhat easier and more reliable (compare P changes at 375 mV with those at 200 mV in Fig. 1). However, since at potentials in the 380 mV region approx. 10 % of the P is chemically oxidized (its E_m is +440 mV, $n = 1$), this has to be taken into account in calculations of total P content.

The extent of absorbance change associated with the flash-induced formation of P^+ was first measured in the intact chromatophore. After this, detergent was added to destroy the chromatophore membrane system and effect the "release" of the reaction center from the chromatophore. Two detergents were used: lauryl dimethylamine-*N*-oxide (a generous gift from Onyx Chemical Co., Jersey City, N.J.) and Triton X-100; the former was added to 1 % (v/v) and the latter to 2 % (v/v)*. Following the addition of detergent, the laser-induced P^+ absorbance change was measured again, the dilution of the suspension resulting from the detergent addition being taken into account so that any $\Delta\varepsilon^{red\text{-}ox}$ differences between P in detergent and P in the intact chromatophore could be calculated. The $\Delta\varepsilon^{red\text{-}ox}$ of P in the detergent was measured using the method of Straley et al. [11]. This relates the absorbance change of P^+ reduction to that of coupled pure mammalian ferrocytochrome *c* oxidation; the $\Delta\varepsilon^{red\text{-}ox}$ at 550 minus 540 nm for cytochrome *c* was taken to be 20.4 $mM^{-1} \cdot cm^{-1}$ (see refs 10 and 11). An excess (over the P content) of the horse heart cytochrome *c* (Sigma Type VI) was then added and the ambient redox state of the system was lowered to about 200 mV to achieve an approx. 80 % level of reduction of the mammalian cytochrome *c* (E_m approx. 280 mV under these conditions; see ref. 13). This reduction was effected with minute additions of dithionite or ascorbate. In the high detergent solution, the flash-oxidized reaction center protein, P^+, is rapidly and completely re-reduced on a second-order basis by the added ferrocytochrome *c* (see ref. 12). Further details are given in Fig. 1 which typifies the experimental quality in showing the flash-induced P and cytochrome *c* changes after the addition of the detergent. The extent of cytochrome *c* oxidation measured at 550 minus 540 nm in relation to absor-

* Two functional effects are revealed on addition of detergent which indicate the "release" of the reaction center bacteriochlorophyll: (a) The reaction center protein is "uncoupled" from efficient light energy transfer from the antenna bacteriochlorophyll proteins as indicated by the requirement for much higher light intensities to effect above 95 % saturation of P^+ formation after addition of detergent; (b) Oxidation of endogenous ferrocytochrome c_2 normally contained within the chromatophore [2, 6, 8] is drastically altered on addition of detergent, becoming very slow (half time tens of milliseconds) and incomplete. This effect is appropriate for the reaction center/cytochrome c_2 reaction becoming second order [12] and for the resultant very low concentrations of the now "free" cytochrome c_2. The reason for use of detergent was to render free access to the reaction center of subsequently added mammalian cytochrome *c*.

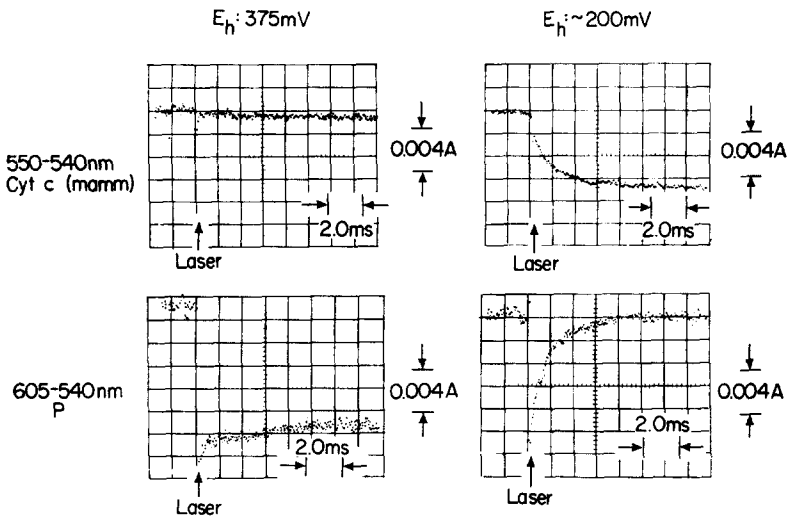

Fig. 1. Laser induced absorbance changes due to reaction center and cytochrome c_2 oxidation and reduction in detergent-treated chromatophores of *Rps. spheroides*. The total bacteriochlorophyll was 27.9 μM with 3.5 μM horse heart cytochrome *c*. Reaction center (P605) was measured at 605–540 nm; cytochrome *c* was measured at 550–540 nm. Studies at a redox potential (E_h) of 375 mV were done (a) to obtain a more reliable flash-induced P605 oxidation absorbance change (lower left) since at this potential it is not rapidly re-reduced by cytochrome *c* because this is already > 95 % oxidised before the flash; and, (b) to ascertain that the use of 550–540 nm for measurement of cytochrome *c*-linked absorbance changes is free of interfering changes from other sources (upper left). Ferri/ferrocyanide (100 μM) gave reliable E_h values at the high potentials both in the presence and absence of detergent. For measurement of redox changes in the 250–200 mV range where cytochrome *c* is mainly reduced, 2–300 μM more ferrocyanide was added and the potential taken down with very small additions of sodium dithionite or ascorbate until complete and suitably rapid re-reduction by cytochrome *c* of P605 was accomplished (bottom right) with concomittant oxidation of cytochrome *c* (upper right). The traces shown are the computer averaged record of four scans. The instrumental response time was 15 μs (90 % of maximum).

bance changes observed for concommitant P^+ reduction (one cytochrome *c* oxidized per P^+ reduced) permits the calculation of the $\Delta\varepsilon^{red\text{-}ox}$ for P at 605 nm (detergent present) using the above $\Delta\varepsilon^{red\text{-}ox}$ of the mammalian cytochrome *c* as a standard. Thus, the $\Delta\varepsilon^{red\text{-}ox}$ for P obtained via the cytochrome *c* method, which requires the presence of detergent, is for the "free" reaction center as determined by Straley et al. [11] and Prince et al. [12]. The $\Delta\varepsilon^{red\text{-}ox}$ values presented here for P605 when in the chromatophore were obtained by comparison of the absorbance change of P605 oxidation after detergent addition with that in the intact chromatophore before detergent addition.

(b) *Cytochrome* c_2. The addition of excess pure *Rps. spheroides* ferrocytochrome c_2 to detergent-treated chromatophores instead of the mammalian cytochrome *c* allowed the determination of the $\Delta\varepsilon^{red\text{-}ox}$ at 550–540 nm for cytochrome c_2 by comparing its laser-induced oxidation absorbance change with that observed for cytochrome *c* under the same conditions. Controls for any effects of lauryl dimethylamine-*N*-oxide or Triton X-100 on the cytochrome *c* or c_2 α-band extinction coefficients showed no detectable alterations, suggesting that their $\Delta\varepsilon^{red\text{-}ox}$ values in detergent are the same as in the chromatophore.

Estimation of cytochrome c_2 and reaction center bacteriochlorophyll content in chromatophores

The absorbance change measured at 550 minus 540 nm over the redox potential range +420 to +200 mV (100 μM ferro/ferricyanide and 10 μM diaminodurol present as redox mediators) was taken as cytochrome c_2. The reaction center content was assessed as described above, using the laser or a 5 μs xenon flash. The molar quantities were calculated using the $\Delta\varepsilon^{red\text{-}ox}$ values determined as described above, and reported in the results section. Total bacteriochlorophyll (antenna and reaction center) was estimated from the in situ absolute absorbance at 865 nm using an extinction coefficient of 95 $mM^{-1} \cdot cm^{-1}$ after Clayton [14].

RESULTS AND DISCUSSION

Difference extinction coefficients

Table I lists the reduced minus oxidized extinction coefficients ($\Delta\varepsilon^{red\text{-}ox}$) obtained for reaction center P605 in the isolated (detergent present) state, or when associated with the chromatophore membrane. The numbers represent the average of at least three separate determinations using different chromatophores and detergent (see Materials and Methods). The individual values varied less than $\pm 5\%$ of the mean. The values in the isolated state are in reasonable agreement with the value for P605 (single beam) reported by Prince et al. [12] using the blue-green mutant of *Rps. spheroides* (R26); their value was 26.4 $mM^{-1} \cdot cm^{-1}$. A significant, approx. 20 % smaller value is apparent for the P605 $\Delta\varepsilon^{red\text{-}ox}$ when associated with the membrane (hypochromism). The same result is observed whether the flash-induced absorbance decrease at 605 nm is measured on the single beam or by dual wavelength analysis at 605 minus 540 nm. The increase in $\Delta\varepsilon^{red\text{-}ox}$ on addition of detergent can neither be ascribed to light scattering effects, nor to a decrease in the actinic flash saturation efficiency due to the abolition of antenna bacteriochlorophyll and carotenoid energy transfer coupling, because both these effects, were they indeed relevant, would have decreased the apparent $\Delta\varepsilon^{red\text{-}ox}$ on addition of detergent. Neither can the $\Delta\varepsilon^{red\text{-}ox}$ increase be the result of an absorption maximum shift of the P605; again, such an effect could only lead to a decrease in the absorption change. Although no conclusive reason for the effect is apparent (it could perhaps be the electronic uncoupling of the reaction center from its antenna bacteriochlorophylls), the results are important from an experimental standpoint.

Thus, the experiments demonstrate that the extinction coefficients for the reaction center obtained in the detergent-isolated state cannot be used for the reaction

TABLE I

EXTINCTION COEFFICIENTS OF *RPS. SPHEROIDES* Ga

	$\Delta\varepsilon^{red-ox}$ ($mM^{-1} \cdot cm^{-1}$)	
	Detergent	Chromatophore
P605 single beam	24.6	19.5
P605–540 nm	37.2	29.8
Cytochrome c_2 551–540	19.0	19.0

Cytochrome c (horse heart) used as standard: $\Delta\varepsilon$ 551–540 taken as 20.4.

center when in the chromatophore membrane environment. The values for the water-soluble cytochrome c_2 seem unaltered by the presence of detergent.

Reaction center and cytochrome c_2 content of Rps. spheroides Ga chromatophores

Cells grown as described in Materials and Methods, using 100 mM KCl/20 mM morpholinopropane sulfonate, pH 7.2, throughout as washing and suspending medium and the French press method for cell disruption, yield chromatophores with cytochrome c_2 and P contents shown in row A in Table II. The ratio of antenna bacteriochlorophyll to cytochrome c_2 is about 50 : 1 (row A; first column), and the

TABLE II

CYTOCHROME c_2 AND REACTION CENTER CONTENT OF *RPS. SPHEROIDES* Ga

Mode of chromatophore preparation*	Total bacteriochlorophyll molecules per unit		Total c_2/P**	Single flash-induced c_2 oxidized/P***	Total kinetically viable c_2/P†
	c_2	P			
(A) French press/KCl/ morpholinopropane sulfonic acid	53††	98	1.84	0.71	1.29
(B) Alumina/KCl/ morpholinopropane sulfonic acid	123	110	0.89	0.41	0.75
(C) Alumina/sucrose/ morpholinopropane sulfonic acid	69	129	1.87	0.40	0.80

* This gives the method of cell breakage and the medium in which the washing and preparative procedures were handled. The pH in all cases was 7.2.

** Obtained by dividing second column (P) by first column (c_2) and gives the ratio of cytochrome c_2 hemes per reaction center bacteriochlorophyll (P).

*** This column gives the ratio of ferrocytochrome c_2 oxidized per total P following a single-turnover laser or xenon flash on the chromatophore poised at a redox potential of 200 mV. This potential is chosen because it is sufficient to establish the cytochrome c_2 over 90 % reduced before flash activation. The test is done in the presence of antimycin A which inhibits the re-reduction of ferricytochrome c_2 and hence makes the measured level of cytochrome c_2 oxidized more reliable. It can be determined by measurement of cytochrome c_2 oxidation extent following a single saturating flash and relating this to the P content or by measuring that extent of reduction of flash-induced P^+ which is kinetically compatible with the rate of cytochrome c_2 oxidation (see Fig. 4). This test is an important indicator of damage incurred on the redox system during the disruptive process of chromatophore preparation. This column multiplied by 100 provides the percent of reaction centers which have functionally associated with cytochrome c_2.

† This is a second test of preparation integrity which gives the amount of cytochrome c_2 that is capable of rapid interaction with P. This is assayed for by multiple flash activating chromatophores at an E_h of 200–220 mV in the presence of antimycin A. Three near-saturating flashes spaced several milliseconds apart are used; 90 % of the maximum is achieved by the second flash and little further oxidation over that established by three flashes is encountered with say eight flashes (see Fig. 6).

†† Data in the table are the average of at least five preparations from different growths of bacteria. Individual values from each preparation fall within 20 % of the mean.

antenna bacteriochlorophyll to P ratio is about 100 : 1 (second column; row A). Therefore, the total number of cytochrome c_2 hemes present in the chromatophore per P approaches 2 : 1 (third column, row A).

However, the expected oxidation of one molecule of ferrocytochrome c_2 per single-turnover flash/generated P^+ (Table II; row A, fourth column) is not realized. The single flash-oxidized c_2/P ratio is always less than one, indicating that some reaction centers do not have an associated cytochrome c_2. We consider this to be a negative effect incurred during the process of cellular disruption, resulting in the dissociation of intact P-c_2 units. In French press preparations thus far, as shown by the fourth column, 70 % of the P population are viably associated with cytochrome c_2; approx. 30 % of the P population are therefore not sufficiently attached to cytochrome c_2 to support normal, rapid, micro to millisecond electron transfer reaction rates ([1, 2]; for relevant P and c_2 kinetic traces, see Fig. 4). Row A, fifth column, however, shows that practically all of the reaction centers found with one associated cytochrome c_2 are capable of rapid oxidation of not only one cytochrome c_2, but of two cytochrome c_2 molecules (for multipulse kinetics, see Fig. 6). The amount of this kinetically viable cytochrome c_2 for the French press preparations shown yield a c_2/P ratio of about 1.3. Since the total c_2/P ratio is nearer 2.0, this reveals some cytochrome c_2 in the chromatophore which is not very effectively oxidizable by P^+. In fact, in the French press preparations, the amount of cytochrome c_2 which is not effectively associated with P is of the same order (approx. 30 %) as the proportion of P which has no associated cytochrome c_2. Thus, we can identify both dissociation products of damaged P-c_2 units in these preparations. It is pertinent that Prince et al. [6], using similar preparative techniques (French press, etc.) have shown that the ratio of total bacteriochlorophyll per cytochrome c_2 is the same in the whole cell as in the chromatophore, because cell breakage occurs very rapidly and only once, so that there is no time for the cytochrome to escape. The cytochrome remaining in the supernatant originates only from partially ruptured cells. This indicates that no loss of the cytochrome c_2 occurs during preparation in this way, and so the values we present for the French press chromatophores probably represent the situation in the intact cell.

We have compared the French press and alumina grinding methods of chromatophore preparation. In our hands, the French press method is superior to the alumina grinding method for the reactions studied here. Two quantitative effects are clear: (1) In the alumina ground preparations the ratio of cytochrome c_2 oxidized per P per single flash (row B, fourth column) is only 0.4, indicating that as much as 60 % of the reaction centers are devoid of attached cytochrome c_2. However, as was found with the 70 % viable P-c_2 units of the French press method, those 40 % intact P-c_2 units do in fact possess two cytochrome c_2 hemes (row B, fifth column), as was clearly shown previously [2]. (2) In addition to the extensive damage to the P-c_2 units during the grinding procedure, there is a net loss of cytochrome c_2 from the chromatophore; total cytochrome c_2/P ratios in the chromatophores are often below one (row B; third column). Thus, once the cytochrome c_2 has become dissociated from the P, it is evidently able physically to escape from the chromatophore during alumina grinding. The cytochrome c_2 loss incurred during the grinding procedure can be largely eliminated by using media of lower ionic strength which would be expected to promote electrostatic binding of the cytochrome c_2 within the chromatophore (row C). How-

ever, although this modification brings the total c_2/P ratio up to approach 2.0, it does not help increase the number of functional P-c_2 units, which remain at 40 %.

Half-reduction potentials of the cytochrome c_2 complement of chromatophores

If there are two populations of cytochrome c_2 as indicated in the last section, and if (a) the half-reduction potential of "free", P-dissociated cytochrome c_2 is measurably different from the P-associated cytochrome c_2, and (b) the exchange rate of cytochrome c_2 between these two states is slow relative to the time taken to perform the titration (usually approx. 20 min for reductive or oxidative phases), then both populations will be resolved in equilibrium redox titrations as two distinct electrochemical species. The fact that our earlier redox titration of cytochrome c_2 [1] did not quite fit an $n = 1$ Nernst curve, together with the above findings, prompted another look at this titration.

Fig. 2a shows redox titrations done on isolated cytochrome c_2 and also in the chromatophore. The half-reduction potential in the isolated state is 345 mV. In the membrane-associated state, the points from two separate chromatophore preparations give the same 295 mV half-reduction potentials as we have previously reported, but at higher potentials careful analysis reveals a second electrochemical species which is just about resolvable. The half-reduction potential of this component can be estimated to be in the +350 mV region and to contribute roughly 30 % of the total cytochrome c_2 complement. This finding is consistent, therefore, with the presence in the chromatophore of P-dissociated cytochrome c_2, which on becoming "free", finds itself in a

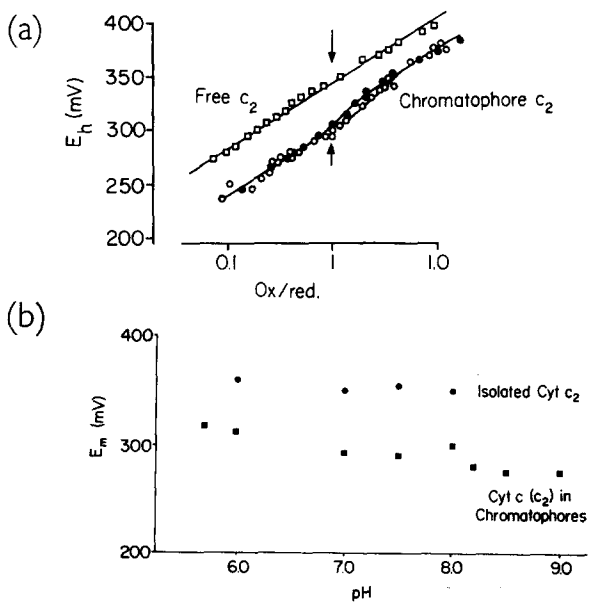

Fig. 2. (a) Redox titration of cytochrome c_2 in the isolated and chromatophore-associated state. Cytochrome c_2 in the isolated state (1.5 μM) or in the chromatophore (approx. 100 μM bacteriochlorophyll) (○ and ● are points from different preparations) was titrated in 100 mM KCl/20 mM morpholinopropane sulfonic acid, pH 7.2, using 550−540 nm as the measuring wavelengths. For further details see Materials and Methods. (b) pH dependence on the half-reduction potentials of isolated cytochrome c_2 and associated with the chromatophore. Details are given in (a) and Materials and Methods.

similar physical environment to the aqueous-solvated, isolated cytochrome c_2. This electrochemical species of "free" cytochrome c_2 (c_{345}) is clearly distinct from that with the 295 mV half-reduction potential (c_{295}) which has previously [1, 2] been firmly identified as capable of micro-millisecond rates of electron transfer to P^+. Recent independent immunological evidence [6] has also demonstrated that the c_{295} and c_{345} are both forms of the same protein, cytochrome c_2. The approximately 50 mV negative shift assumed on changing from the "free" aqueous-solvated state to the membrane-associated state is a similar finding to that of mammalian cytochrome c [13, 15] in which it was concluded [13] and later demonstrated [16] that the oxidized form was bound to the membrane some 7 or 8 times tighter than its reduced form. It may be suggested, therefore, that once the cytochrome c_2 has become dissociated from the P during disruption, it represents a separate electrochemical species and does not exchange with that of the P-associated form. This does not mean that electron transfer between the two states cannot occur. In fact, under sustained flashing or continuous illumination, P-dissociated cytochrome c_2 can become oxidized, but only slowly on a time scale of seconds, possibly by reacting with the oxidized P-associated cytochrome c_2 molecules.

pH dependence of the half-reduction potentials of the cytochrome c_2 complement

Fig. 2b shows that the redox reaction cytochrome c_2, whether isolated or contained in the chromatophore does not involve a proton. The half-reduction potential is essentially pH independent over a 1000-fold change in H^+ concentration. The amount observed is < -10 mV/pH unit compared with the -60 mV/pH unit which would be expected if a proton was exchanged per single electron transfer. Thus, we may anticipate an electrical charge change on the cytochrome undergoing oxidation-reduction which is not countered by proton exchange.

The cytochrome c_2 oxidation and P^+ reduction electron transfer reaction

Single-turnover activation. Fig. 3 shows familiar flash-activated, kinetically derived spectra in the α-band region of *Rps. spheroides* chromatophores. The upper spectrum was taken following single xenon flash activation of chromatophores poised at a redox potential of 390 mV. This potential is sufficiently positive that more than 90 % of the cytochrome c_2 (c_{295}) is chemically oxidized, so that cytochrome c_2 oxidation following a flash will obviously not be evident. However, at 390 mV, the P is still about 90 % chemically reduced (E_m of P/P^+ is 450 mV [1]) before activation. As a consequence, following flash activation at 390 mV, we see P oxidation (complete within the 20 μs chosen as the rise time (90 %) of the instrument) and only a little re-reduction over the following 4 ms. In fact, under these conditions, it takes hundreds of milliseconds for the electron to relax back completely to P^+ via the cyclic electron transfer system. The kinetic traces on the left of Fig. 4 serve to demonstrate the expected kinetics and absorbance changes at the cytochrome c_2 and P measuring wavelengths at the high potentials.

At a redox potential of 240 mV, the cytochrome c_2 (c_{295}) is about 90 % reduced before activation. At this potential we see, from the lower spectrum of Fig. 3 and the kinetics on the right hand side of Fig. 4, that 2 ms after the flash, the course of cytochrome c_2 oxidation is almost complete and that the flash-generated P^+ has become about 65 % reduced. As already discussed, the remaining 35 % represents P^+

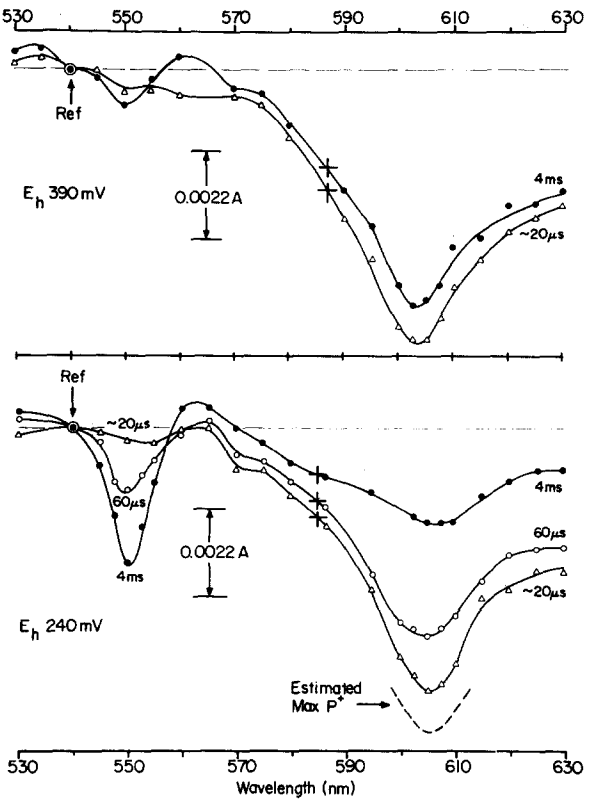

Fig. 3. Flash-induced spectra of *Rps. spheroides* Ga chromatophores. Chromatophores (bacteriochlorophyll 22.6 μM) were poised at a redox potential of 390 mV (upper spectra) using potassium ferri/ferrocyanide (approx. 100 μM) as mediators, or at 240 mV (lower spectra) using diaminodurine (3 μM) and phenazine methosulfate (3 μM) as mediators. The absorbance changes (a downward movement is an absorbance decrease) were measured on the dual wavelength spectrophotometer with 540 nm as the reference. The 10–90 % instrumental rise time was 20 μs. The absorbance changes taken at the indicated times after the flash were taken from the average of 64 kinetic recordings, the quality of which is shown in Fig. 4. 2 μM antimycin A was present to slow down the re-reduction rates of cytochrome c_2 to obtain clearer extents of cytochrome c_2 oxidation. The cross placed on all the spectra at 587 nm is the approximate isosbestic point observed in *Rps. spheroides*. The "estimated maximum P^+" level indicated on the lower spectrum is calculated from the flash-induced P^+ extent at 390 mV. The full extent of P^+ formed at 240 mV is missed due to the limiting 20 μs rise time. The 60-μs spectrum is that which exists after the fast phase of P^+ reduction and cytochrome c_2 oxidation is complete. The 4-ms spectrum is after the slow phase of cytochrome c_2 oxidation and concommitant P^+ reduction. The remaining P^+ after 4 ms is considered to represent damaged P-c_2 units.

of damaged P-c_2 units and goes reduced; partly in the tens, and partly in the hundreds of millisecond time range.

Further examination of Fig. 4 reveals that the character of both cytochrome c_2 oxidation and P^+ reduction within the 2-ms period is biphasic and that the time-course of each event is appropriately the same. The initial fast phase of the cytochrome c_2 oxidation and P^+ reduction is estimated to be more or less complete in 80 μs, with a half-time of 20–30 μs. Thus far, no accurate work has been done on this first phase in the chromatophore, although similar half-times for the rapid phase of cytochrome

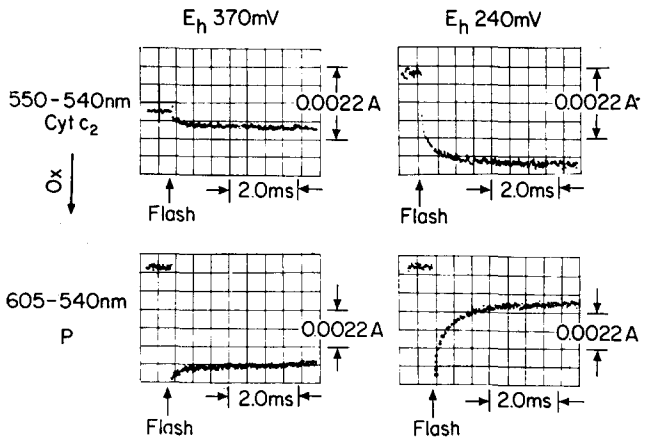

Fig. 4. Kinetics of flash-induced P and cytochrome c_2 oxidation-reduction. Conditions and details are given in Fig. 3.

c_2 oxidation in *Rps. spheroides* cells have been reported [17]. The subsequent slower phase of cytochrome c_2 as shown in Fig. 4 has a half-time of 370 μs and, as is shown in the semilog plot of Fig. 5(a), is first order and very similar to the second phase of P^+ reduction.

The second, slower phase of cytochrome c_2 oxidation exhibits an atypical and therefore important property. The rate of the second phase, in fact, is variable from one chromatophore preparation to another, without any detectable effects on the first phase. Pronounced slowing of the second phase can be induced by preparing the chromatophores in a medium of low ionic strength, with or without sucrose instead of the usual KCl. The reason for the slower rates obtained in low ionic strength is not important for the point being made here; a possible reason for the effect will be discussed later. The important point established in the several plots of kinetics from different chromatophore preparations shown in Fig. 5 is that whatever rate of cytochrome c_2 oxidation is encountered, P^+ reduction is always the same, a mandatory property for the direct coupling of cytochrome c_2 and P in electron transfer.

A second important correlation is that the ratio of the absorbance changes for the cytochrome c_2 oxidation per unit P^+ reduction is proportional to the ratio of the reduced minus oxidized extinction coefficients ($\Delta\varepsilon^{red\text{-}ox}$) we determined for cytochrome c_2 and P in the chromatophore (Table I). The extent of absorbance change accompanying P^+ reduction which is kinetically compatible with that of cytochrome c_2 oxidation can be obtained after the contribution from the third (slowest) phase of P^+ reduction (representing that fraction of the P population which is not coupled to cytochrome c_2) has been subtracted. In Fig. 4, using the $\Delta\varepsilon^{red\text{-}ox}$ values for cytochrome c_2 (19 $mM^{-1} \cdot cm^{-1}$) and for P (29.8 $mM^{-1} \cdot cm^{-1}$) at the wavelength pairs used, the amount of cytochrome oxidized ($\Delta A = 0.00275$) is 0.145 μM and that amount of P^+ reduced ($\Delta A = 0.0044$) is 0.148 μM. This good molar correlation of the amount of cytochrome *c* oxidized per P^+ reduced on a single-turnover basis is extended and presented more graphically in the next section for multiple single-turnover activations.

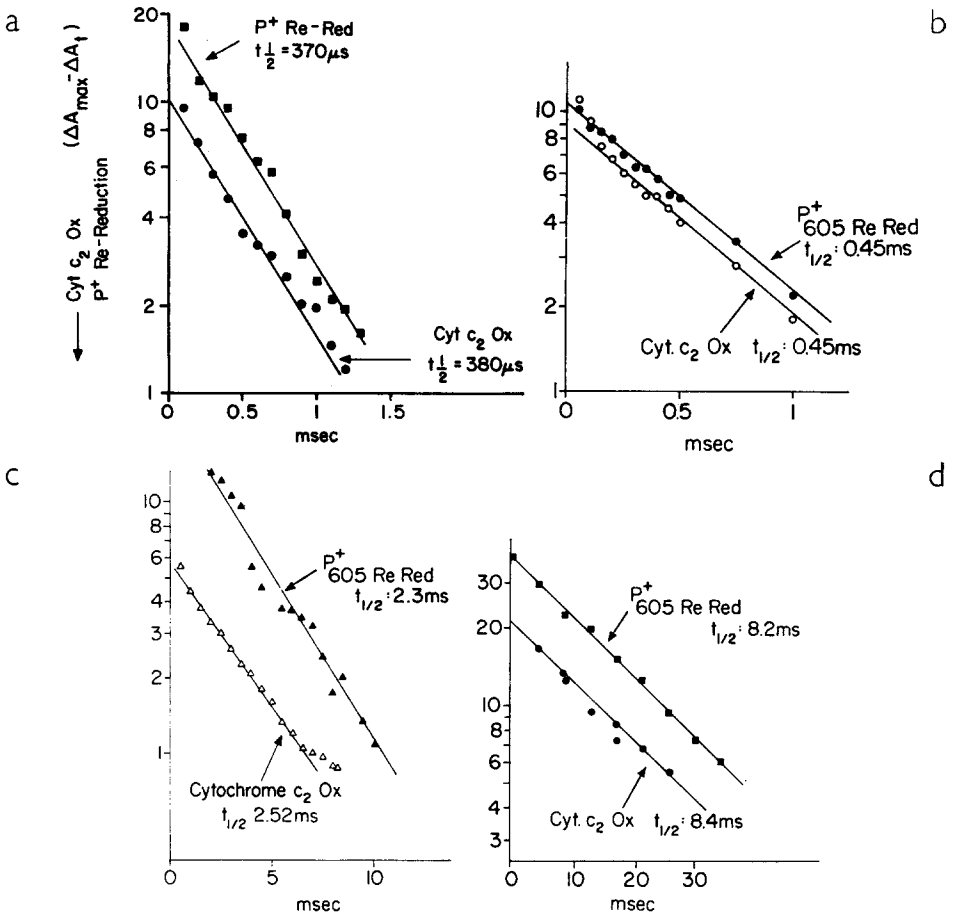

Fig. 5. Semi-log plots of the second phase of cytochrome c_2 oxidation and P^+ reduction. a and b are from chromatophores prepared in 20 mM morpholinopropane sulfonic acid/100 mM KCl, pH 7.2, and c and d are preparations in which the KCl was replaced by sucrose. Conditions as in Fig. 3 with the redox potential at 240 mV before activation.

Multiple single-turnover activation of electron transfer between cytochrome c_2 and P. Our previous work on cytochrome c_2 oxidation [2] following multipulse activation, indicated two distinct turnovers were needed before the cytochrome complement was oxidized. Fig. 6a shows the response of the cytochrome c_2 oxidation following a train of eight flashes spaced 24 ms apart (bottom experimental trace). The first flash is seen to oxidize approximately half of the total rapidly oxidizable cytochrome; after the second flash about 90 % is oxidized and the third flash picks up almost all the remainder. Above the cytochrome c_2 traces in Fig. 6 are shown the P oxidation-reduction reactions under identical conditions. On this slow time-scale we miss the rapid re-reduction of P^+ which is coupled to cytochrome c_2 oxidation; this is because of the 1-ms time constant chosen for the experiment. Thus, in the dark period after the first flash, the earliest measured change is that of the approx. 30 % population of P^+ which remains oxidized for a relatively long period because they had damaged P-c_2

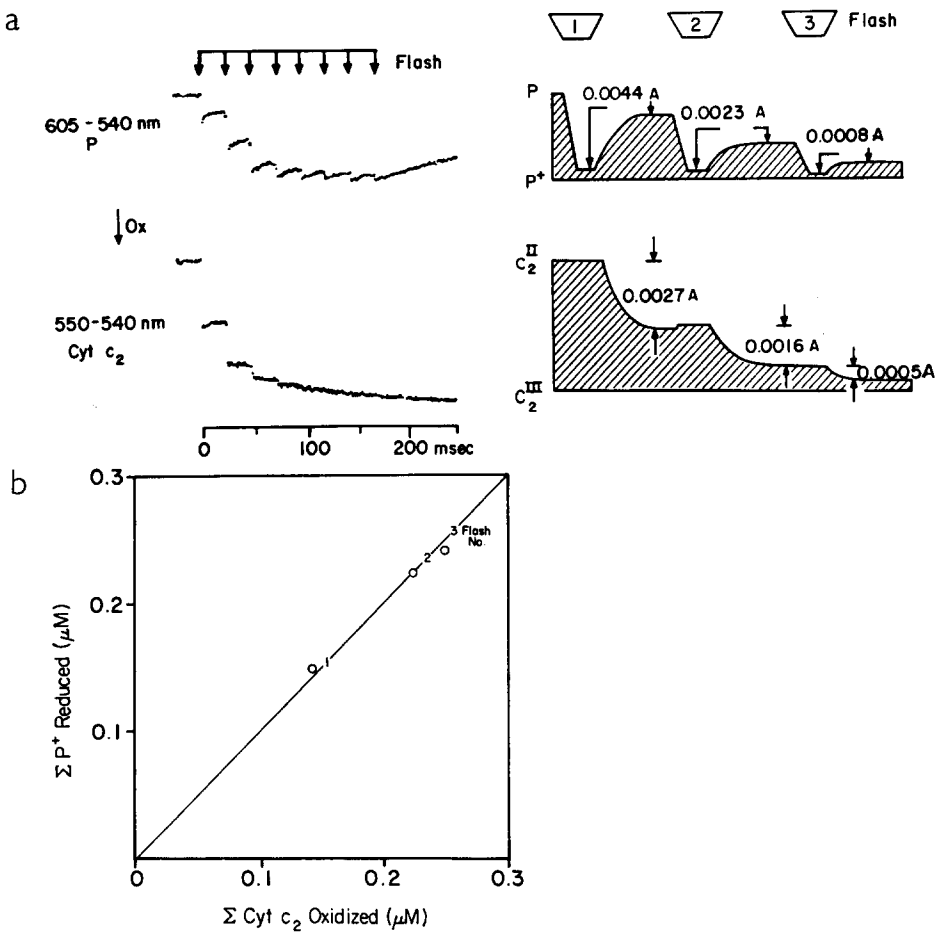

Fig. 6. Multipulsed single-turnover of P and cytochrome c_2. The conditions were as in Fig. 3 with the redox potential at 240 mV before activation. Eight flashes spaced 24 ms apart were used for activation. Part a: The experimental kinetics on the left obtained as an average of four experimental recordings. The 10–90 % rise time was 1.0 ms. On the right is a schematic (showing the experimentally observed absorbance changes for P^+ reduction and cytochrome c_2 oxidation) to make clearer the redox events of P and cytochrome c_2 in the first three flashes; see text for a full description. Part b: A molar correlation curve of the summed amounts of P^+ reduced and cytochrome c_2 oxidized over the first three flashes.

units as shown in Fig. 3. Those of the P^+ population which are reduced by the first cytochrome c_2 heme are then capable of being oxidized again and re-reduced by the second cytochrome c_2 heme. Further flashes render the cytochrome c_2 complement entirely oxidized. Once this situation has been established, P^+ is hardly reduced at all after each successive flash and so, like cytochrome c_2, it achieves a fully oxidized status. On the right of Fig. 6a is a schematic of the events just described to make the redox reactions of the first three turnovers clearer. The absorbance changes for cytochrome c_2 oxidation and P^+ reduction shown on the schematic are taken from analysis of the experimental data on the left. The molar correlation between P^+ reduced

and cytochrome c_2 oxidized is presented graphically in Fig. 6b. Using the extinction coefficients determined above, with the absorbance changes of Fig. 6a, a good correlation is obtained.

Whole cells

Fig. 7 shows multipulse activation of whole cells of *Rps. spheroides*. The same pattern is obtained as was seen in the chromatophores: the first flash elicits 50 % of the total oxidizable cytochrome c_2 complement, the remaining cytochrome being oxidized in the following two flashes. The time-resolved kinetics of the cytochrome c_2 oxidation in the whole cell are shown in the lower part of Fig. 7. The time-course of oxidation is again biphasic like that found with the chromatophores.

These whole cell results add credence to the findings obtained with chromatophores. It may be concluded, therefore, that the two cytochrome c_2 hemes per P relationship, and the biphasic kinetics of the c_2 to P electron transfer are real and functional aspects in the workings of the cell.

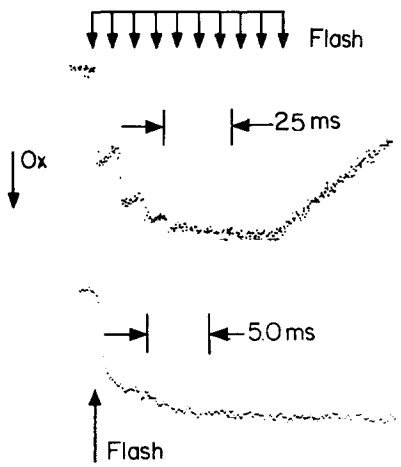

Fig. 7. Multiple and single flash activation of cytochrome c_2 oxidation in whole cells of *Rps. spheroides* Ga. A dilute suspension of whole cells suspended in growth medium under anaerobic conditions was subjected to a train of eight flashes spaced 8 ms apart. The measuring wavelengths were 550–540 nm. The trace shown (upper) is the average of 8 recordings spaced at 10-s intervals. The 10–90 % instrumental rise time was 0.5 ms. The lower trace is the time-course of cytochrome c_2 oxidation. The trace is the average of 64 recordings spaced at 2-s intervals; the 10–90 % instrumental rise time was 150 μs.

Kinetic relationships between the P and the two cytochrome c_2 hemes

In a previous report [2] in which we measured the extent of cytochrome c_2 oxidation following the first and second (and third) flashes as a function of redox potential, it was concluded that each cytochrome c_2 heme should have an equal probability of being oxidized by P^+. A similar relationship has been reported for the two cytochrome c_{555} hemes of *Chromatium* D [10]. Fig. 8 shows the results of the direct kinetic measurement of the oxidation of each cytochrome c_2 heme individually. Fig. 8a shows how the measurement was accomplished. Fig. 8b reveals that the prediction which was based on thermodynamic considerations [6] is true on a mea-

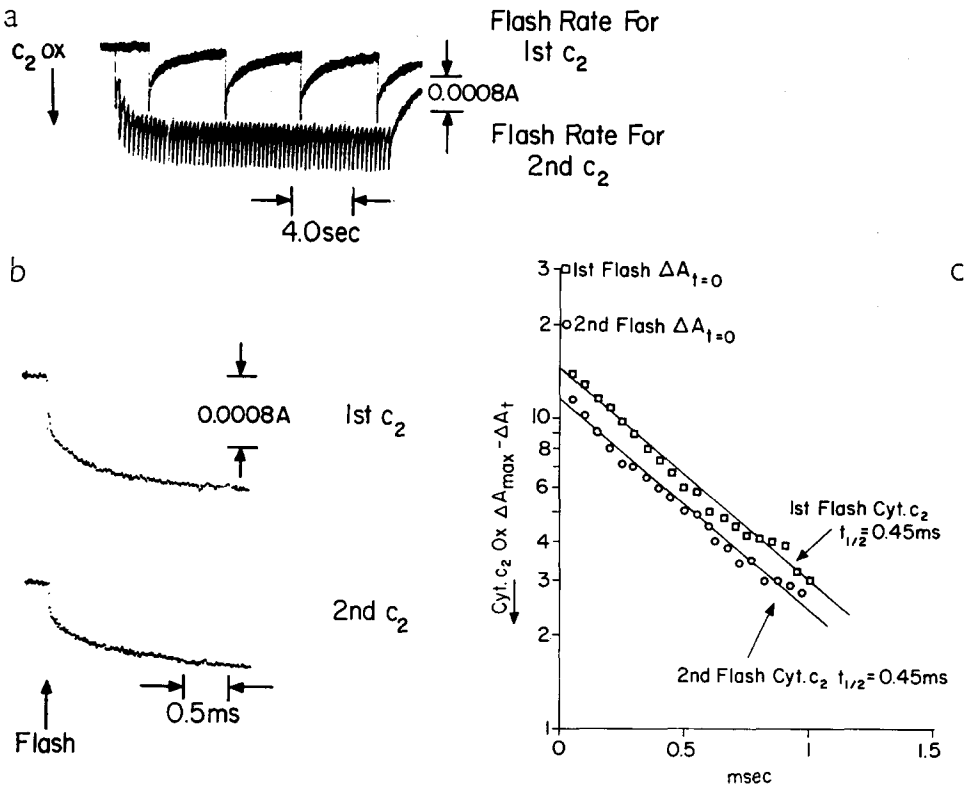

Fig. 8. The individual oxidation kinetics of the two cytochrome c_2 molecules per single P. The conditions were as in Fig. 3. The redox potential was 240 mV. Part a shows the experimental technique used to measure the oxidation rates of the first and second cytochrome c_2 molecules. The measurement of the first cytochrome c_2 oxidation (either of the two cytochromes c_2) was done by averaging 64 single flash recordings; the interval between each flash being sufficient to permit full reduction of the cytochrome c_2 complements. For the second cytochrome c_2 oxidation measurement, the interval between the 64 single flashes was made just short enough to maintain the first cytochrome in an oxidized state. The flash frequency for the first and second cytochrome oxidation was about 10 and 240 per min, respectively. Part b shows the oxidation kinetics of the first and second cytochrome c_2 molecules. Part c is a semilog plot of the second, slower phase of the cytochrome c_2 oxidation.

sured kinetic footing; the time-course of the first cytochrome c_2 oxidation is essentially the same as that of the second. Fig. 8c shows a semilog plot of the second phase of each oxidation. We shall come back to the details of these results later in the discussion.

Correlation of the carotenoid band shift with the redox reactions of cytochrome c_2 and P

In photosynthetic systems, band shifts of the endogenous bulk carotenoids have been considered by several authors to be responses to delocalized transmembrane electric field changes following electron transfer reactions [18–22] assumed to span the width of the membrane. Evidence for this interpretation comes from the induction of the shift by light [18–20] and diffusion potentials by ion gradients in the dark [21, 22]. Recently however, it was demonstrated that carotenoid band shifts could

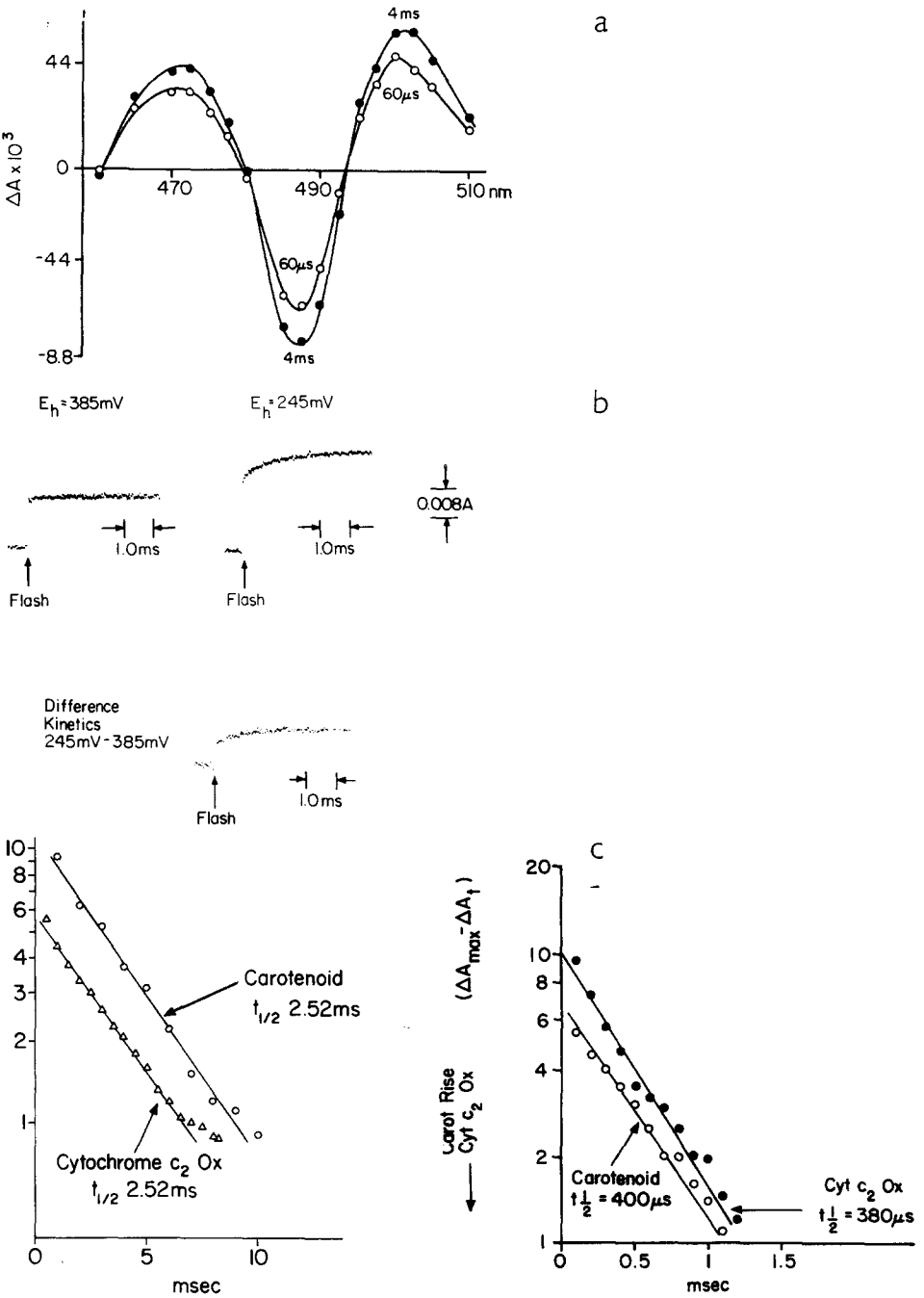

Fig. 9. Time resolved flash-induced spectra and kinetics of the carotenoid band shift in *Rps. spheroides* Ga. chromatophores. The conditions and details were as described in Fig. 3. Part a: The redox potential was 250 mV. The absorbance changes were obtained by dual wavelength measurement using 540 nm as a reference wavelength. The points taken at times shown were obtained from kinetic

also occur at a more localized, intermolecular level within the membrane [10]. This finding has been extended by kinetic studies in the microsecond range [2, 23, 24]. In brief, not only was the band shift identifiable with field alterations induced during the light-driven oxidation of P and reduction of its primary acceptor (photoredoxin; Pd), but also when the cytochrome *c* underwent oxidation. A spherical dielectric model for the chromatophore has been proposed [2] and in such a model the cytochrome would have to be situated on the inner side of the membrane, a suggestion which very recently has gained independent and direct experimental support [6].

Two important experimental facts which were not established in the preliminary kinetic work with *Rps. spheroides* [2] are presented here: (a) The construction of a spectrum of the carotenoid band shift to prove that the cytochrome c_2 oxidation/P^+ reduction-induced absorbance change measured at 490 minus 475 nm is, in fact, part of a legitimate carotenoid band shift, and (b) the proper time resolution of the presumed carotenoid band shift in question; this should follow the time-course of cytochrome c_2 oxidation/P^+ reduction.

Fig. 9a shows that the spectrum of the carotenoid band shift up to 60 μs and then up to 4 ms is indeed part of the carotenoid band shift. Fig. 7B shows the kinetics of the carotenoid band shift at 380 and 240 mV. At the higher potential, the shift emanates only from intra-reaction center protein electron transfer between P^+ and Pd^-, since cytochrome c_2 is already oxidized before the flash. At 240 mV, where cytochrome c_2 is reduced before the flash, the band shift is enhanced as cytochrome c_2 becomes oxidized and P^+ reduced. Subtraction of these two kinetic traces would be expected to reveal the shifts induced by the cytochrome c_2 oxidation and P^+ reduction alone. The difference kinetics shown at the bottom of Fig. 9b are, in fact, strongly biphasic and similar in rate to those of cytochrome c_2 oxidation and P^+ reduction (Fig. 4). Fig. 9c shows semilog plots of the second phase of carotenoid band shift and cytochrome c_2 oxidation in two different preparations. At two widely differing rates of the variable second phase of cytochrome c_2 oxidation (and P^+ reduction), the carotenoid band shift kinetics are the same. This confirms earlier indications that membrane carotenoids are able to register events occurring during cytochrome c_2 oxidation/P^+ reduction electron transfer in *Rps. spheroides*; it is not unrealistic to consider that the effects result from electric field alterations within the membrane dielectric.

Concluding remarks

Figs 10a and 10b summarize the properties of P and cytochrome c_2 in current French press preparations. From the work of Prince et al. [6] it seems clear that little or no cytochrome c_2 is lost from chromatophores prepared in this way, but rather that the soluble cytochrome found in the chromatophore supernatant is liberated from partially ruptured cells which have not broken sufficiently to free their chromato-

traces obtained by averaging 32 recordings; the typical quality of the kinetics are shown in Part B. Part b shows kinetics of the carotenoid band shift in chromatophores poised at 385 and at 245 mV. The measurements were obtained at 490 minus 475 nm. The difference kinetics shown at the bottom are a simple subtraction of the 385 mV kinetics from the 245 mV kinetics performed by the computer. Part c shows the semilog plots of the slow phases of cytochrome c_2 oxidation and the carotenoid band shift. The chromatophores were prepared in KCl (left) or sucrose (right) to obtain the variation in the second phase of cytochrome c_2 oxidation.

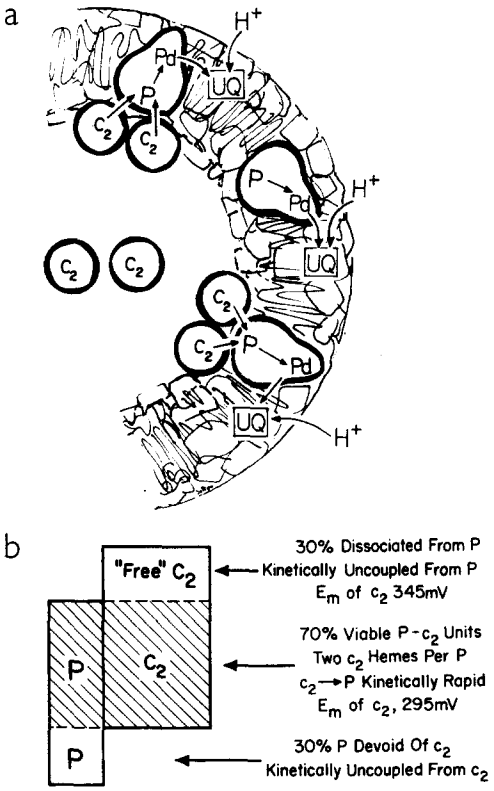

Fig. 10. (a) Schematic representation of a segment of the *Rps. spheroides* chromatophore membrane and interior. The reaction center protein, P, resides in the membrane hydrophobic lipoprotein matrix. Approximately two-thirds (70 %) of the reaction center population are associated with two cytochrome c_2 molecules at the membrane-aqueous interface on the inner side-forming intact P-c_2 units. Approximately one-third of the population of P-c_2 units is damaged during chromatophore preparation; this is symbolized by a tilted reaction center and "free" cytochrome c_2. The reaction center is presented in a "tilted" fashion to indicate that it is not simply inverted through 180° in the membrane, since the addition of up to millimolar quantities of reduced bovine cytochrome *c* did not affect the rate of the re-reduction of the P^+ entities in question. Complementary evidence suggests that in all cases the primary acceptor (designated photoredoxin; Pd) is near the outside of the chromatophore membrane and delivers an electron to ubiquinone. This conclusion for the position of the primary acceptor comes from the fact that, following a single-turnover flash, there is 1.0 H^+ taken up from the external aqueous phase for every P [28]. This should only happen if the electron transferred from each P to the primary acceptor was directed to the outer surface of the chromatophore membrane irrespective of damaged P-c_2 units. (b) Summary of physical properties of the P and cytochrome c_2 in *Rps. spheroides* chromatophore.

phores. The identification of two cytochrome c_2 hemes per P, both on a functional basis as well as on a total basis in the chromatophore and whole cell, permits the conclusion that in the intact, living system the entire cytochrome c_2 population is associated with the reaction center protein, and little or none operates as a free "pool" of redox equivalents. The 2 : 1 cytochrome c_2/P ratio in *Rps. spheroides* is not a unique finding with regard to *c*-type cytochromes and their membrane-bound electron donors or acceptors. For example, two cytochrome c_{555} hemes operate into one P

in the photosynthetic bacterium *Chromatium* D [4] and two cytochrome *c* hemes have been reported per oxidase in the mitochondria of higher organisms (see refs 7 and 25).

Although it has long been accepted in *Rhodospirillum rubrum* and *Rps. spheroides* that cytochrome c_2 is the principal physiological electron donor to P^+, there have always been kinetic anomalies in this transfer [1–3, 5, 25]. These anomalies appear to be adequately accounted for by the recognition of biphasic cytochrome c_2 oxidation and matching biphasic re-reduction properties of P^+, the recognition of two kinetically similar cytochrome c_2 hemes per P (see also ref. 2), and also the realization of the fact that some P-c_2 units are damaged, leaving approx. 30 % of the P^+ population devoid of any cytochrome c_2 and hence not in possession of an immediate physiological electron donor (see legend of Fig. 10).

We interpret the biphasic nature of the cytochrome c_2 oxidation in intact P-c_2 units as indicating the existence of a two-state equilibrium of the cytochrome c_2 and a binding site on the membrane (see also refs 27 and 28). We envisage the equilibrium as being roughly equal populations of cytochrome c_2 hemes orientated favorably (fast phase) or unfavorably (variable slow phase) to the site on the reaction center. The variable slow phase might be the result of a rotational diffusion, as originally suggested by Chance and Williams [29] or a restricted on/off diffusion. That such a proposed equilibrium at the aqueous-membrane interface may play an important role in handling electrons from the membrane-bound electron donor (cytochrome *b* complex) to the membrane-bound reaction center is currently under investigation.

ACKNOWLEDGEMENTS

We are grateful for financial support from the National Science Foundation grant number GB 28125 and to the National Institutes of Health grant GM 12202. P.L.D. acknowledges his Research Career Development Award No. 1.K04. GM. 70771. We are also indebted to Dr Roger Prince for his thoughtful advice in the preparation of this paper.

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