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THE ELECTROSTATIC INTERACTION BETWEEN THE REACTION-CENTER BACTERIOCHLOROPHYLL DERIVED FROM *RHODOPSEUDOMONAS SPHEROIDES* AND MAMMALIAN CYTOCHROME *c* AND ITS EFFECT ON LIGHT-ACTIVATED ELECTRON TRANSPORT*

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SUMMARY

1. By means of Q-switched ruby-laser flash excitation, the photooxidation of P870 in the reaction-center complex isolated from *Rhodopseudomonas spheroides* takes place within 1 μsec. The reduction of photooxidized P870 in the dark follows a first-order kinetics, with a pseudo first-order rate constant of $1.85 \cdot 10^8$ l·mole^{-1}·sec^{-1} and an activation energy of 6 kcal/mole.

2. Through an electrostatic interaction of the bacteriochlorophyll reaction-center complex and mammalian cytochrome *c*, an intimate contact between the two components resulted, and a collision-independent electron-transfer with a halftime of 25 μsec can be attained by laser-flash excitation. The absorbance changes at 870 and 550 nm indicated a good stoichiometry of the reaction. The oxidation of the *c*-type cytochrome in cells of *Rps. spheroides* (R-26 mutant) has a halftime of 12 μsec.

3. The portion of P870 which recovered rapidly was closely related to the mole ratio of cytochrome/P870. Complete recovery with a halftime of 25 μsec occurred when the cytochrome/P870 ratio was above approx. 10. At cytochrome/P870 ratios lower than 10, only the fraction of the reaction-center complex which have cytochromes bound at the active site can recover with the rapid decay time. Ultrafiltration measurements showed that each particle of the reaction-center complex can bind approx. 24 cytochrome molecules.

4. An electro static interaction is expected simply from the large difference between the isoelectric points of cytochrome *c* ($\geqq$ 10) and that of the reaction-center complex (4.1 measured by electro-focusing). The electro static interaction was further evidenced by the effects of pH, ionic strength, and by polylysine displacement of binding sites on the coupled oxidation of ferrocytochrome *c* by P870. From the limiting polylysine concentration giving complete blocking of cytochrome coupling, it was calculated that each reaction-center complex with a particle weight of $6.5 \cdot 10^5$ contained approx. 500 negative charges.

5. Arrhenius plot of the first-order rate constants *vs.* the reciprocal absolute temperature yielded an activation energy of 12 kcal/mole for the cytochrome/P870 reaction, which is presumably the energy needed for cytochrome to achieve the

Abbreviation: PMS, phenazine methosulfate.

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most favorable orientation for the rapid electron transfer. Below the freezing temperature of the sample, the cytochrome reaction appeared to be uncoupled. The temperature dependence is consistent with the effect of viscosity on the reaction rate.

6. Double flash excitations spaced 200 μsec apart showed that at a cytochrome/P870 ratio of 24, the first flash caused maximum oxidation, indicating that all the reaction-center particles have at least one cytochrome attached to the active site. However, only 60% of the particles have a second cytochrome closely attached and capable of undergoing the rapid electron transport.

INTRODUCTION

The functionality of cytochromes in photosynthetic electron transport has been well established and documented (see ref. 1 for a recent review). CHANCE AND NISHIMURA[2] have found that cytochrome 552 in *Chromatium* cells can be oxidized at 77° K with a halftime of 2 msec. A study of the temperature dependence of cytochrome oxidation in *Chromatium* by DEVAULT AND CHANCE[3] subsequently has shown that below 120° K the reaction is temperature independent. More recently KIHARA AND CHANCE[4] have found that low-temperature electron transfer involving *c*-type cytochromes is a general phenomenon among many photosynthetic bacteria, with *Rhodopseudomonas spheroides* and *Rhodospirillum rubrum* being the exceptions. Oxidation of endogenous[5] as well as exogenous *c*-type cytochromes[6,7] occurs with a high quantum yield near unity.

The high quantum efficiency and temperature insensitivity for these cytochrome oxidations indicate that the *c*-type cytochromes react near the reaction-center bacteriochlorophyll and implicate an intimate structural relationship between these two molecules. The first kinetic evidence for the role of the reaction-center bacteriochlorophyll in bacterial electron transport was obtained by PARSON[5] who showed that cytochrome oxidation in *Chromatium* chromatophores was directly coupled to the dark re-reduction of the reaction-center bacteriochlorophyll, P870.

To account for the relatively slow, activationless electron transport involving cytochrome-552 in *Chromatium* cells, DEVAULT AND CHANCE[3] have advanced the 'quantum-mechanical electron-tunnelling' mechanism. Thus, the primary photochemical reaction may be viewed as consisting of the formation of an excited state of chlorophyll by light activation which leads to a charge separation with the formation of a reduced acceptor and oxidized chlorophyll. In the following dark reaction, the cytochrome donates an electron to the oxidized chlorophyll. The electron-tunnelling mechanism assumes matched electronic levels of the donor and acceptor molecules. The activation energy of 3–4 kcal/mole required for the cytochrome oxidation at higher temperatures has been viewed as necessary for small-scale rotational and vibrational diffusion of the heme to achieve a preferred orientation relative to the electron acceptor.

Further studies into the effect of various molecular parameters on the electron-transport reaction between light-activated bacteriochlorophyll and cytochrome affords a better understanding of the reaction mechanism. In this paper we wish to report studies of the interaction of mammalian cytochrome *c* with the bacteriochlorophyll reaction-center complex isolated from the blue-green mutant strain

R-26 of *Rps. spheroides*[6] and the effect of this interaction on the light-activated electron transport. Present data indicate that through an electrostatic interaction between the two reagents, a contact-type electron transport can be activated by light pulses and a transport time nearly half as rapid as that of the endogenous *c*-type cytochrome in intact *Rps. spheroides* cells can be approached.

MATERIALS AND METHODS

The reaction-center complex from *Rps. spheroides* R-26 mutant cells was prepared according to REED AND CLAYTON[8]. Cytochrome *c* (Type III) was purchased from the Sigma Chemical Co. The reaction-center complex prepared by this procedure is devoid of the endogenous cytochrome c_2. Reduced cytochrome *c* was prepared by catalytic hydrogenation and assayed spectrophotometrically using an extinction coefficient of $2.77 \cdot 10^4$ $l \cdot mole^{-1} \cdot cm^{-1}$ at 550 nm. Polylysine of 150000 mol. wt. was purchased from K and K Laboratories. Other chemicals were of reagent grade and were used without further purification.

A Cary 14R spectrophotometer was used for spectral measurements. Steady-state light bleaching of the reaction-center bacteriochlorophyll was examined in the IR-2 mode of this spectrophotometer. The basic apparatus for flash kinetic spectrophotometry was described previously[9]. The absorbance-change transients induced by Q-switched ruby-laser pulses were processed through a Fabri-tek Model 1062 signal averager together with a Model 952 rapid digitizer at a time resolution of 1 μsec. For double-flash excitations, a Suntron-1 xenon flash lamp (Xenon Corp., Medford, Mass.) was used in conjunction with the giant-pulse laser flash. All reaction mixtures were 3 ml in a 1-cm cuvette, and, unless otherwise stated, contained 10 mM Tris buffer (pH 7.5), 0.5 mM ascorbate and 1 μM phenazine methosulfate (PMS), in addition to the P870 preparation and ferrocytochrome *c*. Low-temperature experiments were conducted in a cuvette–Dewar assembly similar to that described previously[10]. The Centriflow membrane ultrafilter (Amicon Corp.) with a cut-off at mol. wt. 50000 was used for determining cytochrome *c* binding to the reaction-center complex. The LKB electro-focusing column was used for determining the isoelectric point of the reaction-center complex.

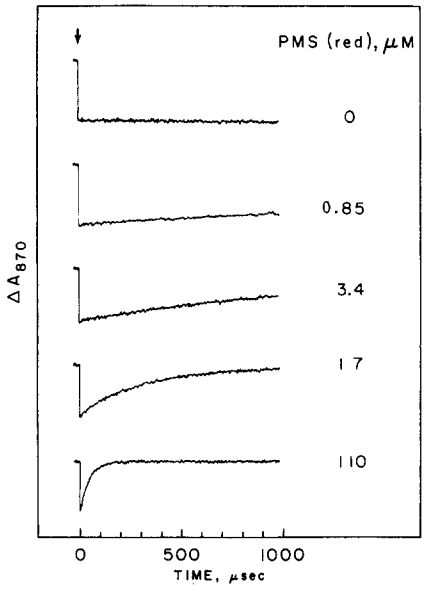
RESULTS

Dark reduction of photooxidized P870 by reduced phenazine methosulfate (PMS)

The reaction-center complex of *Rps. spheroides*, or P870, can be reversibly photobleached, as shown previously by REED AND CLAYTON[8]. In the absence of added electron donors, its recovery (re-reduction) in the dark takes 1–2 sec. By means of giant-pulse laser-flash excitation, photooxidation of P870 takes place within 1 μsec and no decay occurs within the 1-msec span, as can be seen from the first absorbance-change transient in Fig. 1. With increasing concentrations of reduced PMS, the re-reduction of photooxidized P870 became accelerated. At 0.1 mM reduced PMS, the decay halftime is 36 μsec (Fig. 1).

The reduction of P870 in the dark follows a first-order kinetics, as shown by the linear kinetic plots (Fig. 2) obtained from absorbance-change transients at several PMS concentrations. The decay halftime ($t_{1/2}$) derived from these plots was related

to the PMS concentration in a log–log plot in Fig. 3. A pseudo first-order rate constant of $1.85 \cdot 10^8$ l·mole^{-1}·sec^{-1} was obtained for the dark reduction of photooxidized P870 by reduced PMS. Between room temperature and slightly below the freezing temperature of the reaction mixture, an activation energy of 6 kcal was obtained for the P870–PMS reaction. It is of interest to note that a pseudo first-order rate constant determined for a similar reaction for the green-plant reaction-center complex P700 and reduced PMS was $1.5 \cdot 10^7$ l·mole^{-1}·sec^{-1}, and the activation energy was about 4 kcal (ref. 11).



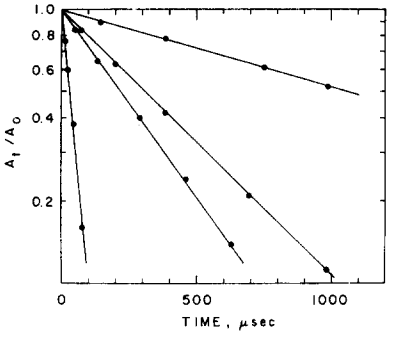


Fig. 1. Decay kinetics due to P870 re-reduction at different concentrations of reduced PMS. Reaction-center complex, 0.88 μM.

Fig. 2. First-order plot for the decay kinetics of P870 re-reductions at 3.4, 8.5, 17 and 85 μM of reduced PMS (from upper right to left curve).

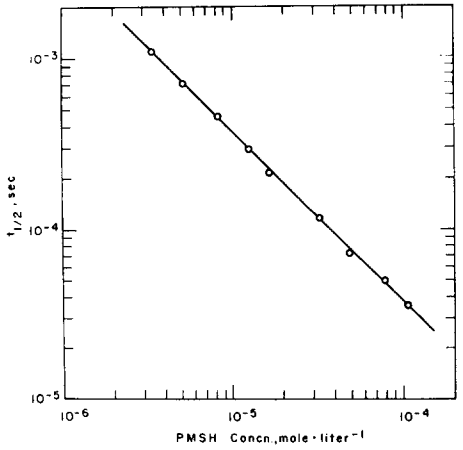


Fig. 3. Decay halftime of P870 re-reduction as a function of the concentration of reduced PMS.

Dark reduction of photooxidized P870 by ferrocytochrome c

The photooxidized P870 can also be very efficiently reduced by ferrocytochrome *c*. The 870-nm absorbance-change transients of P870 in the absence and in the presence of ferrocytochrome *c* are shown on the left side of Fig. 4. At sufficiently high concentrations of cytochrome *c* relative to P870 the decay of the P870 signal was almost

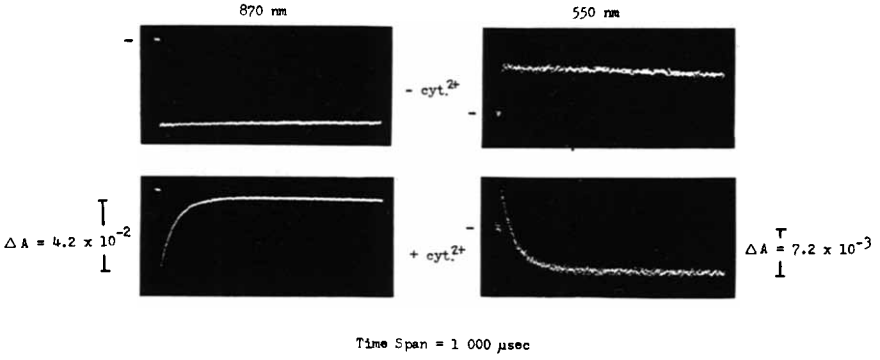
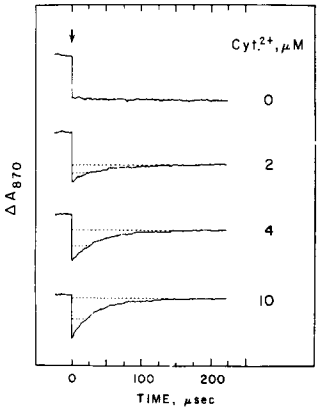


Fig. 4. Kinetics of the absorbance-change transients at 870 and 550 nm in the absence and presence of ferrocytochrome *c*. Reaction-center complex, 0.53 μM; ferrocytochrome *c*, 15 μM.

complete within the 1-msec span and the halftime was 25 μsec. In the absence of ferrocytochrome *c*, the absorbance at 550 nm increased within 1 μsec after the flash and decayed slowly (see right side of Fig. 4) with kinetics similar to those at 870 nm. In the presence of ferrocytochrome *c*, the decay of the positive 550-nm change was accelerated and the net result was a negative absorbance change attributable to the oxidation of ferrocytochrome *c*. The absorbance changes at 870 and 550 nm were $4.2 \cdot 10^{-2}$ and $7.2 \cdot 10^{-3}$, respectively. Taking the differential molar extinction coefficients for the reduced *minus* oxidized forms of P870 and cytochrome *c* to be 97 and



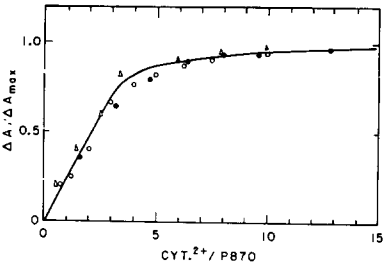


Fig. 5. Decay kinetics of P870 re-reduction at different concentrations of ferrocytochrome *c*. Reaction-center complex, 1 μM.

Fig. 6. Fraction of recovery of photooxidized P870 by ferrocytochrome *c* as a function of ferrocytochrome *c*-to-P870 ratio. Concentration of reaction-center complex, 0.7 (△), 1.1 (●) and 1.32 (○) μM.

18.7, respectively, a stoichiometry of 1.1:1 for the reaction between P870 and cytochrome *c* was indicated.

Effect of the ferrocytochrome c concentration. The portion of the P870 undergoing rapid recovery, *i.e.* with a halftime of 25 μsec, was proportional to the concentration of ferrocytochrome *c*, as can be seen from Fig. 5. Also, this rapid recovery was complete in about 150 μsec. The portion of P870 which recovered rapidly was closely related to the mole ratio of cytochrome to P870 rather than to the P870 concentration, as shown by the composite plot in Fig. 6. Complete recovery with a halftime of 25 μsec occurred when the mole ratio of cytochrome to P870 was above approx. 10 (*cf.* Figs. 4–6).

The decay profile of P870 coupled to ferrocytochrome *c* was quite different from when it was coupled to reduced PMS. In the case of the PMS coupling, within the concentration range of 1–100 μM PMS, the entire decay within the 1-msec period can be described by a pseudo first-order kinetics (see Fig. 1). In the case of cytochrome *c* coupling, depending on the cytochrome/P870 ratio, clearly only a certain fraction decayed within 1 msec and the halftime of the decay was constant. These results suggest that the fraction of P870 decaying within 1 msec represents those P870 particles which have one or more cytochromes bound at the active site, and the constant decay time is attributable to the bound state of the reacting components. The requirement of a high cytochrome/P870 ratio for maximal extent of the decay may reflect binding of several cytochrome molecules to each P870 particle and/or binding of cytochrome molecules at inactive sites. The double-flash experiments to be described later provided more information related to this question.

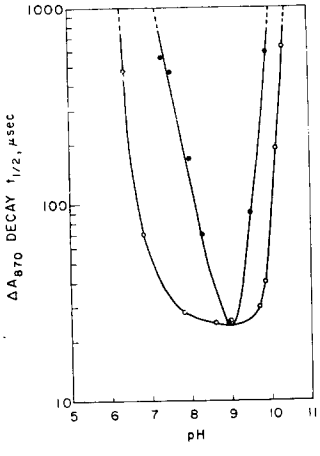
Determination of the amount of cytochrome c bound to the reaction-center complex. Ultrafiltration of reaction mixtures containing varying amounts of cytochrome *c*, P870, ascorbate, and PMS, showed that no cytochrome *c* was detectable in the filtrate until the cytochrome/P870 ratio exceeded approx. 24. The cytochrome *c* in solution without the P870 preparation readily passed through the ultrafilter.

Origin of the electrostatic interaction; effect of pH and ionic strength. Cytochrome *c* has a highly basic isoelectric point and would thus behave as a polycation at the pH near neutrality used in the kinetic experiments. The isoelectric point of the reaction-center complex was determined by the electro-focusing technique to be 4.1. Thus, these P870 particles would behave as a polyanion. At pH 7.5, these two components would exhibit a strong electrostatic interaction.

The presence of an electrostatic interaction between these oppositely charged polyions is further supported by the effect of pH and ionic strength on the coupled oxidation of ferrocytochrome *c* by P870, as shown in Fig. 7. The effect of varying the pH of 0.01 M buffers on the decay halftime of the P870 signal showed that the fastest decay occurs between pH 7.5 and 9.5. On either side of this pH range, the decay time increased rather sharply. At pH values of 6.5 and 10, for instance, the decay halftime increased from 25 μsec to approx. 500 μsec. At buffer concentrations of 0.1 M, the pH plot has nearly the same minimum point, but becomes much narrower, *i.e.* the decay time becomes much more sensitive toward changes in the pH of the buffer.

Inhibition of cytochrome c oxidation by polylysine. Additional experimental evidence supporting the electrostatic interaction between the oppositely charged polyions, *i.e.* ferrocytochrome *c* and P870, was sought by determining the possible

inhibition of their coupling by other polyionic molecules. We therefore used the synthetic polycation, polylysine, to test this point. As shown in Fig. 8, the percentage of cytochrome *c* oxidation completed within 1 msec decreased sharply as the polysine concentration was increased. From the limiting polylysine concentration



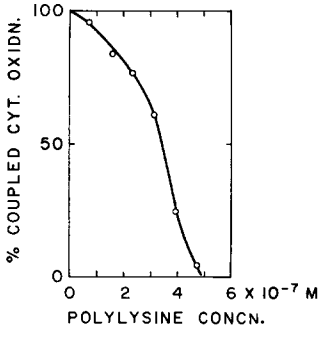


Fig. 7. Reaction halftime of P870 reduction coupled to ferrocytochrome *c* oxidation as a function of pH and ionic strength. Reaction-center complex, 1 μM; ferrocytochrome *c*, 10 μM. Buffers are: Tris–maleate (pH from 4.9–6.4), Tris (6.8–8.6), glycine (8.9–10.15). Ionic strength: 0.01 M (○) and 0.1 M (●).

Fig. 8. Effect of polylysine (mol. wt. 150000) on the coupling of ferrocytochrome *c* to P870 re-reduction. Reaction-center complex, 1 μM; ferrocytochrome *c*, 10 μM.

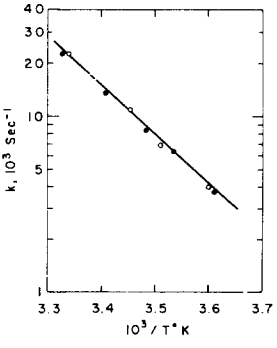
giving complete blocking of cytochrome coupling, it was calculated that each reaction-center complex of particle weight near $6.5 \cdot 10^5$ (ref. 6) contained approx. 500 negative charges. This calculation accounts for the multiple binding of ferrocytochrome *c* to the P870 particle as indicated by both the kinetic measurements (Fig. 6) and the ultrafiltration data. Ultrafiltration data showed the limiting value of 24 for the cytochrome/P870 ratio, beyond which cytochrome becomes detectable in the filtrate.

Temperature dependence. Between room temperature and the freezing temperature of the reaction mixture, the coupled oxidation of cytochrome *c* by P870 was temperature dependent. The first-order rate constants derived from the decay halftimes of the signals from both P870 and the cytochrome are plotted against the reciprocal of the absolute temperature according to the Arrhenius equation in Fig. 9. The data from these two sets of experiments fall on a single straight line over this temperature range. According to the Arrhenius equation, this temperature dependence yielded an activation energy of 12 kcal for the coupled reaction between P870 and ferrocytochrome *c*.

It should also be noted that below the freezing point of the sample, the cytochrome reaction appeared to be completely uncoupled. Although absorbance changes still persisted, they did not appear to be different from those measured in the absence of cytochrome.

Viscosity dependence. In a reaction medium containing 66 % glycerol, the halftime for cytochrome oxidation was slowed to 200–250 μsec, and the halftime for reduction of the cytochrome in the dark was slowed from 80 msec to 1.5 sec.

Mode of cytochrome binding studied by double-flash experiments. The mode of cytochrome binding to the reaction-center complex, *i.e.* the proof of multiple binding, the question of active sites near P870, *etc.* were explored in more detail by successive flash excitations separated by a short time interval. Results from one set of double-flash experiments performed on a reaction mixture with a cytochrome/P870 ratio



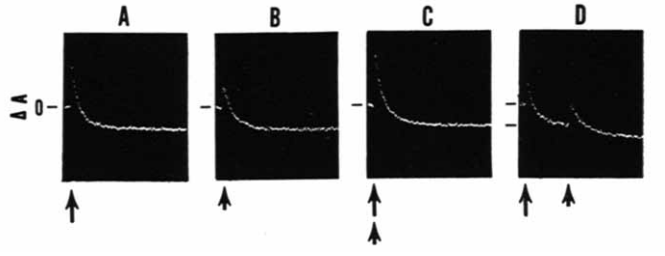


Fig. 9. First-order rate constant for cytochrome *c* oxidation plotted against the reciprocal temperature. Data points were taken from the P870 signal (●) as well as the cytochrome signal at 550 nm (○). Reaction conditions the same as in Fig. 4.

Fig. 10. Cytochrome *c* oxidation measured at 550 nm. (A) by laser flash (long arrow) alone; (B) by xenon flash (short arrow) alone; (C) by laser and xenon flashes together; (D) by laser flash, then by xenon flash 200 μsec later. Reaction-center complex, 0.5 μM; ferrocytochrome *c*, 12 μM. See text for details.

of 24 are shown in Fig. 10. Fig. 10A shows the absorbance change at 550 nm, induced by the laser flash of saturating intensity. The net negative change is taken as a measure of the amount of cytochrome *c* oxidized. Fig. 10B shows the 550-nm change caused by the xenon flash through a red cut-off filter (Corning 2-58). Fig. 10C shows the absorbance change caused by both the laser and xenon flashes together. In all three cases, the net negative changes are nearly quantitatively identical; the magnitude of the absorbance changes at 870 nm were also the same by the different saturating flashes. The laser-induced absorbance decrease at 870 nm is approx. 80 % of the total absorbance decrease of reaction-center P870 measured by the IR-1 and IR-2 modes in the Cary-14 spectrophotometer. Since flashes of higher intensity did not further increase this magnitude, it was assumed that essentially all of the active P870 was bleached by the first laser flash. However, depending on whether the laser, the xenon, or both flashes were used, the rapid positive absorbance increase at 550 nm appears to be dependent on the absolute intensity of the flashes, and it was apparently not yet saturated even when both flashes were used together.

In Fig. 10D, the laser flash was followed by the xenon flash at about 200 μsec later. This time interval was chosen because it is known that under the experimental conditions used P870 is largely re-reduced and should be ready to participate in photooxidation again at the time of the xenon flash. It is known, by separate measurements, that the cytochrome *c* oxidized by P870 in the first flash is not re-reduced by ascorbate–PMS until about 100 msec later. Thus the net negative absorbance change attributable to cytochrome oxidation by the second flash has

to come from cytochrome molecules other than those which were oxidized by the first laser flash, but bound at or near the active site of P870.

As seen from Fig. 10D, the net negative absorbance change caused by the xenon flash was only about 60 % of that caused by the first (laser) flash. These data are taken to mean that only 60 % of the P870 molecules have a second cytochrome molecule bound at or near the active site capable of carrying out the rapid electron transfer.

Note that the decay time of the signal caused by the second flash was also slightly slower than the first one. According to the tunnelling–barrier concept advanced by DEVAULT AND CHANCE, these data could indicate that these cytochrome molecules are slightly more loosely bound at the active site of P870. In theory, multiple-flash experiments could also yield additional information about the distribution of the cytochrome molecules as well as the relative geometry of these cytochromes to the P870 active site.

DISCUSSION

In photosynthetic bacteria, oxidation of *c*-type cytochromes is the fastest electron-transport reaction measured for any cellular component other than the photooxidation of the reaction-center bacteriochlorophyll. The halftimes for cytochrome oxidations in whole cells range from 0.5 μsec in *Rps. gelatinosa*, 2 μsec in *Chromatium*, 8 μsec in *Rps. spheroides*, to 25 μsec in *Rps. capsulata* or *Rps. rubrum*[4]. The rapid oxidation time in *Chromatium* cells is retained in both chromatophores[5] and subchromatophore particles[12] isolated from this bacterium. The variation in the measured oxidation times for different bacterial species has been attributed to a small difference in the molecular arrangements[4] and reflects the extreme sensitivity of the electron-transfer time to the tunnelling–barrier width.

In *Rps. spheroides* cells, a halftime of 8 μsec for the oxidation of the *c*-type cytochrome has been reported[4]; and in aerobic cells of the R-26 mutant, we have obtained 12 μsec. The 25-μsec halftime for the oxidation of exogenous cytochrome *c* by the reaction-center complex is only twice that for the endogenous cytochrome oxidation rate in whole cells and is most likely the result of a contact-type electron transport. The close association of the cytochrome molecules and the reaction-center complex particles necessary for this rapid coupling results from attractive forces which are predominantly electrostatic.

Several complexes formed by electrostatic interaction of mammalian cytochrome *c* with other proteins or lipoproteins have been reported[13], and an active electron-transport complex was formed with cytochrome oxidase[14]. From the large difference between the isoelectric points of mammalian cytochrome *c* and the bacteriochlorophyll reaction-center complex, electrostatic interaction between these polyions of opposite net charges would be expected. This expectation is supported by the effects of both pH and ionic strength on the rate of coupling between these two components (Fig. 7). The fastest cytochrome oxidation time occurs in the pH range intermediate between the isoelectric points of the reactants. Outside this optimum range, the reaction is quite sensitive toward pH changes, especially in buffers of higher ionic strength. Also, from the theory of electrolyte solutions, the rate of a reaction

between two ions depends not only on the product of the ionic charges, but the reaction rate between ions of opposite charges should decrease with increasing ionic strength.

The presence of electrostatic interaction between cytochrome *c* and the reaction-center complex is further supported by the effect of polylysine on the reaction between these two components (Fig. 8). The polycationic polylysine binds to the negatively charged reaction-center complex, and thus prevents binding of the cytochrome. The net negative charges on each reaction-center complex particle can be estimated as approx. 500 from the limiting polylysine concentration for complete inhibition. Direct ultrafiltration measurements showed that each reaction-center particle binds about 24 cytochromes, which is consistent with the total number of charges on the particle, considering that each cytochrome has 21 positively charged groups. However, it should be noted that each cytochrome only has 8 net positive charges at the optimum pH used for the reaction. These apparent discrepancies in charge stoichiometry may arise from the distribution of charged amino acids in the cytochrome peptide chain, the steric conformation of the protein molecule or the effects of other electrolytes in the reaction medium. The requirement for a ratio of 10 cytochromes *c* to one P870 for maximal dark recovery of P870 (Fig. 6) is consistent with the ultrafiltration data of cytochrome binding. Evidently complete P870 recovery does not require saturation binding of 24 cytochrome molecules.

Although the cytochrome is bound to the bacteriochlorophyll reaction-center complex and the light-activated electron transfer is probably a collision-independent reaction, the temperature dependence of the coupled oxidation of cytochrome *c* by P870 and the required activation energy of 12 kcal/mole indicate that this rapid electron transfer does involve a thermal activation. This energy requirement may be necessary for the two reacting components to achieve the most favorable orientation. The temperature dependence is also consistent with the effect of viscosity on the reaction rate. In 66% glycerol, the oxidation time is comparable to that at approx. 4° in Tris buffer without glycerol. It is of interest to note that the oxidation time of 2 μsec for the oxidation of endogenous cytochrome-555 in *Chromatium* subchromatophore particles is unaffected by 66% glycerol[12].

Although we have demonstrated here that, through electrostatic interactions, an electron-transfer complex can be formed which reacts in a time approaching that of the reaction *in vivo*, it is not yet clear whether the electrostatic interaction plays a similar role *in situ*. The isoelectric point of the high-potential *c*-type cytochrome isolated from *Rps. spheroides* has been measured as 5.5 (ref. 16). Thus, the relative molecular arrangement in intact cells is presumably influenced by some structural factors in addition to these electrostatic interactions. Nevertheless, the complex formed by electrostatic interactions appears to furnish a reaction system in which the various parameters affecting photosynthetic electron transport may be further studied.

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