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NEW EXPERIMENTAL APPROACH TO THE ESTIMATION OF RATE OF ELECTRON TRANSFER FROM THE PRIMARY TO SECONDARY ACCEPTORS IN THE PHOTOSYNTHETIC ELECTRON TRANSPORT CHAIN OF PURPLE BACTERIA

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SUMMARY

A method for calculating the rate constant ($K_{A_1A_2}$) for the oxidation of the primary electron acceptor (A_1) by the secondary one (A_2) in the photosynthetic electron transport chain of purple bacteria is proposed.

The method is based on the analysis of the dark recovery kinetics of reaction centre bacteriochlorophyll (P) following its oxidation by a short single laser pulse at a high oxidation-reduction potential of the medium. It is shown that in *Ectothiorhodospira shaposhnikovii* there is little difference in the value of $K_{A_1A_2}$ obtained by this method from that measured by the method of Parson ((1969) Biochim. Biophys. Acta 189, 384–396), namely: $(4.5\pm1.4)\cdot10^3\ s^{-1}$ and $(6.9\pm1.2)\cdot10^3\ s^{-1}$, respectively.

The proposed method has also been used for the estimation of the $K_{A_1A_2}$ value in chromatophores of *Rhodospirillum rubrum* deprived of constitutive electron donors which are capable of reducing P^+ at a rate exceeding this for the transfer of electron from A_1 to A_2. The method of Parson cannot be used in this case. The value of $K_{A_1A_2}$ has been found to be $(2.7\pm0.8)\cdot10^3\ s^{-1}$.

The activation energies for the A_1 to A_2 electron transfer have also been determined. They are 12.4 kcal/mol and 9.9 kcal/mol for *E. shaposhnikovii* and *R. rubrum*, respectively.

INTRODUCTION

A number of investigators have attempted to detect the photoinduced oxidation-reduction reactions of the primary and secondary electron acceptors and to identify these carriers of the photosynthetic electron transport chain in purple bacteria.

Analysis of the difference spectra "light-minus dark" of the photosynthetic reaction centre preparations made from the cells of *Rhodopseudomonas spheroides* and *R. rubrum* suggests that the absorption bands centred at 285 nm [2] and 420–450 nm [3]

may be ascribed to the photoreduced products, most probably, to ubisemiquinone [2]. The obtained data, however, are not the reason for solving unequivocally the question whether or not ubiquinone plays the role of the primary or secondary acceptor.

ESR investigation of the photosynthetic reaction centre preparations from *Rps. spheroides*, strain R-26, at temperatures close to 4 K enabled Dutton et al. to find a signal with g-factor of 1.82 which was produced by the photochemical act or chemical reduction [4].

It has been concluded that the primary electron acceptor is responsible for this signal. Apparently this acceptor is an iron-sulphur protein.

A photo-induced ESR signal of semiquinone with $g = 2.005$ has been observed in the preparations of the photoreceptor subunits from *R. rubrum* [5] and photosynthetic reaction centres from *Rps. spheroides* strain R-26 [6], from which most of iron has been extracted (Fe/*P*-870 < 1).

It has been suggested that the primary electron acceptor is an iron-ubiquinone complex [7]. Evidence for this is the fact that the complete extraction of ubiquinones blocks the photochemical oxidation of P-870 in photosynthetic reaction centres from *Rps. spheroides*, R-26 [8], as well as that the chemical reduction of the primary acceptor in these preparations does not lead to the change in the valent state of iron but is attended by an ESR signal with $g = 1.84$ [9]. This is explained by the change of the spin state of the iron atom whenever a non-paired electron is localized on ubiquinone.

Though further study is required to elucidate the chemical nature of the primary acceptor, the kinetics and the mechanisms of oxidation-reduction photochemistry can be investigated in an indirect way.

The method of Parson [1] for the determination of the rate constant for electron transfer from the primary electron acceptor to the secondary one is based on the measurement of the extent of the oxidation of *P*-870 or cytochrome *c* exposed to two successive laser pulses with the interval between them being varied. Using this method Case and Parson [10] have measured the activation energy for this reaction, the redox potentials for the primary and secondary acceptors, as well as dependence of these potentials on temperature and pH in *Chromatium* chromatophores.

The method of Parson, however, is only applicable for an investigation of the preparations with electron donors capable of reducing reaction centre bacteriochlorophyll at a rate higher than that for the A_1 to A_2 electron transfer.

This method cannot be used for the investigation of bacterial chromatophores deprived of secondary electron donors (for example, *R. rubrum*) or photosynthetic reaction centre preparations.

In the present work we propose a method for calculating the rate constant for electron transfer from the primary to the secondary acceptor ($K_{A_1A_2}$) which is based on the analysis of the dark recovery kinetics of the reaction centre bacteriochlorophyll following its oxidation by a single laser pulse at a high oxidation-reduction potential of the medium; that is, under conditions where photooxidized bacteriochlorophyll can only take an electron from photoreduced acceptors.

This rate constant measured by the proposed method was found to be $(4.5\pm 1.4)\cdot 10^3\ s^{-1}$ and $(2.7\pm 0.8)\cdot 10^3\ s^{-1}$ in *E. shaposhnikovii* and *R. rubrum* chromatophores, respectively. For *E. shaposhnikovii* chromatophores we also measured the $K_{A_1A_2}$ value by the method of Parson and found it to be $(6.9\pm 1.2)\cdot 10^3\ s^{-1}$.

MATERIALS AND METHODS

Photosynthesizing bacteria *E. shaposhnikovii* and *R. rubrum* were grown and chromatophores were prepared as described elsewhere [11]. Stock suspensions were stored under argon at ice in a medium containing Tris–HCl buffer, 50 mM (pH 7.8), sucrose, 250 mM and $MgCl_2$, 5 mM. The concentration of bacteriochlorophyll in the chromatophore samples has been determined from the absorbance values at 880 nm in *R. rubrum* and at 860 nm in *E. shaposhnikovii*. The effective extinction coefficients $\varepsilon_{880} = 140\ mM^{-1} \cdot cm^{-1}$ and $\varepsilon_{860} = 101\ mM^{-1} \cdot cm^{-1}$ have been determined using the method of Clayton [12] by extracting the chromatophores with an acetone-methanol mixture and measuring the bacteriochlorophyll concentration using an extinction coefficient as given in ref. 12. The redox potential of the reaction mixture was measured with the aid of platinum electrode and Ag/AgCl reference electrode. The potentiometric electrodes were calibrated against saturated solution of quinhidrone at different pH. Potassium ferricyanide has been added to increase the redox potential of the medium and sodium ascorbate to decrease it. 2,6-Dichlorophenolindophenol (Cl_2Ind), 100 μM or potassium ferricyanide, 100 μM, were used as mediators to provide redox equilibration between electron carriers in the chromatophore membranes and the platinum electrode.

The bacteriochlorophyll photoreactions in chromatophores were followed by measuring absorbance changes at 440 nm for *E. shaposhnikovii* [13, 14] and at 430 nm for *R. rubrum* [14].

A single beam spectrophotometer employed for recording the kinetics of absorbance changes induced by a short laser pulse has been described elsewhere [15].

In order to reject the scattered actinic light from a laser pulse and luminescence from the sample, a cut-off filter with a long wavelength passband confined to 600 nm was placed between the sample and photodetector during the measurements in the wavelength region of 400 to 450 nm.

By adjusting the concentration of dye in a photochemical shutter and by changing the pumping voltage the successive laser flashes may be produced with 20 to 500 μs apart. The energy density of a laser flash at 694.3 with a pulse duration of 30 ns was 3 mJ cm^{-2}.

For the spectrophotometric measurements at room temperature the mixture of chromatophores was diluted with 0.15 M phosphate buffer (pH 7.0) to give an absorbance of 0.9 at 590 nm. The chromatophores in 80 % glycerol were used for measurements at low temperatures. Such a concentration of glycerol did not affect the kinetics of light-induced changes in chromatophores. After a slow freezing such samples remained as clear as glass throughout the experiment over the temperature range of 298 K to 120 K. A special cuvette was used for low temperature measurements, the design of which was described in ref. 16. A calibrated copper-constantan thermocouple adjacent to the sample was employed to monitor the temperature.

RESULTS AND DISCUSSION

Photochemical reactions induced by successive laser flashes in E. shaposhnikovii chromatophores

The sequence of the primary reactions induced by a single laser pulse in the photosynthetic reaction centre may be presented in the following manner.

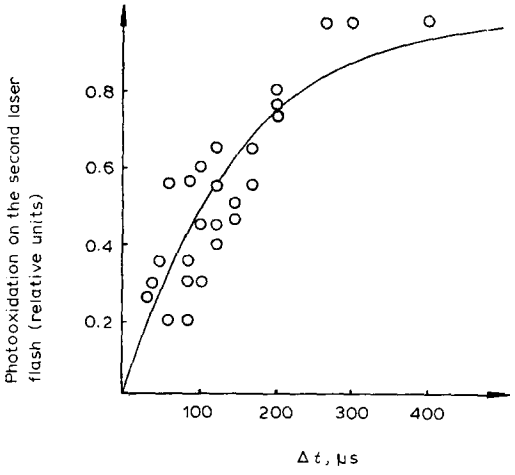

Fig. 1. Dependence of the extent of cytochrome C_{290} oxidation following the second laser pulse on the dark time interval between two successive actinic pulses in chromatophores of *E. shaposhnikovii*. Wavelength of measuring light, 424 nm. Chromatophores were suspended in 0.15 M phosphate buffer, pH 7.0. Bacteriochlorophyll concentration, 12 μM. 100 μM Cl_2Ind added in a reaction mixture as a redox mediator, $E_h = +160$ mV. Solid line is a theoretical curve $1-e^{-t/\tau}$, where $\tau = 145$ μs.

The photo-excited reaction centre bacteriochlorophyll donated an electron to the primary acceptor (A_1). According to a number of authors this reaction completes in picosecond time range [17, 18]. The oxidized bacteriochlorophyll then receives an electron from the secondary donor. In chromatophores of *E. shaposhnikovii* the function of the secondary donor is fulfilled by cytochrome *c* named because of its kinetic and potentiometric characteristics as "fast reducing" or "high potential". We shall adopt the cytochrome designation as C_{290} for its midpoint redox potential which is +290 mV as determined previously [14]. The half-time for C_{290} oxidation in *E. shaposhnikovii* chromatophores is 2.5 μs. Thus, a single laser pulse induces electron transfer from cytochrome C_{290} to A_1 with a half-time of 2.5 μs.

Fig. 1 shows the dependence of the extent of cytochrome C_{290} oxidation, at an instant of exposure to the second flash, on the time interval between the two flashes which varies between 20 and 450 μs. It can be seen that the extent of C_{290} oxidation is enhanced with an increase in the interval between the flashes and is maximal at an interval of 300 μs. The experimentally obtained points may be approximated by a theoretical curve $1-e^{t/\tau}$, where $\tau = 145\pm30$ μs. Since the transfer of an electron from C_{290} to A_1, when sensibilized by reaction centre bacteriochlorophyll, occurs more rapidly ($t_{\frac{1}{2}} = 2.5$ μs), an increase of the extent of C_{290} oxidation is due to reoxidation of A_1 during the transfer of an electron from A_1 to the secondary electron acceptor (A_2), and $\tau = 145\pm30$ μs is the characteristic time of this reaction, which corresponds to the value of $K_{A_1A_2} = (6.9\pm1.2)\cdot10^3$ s^{-1}.

Analysis of dark recovery kinetics of reaction centre bacteriochlorophyll following its oxidation induced by laser flash at low temperatures

1. *Chromatophores of E. shaposhnikovii.* The dark recovery kinetics of bacterio-

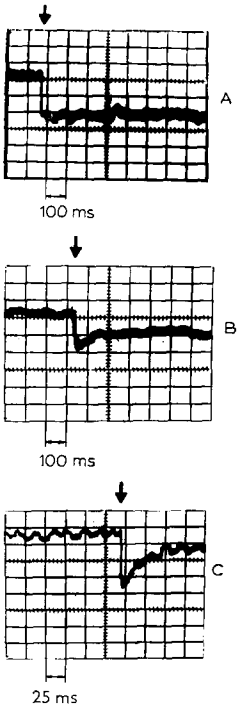

Fig. 2. Oscillograms of the dark recovery kinetics of reaction centre bacteriochlorophyll following its oxidation by a single laser pulse in chromatophores of *E. shaposhnikovii*. Wavelength of measuring light, 440 nm. Chromatophores were suspended in a medium containg 0.15 M phosphate buffer, pH 7.0 and glycerol in a 1 : 4 ratio. Bacteriochlorophyll concentration, 61 μM. 100 μM potassium ferricyanide added as a redox mediator, $E_h = +380$ mV. Arrow indicates laser pulse. A, at 300 K; B, at 245 K; C, at 150 K.

chlorophyll oxidized by a laser flash has been studied over the temperature region of 300 K to 120 K. At 300 K the half-time for its reduction is 7 ± 3 s, which corresponds to the characteristic time $\tau = 10\pm4$ s (Fig. 2A).

When the temperature is lowered to 250 K, along with this relatively slow component, a faster kinetic component appears (Fig. 2B). With further temperature lowering the contribution of the fast component into the dark recovery kinetics increases (Figs. 2 and 3). The characteristic time for the fast component (35 ± 7 ms) was found to be independent of temperature throughout the temperature range of 250 to 120 K.

Addition to the chromatophore suspension 10^{-2} M of *o*-phenanthroline which is known to be an inhibitor for electron transfer from A_1 to A_2 [19], brings about the appearance of the fast component of bacteriochlorophyll reduction already at room temperature. In this condition the characteristic time for this reaction is 60 ± 10 ms.

Since the fully reversible photooxidation of bacteriochlorophyll over the entire temperature range studied in the absence of exogenous electron donor can be reproduced repeatedly, bacteriochlorophyll (P) must receive an electron from the photoreduced acceptors of the reaction centre.

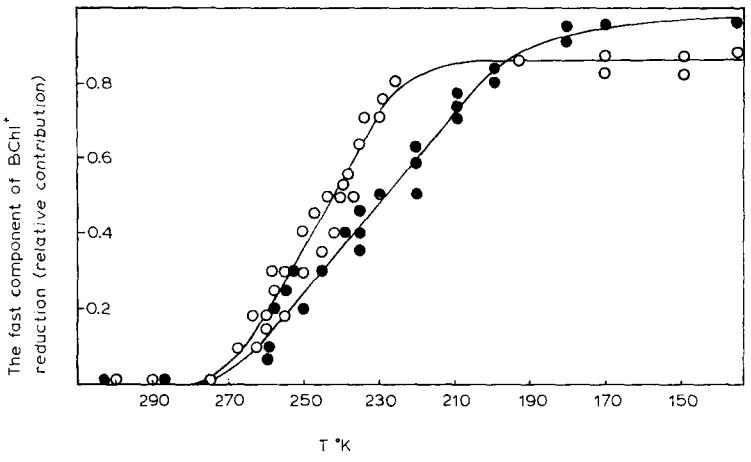

Fig. 3. Temperature dependence of the relative contribution of the fast component of the dark reduction of reaction centre bacteriochlorophyll following its oxidation by a single laser pulse in chromatophores of *E. shaposhnikovii* (○–○) and *R. rubrum* (●–●). Wavelength of measuring light, 440 nm in the experiments with *E. shaposhnikovii* and 430 nm in the experiments with *R. rubrum*. Other conditions the same as in Fig. 2, except for: in *R. rubrum* chromatophores bacteriochlorophyll concentration, 83 μM.

The fast component which appears in the presence of *o*-phenanthroline as well as at low temperature, when the photochemical reactions are confined to the P–A_1 interaction [20], is, apparently, associated with the reverse transfer of an electron from A_1 to pigment. At high temperature, due to the effective transfer of an electron from A_1 to A_2 the reduction of bacteriochlorophyll occurs from the photo-reduced A_2 with a characteristic time $\tau = 10$ s.

On exposure to a short laser pulse (30 ns) the electrons that have left the bacteriochlorophyll reaction centre are localized in the primary electron acceptor. Their pathway is further conditioned by the ratio of the rate constants for electron transfer from A_1 to A_2 and to P^+. Thus, the contribution of the fast component (X) in P^+ reduction shall be determined by the following relationship:

$$x = \frac{K_{A_1P}}{K_{A_1P} + K_{A_1A_2}} \tag{1}$$

where from:

$$K_{A_1A_2} = K_{A_1P} \frac{1-x}{x} \tag{2}$$

where $K_{A_1A_2}$ and K_{A_1P} are the rate constants for electron transfer which are the reciprocal of the characteristic times of the respective reactions.

In the experiment it was possible to measure both the contribution of the fast component and its half-time at each temperature. Using these data the value of the constant $K_{A_1A_2}$ may be found from Eqn. (2). The temperature dependence of $K_{A_1A_2}$, calculated in such a manner, is presented as an Arrhenius plot in Fig. 4.

The experimental points may be approximated by a curve analytically expressed as:

$$K_{A_1A_2} = K_0 + K_1 e^{-E_{act.}/RT}$$

where $K_0 = 3.4\ s^{-1}$, $K_1 = 3.54\ 10^{12}\ s^{-1}$, $E_{act.} = 12.4$ kcal/mol. The dispersion of the experimental data corresponds to the variation of the activation energy within 11.6 kcal/mol to 12.8 kcal/mol. The values of constant $K_{A_1A_2}$, obtained by extrapolation of its temperature dependence to 300 K, range from $3 \cdot 10^3\ s^{-1}$ to $5.9\ 10^3\ s^{-1}$. This corresponds to $\tau = 170$–$330\ \mu s$. This calculated value of the rate constant for the A_1-A_2 electron transfer — $(4.5 \pm 1.4) \cdot 10^3\ s^{-1}$ differs little from that measured by the method of Parson — $(6.9 \pm 1.2) \cdot 10^3\ s^{-1}$. Some discrepancy in the obtained results may be due, first of all, to considerable experimental errors inherent in both methods. Besides, measurements by our methods unlike those of Parson's method were done at somewhat higher oxidation-reduction potential of the medium and in the presence of glycerol. These also might account for the discrepancy observed.

2. *Chromatophores of R. rubrum.* A similar investigation of the temperature dependence of the dark recovery of reaction centre bacteriochlorophyll were carried out on a *R. rubrum* chromatophore preparation with cytochrome *c* content not exceeding that in whole bacterial cells by 10–15 %. This was determined by measuring the "oxidized minus reduced "difference spectra for all fractions of the disintegrated cells. A similar "washing" of cytochrome *c* during the chromatophores isolation

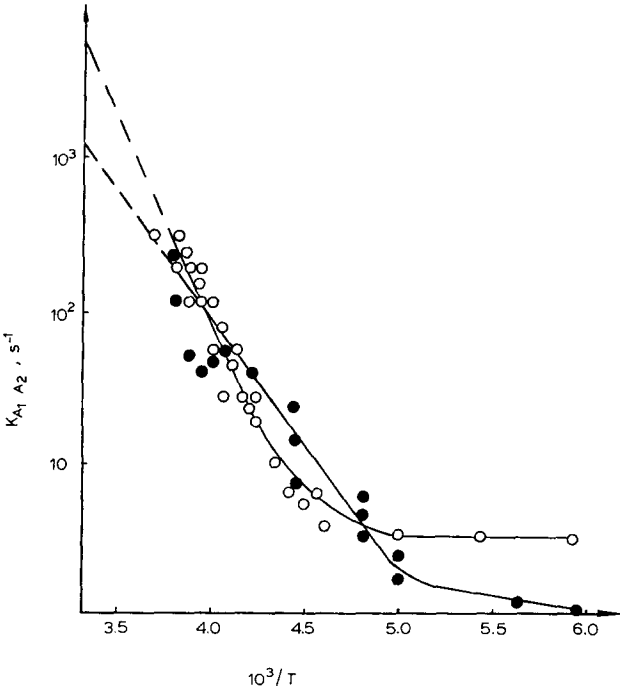

Fig. 4. Temperature dependence of constant $K_{A_1A_2}$ as an Arrhenius plot: ○—○, in chromatophores of *E. shaposhnikovii*; ●–●, in chromatophores of *R. rubrum*.

procedure has been described by Smith et al. [21]. Because of the absence of cytochrome *c* in such chromatophores, the method of Parson cannot be applied for the investigation of electron transfer from A_1 to A_2.

At 300 K reaction centre bacteriochlorophyll oxidized by a laser pulse reduces with a characteristic time of 5 ± 2 s. With temperature lowered to 260 K the fast component of dark recovery is exhibited. As in chromatophores of *E. shaposhnikovii*, its contribution increases as temperature is lowered (Fig. 3). The characteristic time (85 ± 15 ms) of this component measured over the range from 260 K to 120 K is independent of temperature.

Addition of 10^{-2} M of *o*-phenanthroline to the chromatophore suspension causes the appearance of the fast component ($\tau = 85\pm15$ ms) at room temperature. It is seen that in chromatophores of *R. rubrum* the rate of the reaction proceeding in the presence of *o*-phenanthroline or at low temperatures is practically the same.

Fig. 3 shows the temperature dependence of the fast component contribution to the oxidized reaction centre bacteriochlorophyll reduction in *R. rubrum* chromatophores. The values of $K_{A_1A_2}$ were calculated from Eqn. (2). Temperature dependence of this constant is given in Fig. 4. When extrapolated to 300 K, the value of the rate constant for the A_1 to A_2 electron transfer appears to be $(2.7\pm0.8)\cdot10^3\ s^{-1}$. This corresponds to the characteristic time of 280–530 μs. The activation energy for this reaction is 9.4–10.4 kcal/mol.

Both for *E. shaposhnikovii* and *R. rubrum* the curve of $K_{A_1A_2}$ plotted as a function of temperature (Fig. 4) has a temperature-independent portion ($E_{act.} < 1$ kcal/mol) and a portion where $\lg K_{A_1A_2}$ is linearly dependent on $1/T$.

A similar temperature dependence of the rate constant for electron transfer from cytochrome *c* to photooxidized bacteriochlorophyll was observed earlier [22–23]. These results are interpreted [23, 24] as indicating a tunneling mechanism for electron transition coupling with energy transfer to normal vibrations of the medium, since the A_1 to A_2 electron transfer is attended by the loss of energy. At high temperatures the tunneling transfer coupled with the energy change of the thermally-induced normal vibrations of the medium is predominant. The rate of this process decreases exponentially as the temperature is lowered. At rather low temperatures, the temperature-independent tunneling transfer becomes predominant attended by the vibration quantum emission.

Tunneling mechanism may also be proposed for the temperature-independent reversion of the photochemical act as was demonstrated by McElroy and co-workers [25]. However, there is another possible explanation for this. One might also postulate, for example, that there is no energy barrier at all between photoactive bacteriochlorophyll and the primary acceptor so that these form a common potential well. In this case an electron can be transferred from the first excited singlet level of the pigment on a common metastable energy level, locating, however, mainly in the primary acceptor. From this point of view the rate constant K_{A_1P} is a measure of the probability for an electron transition from this metastable level on the ground electron level of reaction centre bacteriochlorophyll.

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