MICROSECOND PHOTOOXIDATION KINETICS OF CYTOCHROME c_2 FROM *RHODOPSEUDOMONAS SPHAEROIDES*: IN VIVO AND SOLUTION STUDIES

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1. Introduction

In the photosynthetic bacteria, stabilization of the primary charge separation between the bacteriochlorophyll primary donor and the quinone acceptor proceeds almost universally by the rapid photooxidation of a *c*-type cytochrome [1]. In some species, the cytochrome is tightly bound to the reaction center and oxidation is roughly monophasic and extremely rapid, with halftimes of 0.1–1 μs [1]. In *Rhodopseudomonas sphaeroides*, a species otherwise better characterized than any other, the kinetics of cytochrome c_2 photooxidation are biphasic and are currently not well understood. As was first shown [1,2] the in vivo kinetics can be well reproduced in form using isolated reaction centers incorporated into phospholipid vesicles. We have found the kinetics to be very similar for isolated reaction centers either in detergent solubilized form or incorporated into phospholipid vesicles ([3], unpublished observations). The correspondence between the reconstituted kinetics and those in vivo has been uncertain, however, because the fast oxidation phase has never been sufficiently resolved, although a halftime of 10–30 μs has been estimated [4,5].

We report here on the resolution of this rapid process which proves to be considerably faster than has been supposed for this species [4,5]. We also report some observations on the slow phase which suggest a kinetic scheme for the photooxidation of cytochrome both in vivo and in vitro.

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2. Materials and methods

Reaction centers were prepared from the carotenoidless mutant of *Rps. sphaeroides*, strain R26, by fractionation of the chromatophore membrane with Ammonyx-LO (LDAO, a mixture of lauryl and myristyl dimethylamine *N*-oxide, a generous gift of the Onyx Corporation, Division of Millimaster, Jersey City, NJ 07023), essentially as in [6], except that the detergent concentration used in the fractionation step was 0.35% and the reaction centers were eluted from the DEAE column with 0.25 M NaCl, 0.1% LDAO, 10 mM Tris (pH 8.0). Reaction center purity was judged by the ratio of absorbance at wavelengths (nm) 800/870, which was 2.2, and 280/800, which was typically 1.3.

Cytochrome c_2 was purified by the method in [7,8]. The final preparation met the purity criterion of [8] ($A_{275/417}$ (reduced) = 0.21).

The slower kinetic experiments (resolution > 20 μs) were done in dualbeam mode in an anaerobic cuvette. Buffer solution was deoxygenated with argon and the pH and ambient redox potential (E_h) were monitored and controlled continuously in the cuvette. Naphthoquinone was used as a redox mediator and slow electron shuttle from the reaction center acceptor region to cytochrome c_2. A dye laser (Phase-R, Model 1100, New Durham, NH 03855) with an emission wavelength of 585 nm (rhodamine 6-G in ethanol) and pulsewidth of 0.4 μs was used to activate the reaction centers with > 95% saturation. The faster kinetic experiments were done in singlebeam mode with a near-saturating Q-switched ruby laser excitation source (pulsewidth 30 ns). The probe light was a

pulse-boosted xenon arc, based on the design in [9]. Details of the spectrophotometer appear in [21]. In these experiments, which were not performed anaerobically, 2 μM *N,N,N',N'*-tetramethyl-*p*-phenylene diamine (TMPD) and 1 mM ascorbate were also included to keep the cytochrome in the reduced state in the dark.

In all experiments with isolated reaction centers, the detergent was kept at < 0.02%. We have found LDAO to markedly affect the kinetics at > 0.1% and a massive shift has been reported in the midpoint potential of cytochromes *c* and c_2 at similar detergent levels [10].

3. Results

3.1. *Fast kinetics in solution*

The kinetics of photooxidation of cytochrome c_2 by reaction centers in solution at equimolar concentration are shown in fig.1. At low concentration, the kinetics were all slow and second order. Second order kinetics were also reported [11] in 2% LDAO. At higher concentrations an additional fast phase of oxidation appeared with an app. K_b 10^5 M^{-1}. The time base in figs.1,3 covers 3 orders of magnitude. This resolution was necessary to distinguish the biphasic kinetics of cytochrome oxidation. The initial downward absorbance change is due to the rapid creation of P^+. The signal was limited to 30 ns risetime by a low pass filter. The subsequent upward deflection corresponds to the oxidation of cytochrome c_2 and the concomitant rereduction of P^+. At equimolar concentrations of cytochrome and reaction centers $\lesssim$ 1 μM the cytochrome oxidation was distinctly biphasic. As the concentration was raised the proportion of fast phase increased. In fig.1 this proportion is $\leqslant$ 30%. As much as 50% fast phase, but not more, could be obtained by titrating reaction centers with excess cytochrome c_2. When first detectable, the fast phase exhibited a halftime of 2 μs but, as the concentration of each reactant was increased, it accelerated slightly and became > 1 μs (fig.2a). As the ionic strength was increased the fast phase slowed and decreased in magnitude. At 50 mM NaCl the halftime was ~4 μs and the fast phase app. K_b ~10^4 M^{-1}.

3.2. *Slow kinetics in solution*

The slow phase of oxidation was studied more extensively on the dualbeam machine. The inverse halftime of the slow phase increased linearly at first with increasing equimolar concentration of reactants, indicating a second order process (fig.2b). The second order rate constant given by the slope of this line is 8×10^8 M^{-1} $.s^{-1}$. The second order phase of oxidation was found to limit to a pseudo first order reaction at ~6 μM of each reactant. The lower limit to the halftime of the slow phase of oxidation at high concentrations was 400 μs.

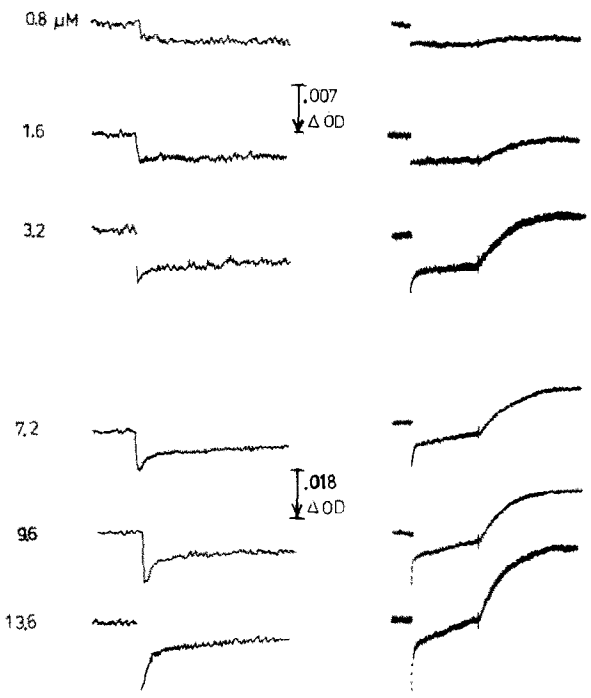

Fig.1. Solution kinetics of cytochrome c_2 oxidation and P^+ rereduction at 417 nm. Reaction centers in low LDAO and cytochrome c_2 were present at the equimolar concentration shown at the left. The traces on the left are 20 μs fullscale. Those on the right show the first 200 μs and the next 2 ms for the same flash. The buffer was 10 mM Tris (pH 8.0) with 1 mM ascorbate and 20 μM naphthoquinone. Excitation was by 30 ns laser pulse.

3.3. *In vivo kinetics*

The in vivo kinetics were very similar to the in vitro kinetics observed at high concentrations. This is particularly evident from a comparison of the kinetics

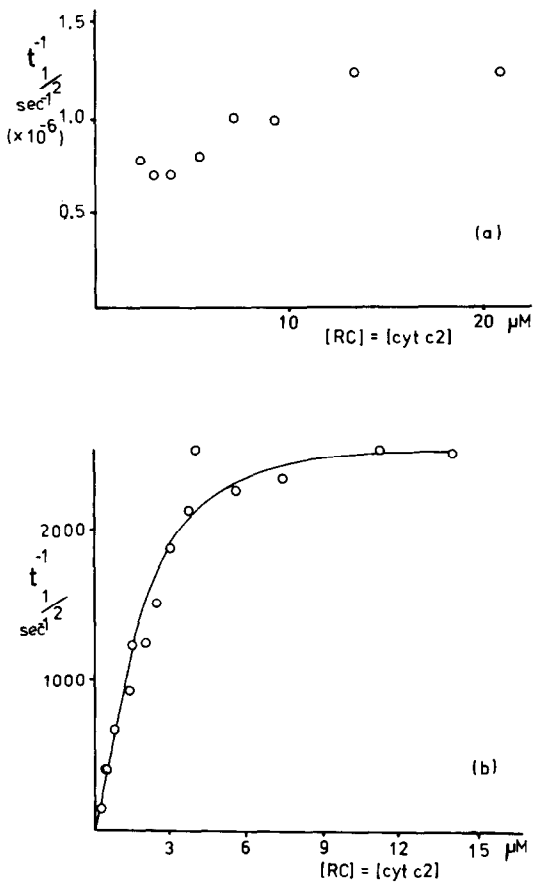

Fig.2. Kinetic constants of cytochrome c_2 photooxidation in solution. (a) Inverse halftime of resolved fast phase determined as in fig.1. (b) Inverse halftime of slow phase measured at 550–540 nm. Reactants were in equimolar concentrations as in fig.1.

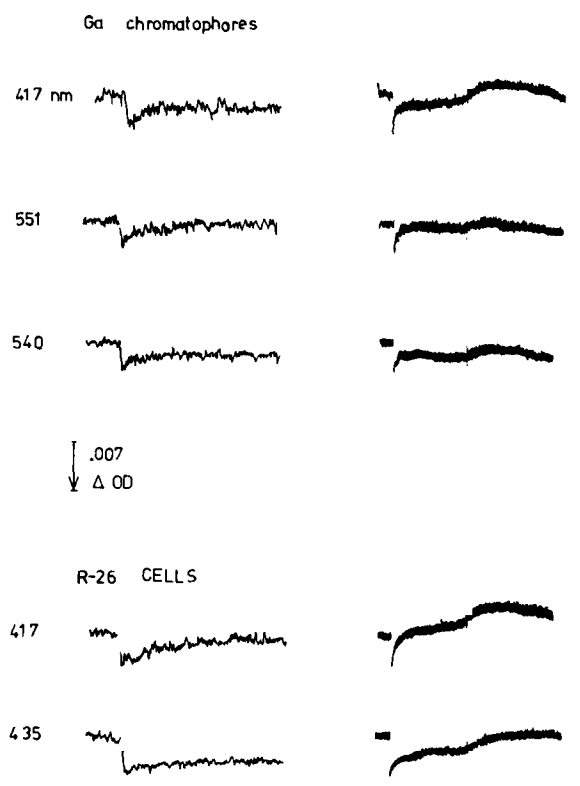

Fig.3. In vivo kinetics of cytochrome c_2 oxidation and P^+ rereduction. Measurements were performed at the wavelengths indicated on the left. Ga chromatophores (A_{590} = 1.3) were suspended in 100 mM KCl, 5 mM $MgCl_2$, 10 mM Tris (pH 8.0), 1 mM ascorbate, and 50 μM TMPD. R-26 whole cells were suspended in similar buffer with the addition of 25% (w/v) Ficoll to minimize light scattering. The time bases for the traces on the left were 50 μs fullscale for the chromatophores and 20 μs fullscale for the whole cells. Time bases on the right were as in fig.1.

at 417 nm for reaction centers in solution (fig.1) with those for Ga chromatophores and R-26 whole cells (fig.3). The halftime of the fast phase was found to be 3 μs for chromatophores and whole cells. The halftime of the slow phase of oxidation in vivo was found to be 200–400 μs. Very similar kinetics were seen for P^+ rereduction at the cytochrome isosbestic wavelengths of 540 nm for Ga chromatophores at 435 nm and 540 nm for R-26 whole cells.

3.4. *Mobility of the cytochrome*

Although, the data of figs.1–3 demonstrate a strong similarity between the in vivo and in vitro kinetics of cytochrome c_2 photooxidation, recent results [12] have suggested that the cytochrome in vivo is not freely mobile between different reaction centers, in contrast to the clearly second order kinetics (i.e., diffusional) of the slow phase in vitro.

The in vitro mobility of the cytochrome was probed by the light saturation characteristics of cytochrome oxidation after a single, variably attenuated flash following the method in [13] and as used in [12]. Figure 4 shows that the extent of oxidation of cytochrome c_2 fell off linearly with the extent of reaction

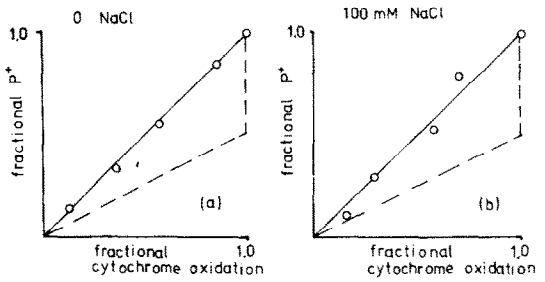

Fig.4. Light saturation characteristics of reaction center and cytochrome c_2 photooxidation. The fraction of cytochrome oxidized on a single, variably attenuated laser flash is plotted against the fraction of P^+ created. P^+ was measured at 540 nm and cytochrome oxidation was measured at 550–540 nm on alternate flashes. Solid lines are expected for a long lived complex, dashed lines if cytochrome c_2 is fully mobile. Reaction centers at 1.0 μM and cytochrome c_2 at 0.5 μM in: (a) 10 mM Tris (pH 8.0); (b) 100 mM NaCl and 10 mM Tris (pH 8.0). Naphthoquinone (20 μM) was present in both cases.

center oxidation, indicating that, within the lifetime of P^+ (0.1 s), the cytochrome was not free to diffuse from an unactivated reaction center to an activated one. This is the same result as obtained in vivo [12] and we conclude that the reaction center and cytochrome form a long-lived complex even when they are not tightly bound in an equilibrium sense. Using the technique of equilibrium chromatography we have not been able to detect binding of cytochrome c_2 to reaction centers at high or low ionic strength. We estimate the limit of resolution of this technique to be $\sim 10^5\ M^{-1}$. A recent study using equilibrium dialysis has reported $K_b \sim 3 \times 10^5\ M^{-1}$ at low ionic strength [14].

4. Discussion

4.1. *The reaction in solution*

We have shown here that the kinetics of cytochrome c_2 oxidation are truly biphasic at high concentrations, and are very similar in solution and in vivo. The general form of the kinetics can be accounted for by the following scheme:

$$RC^+ + c_2 \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} RC^+ \cdot\cdot c_2 \xrightarrow{k_{23}} RC^+ \cdot c_2 \xrightarrow{k_{34}} RC \cdot c_2^+$$

$$h\nu \uparrow\downarrow k_b \qquad h\nu \uparrow\downarrow k_b \qquad h\nu \uparrow\downarrow k_b$$

$$RC + c_2 \underset{k'_{21}}{\overset{k'_{12}}{\rightleftharpoons}} RC \cdot\cdot c_2 \underset{k'_{32}}{\overset{k'_{23}}{\rightleftharpoons}} RC \cdot c_2$$

In the dark, an equilibrium is set up between the dissociated reactants RC and c_2 and the bound states $(RC \cdot\cdot c_2)$ and $(RC \cdot c_2)$. The reaction centers are then oxidized by a short flash and the upper states are created. The reaction proceeds to the product state $(RC \cdot c_2^+)$ with competition from the back reaction of P^+ to P. This latter pathway is slow ($k_b \simeq 10\ s^{-1}$), however, and does not normally interfere with the measured cytochrome kinetics. Values for the other rate constants can be obtained from the present work, as follows:

$$k_{12} \simeq 8 \times 10^8\ M^{-1}\ .s^{-1}$$
$$k_{21} \simeq 10^3\ s^{-1}$$
$$k_{23} \simeq 3 \times 10^3\ s^{-1}$$
$$k_{34} \simeq 10^6\ s^{-1}$$

$$k'_{12} \leqslant 10^6\ M^{-1}\ .s^{-1}$$
$$k'_{21} \leqslant 10\ s^{-1}$$
$$k'_{23} \simeq k'_{32}$$

The (net) forward rate constants k_{34} and k_{23} were obtained from the halftimes of the fast phase and the pseudo first order limit to the slow phase, respectively. The association rate constant in the light, k_{12}, was obtained from fig.2b. The dissociation rate constant for the unactivated reaction center and reduced cytochrome, k'_{21}, was estimated to have an upper limit of $10\ s^{-1}$ from the light saturation experiment.

The existence of the state $(RC^+ \cdot c_2)$, formed from $(RC \cdot c_2)$, is indicated by the fast phase of oxidation of cytochrome c_2 at high concentrations. The pseudo first order limitation in the second order phase of oxidation indicates another state, $(RC \cdot\cdot c_2)$. At high concentrations the kinetics are half fast ($t_{1/2} \simeq 1$ μs) and half slow (pseudo first order, $t_{1/2} \simeq 400$ μs). Thus, the dark equilibrium constant between the states $(RC \cdot c_2)$ and $(RC \cdot\cdot c_2)$ is ~1 and $k'_{23} \simeq k'_{32}$. This is fully consistent with the apparent binding constant for the fast phase of $10^5\ M^{-1}$, which is very similar to the net binding constant of $3 \times 10^5\ M^{-1}$

for cytochrome c_2, determined directly [14]*. Taking 10^5 M^{-1} for the elementary binding step in the dark and a dissociation rate constant (k'_{21}) $\leqslant 10$ s^{-1}, we obtain $k'_{12} \leqslant 10^6$ M^{-1} s^{-1}.

The redox midpoint potentials of cytochromes c and c_2 are both about 50 mV lower in vivo compared to in vitro, indicating a 5–7-fold stronger binding of the oxidized form to some membrane component [4,15–17]. For cytochrome c this has been confirmed by direct binding studies on stripped mitochondrial membranes [18]. On the other hand, there is no indication that the redox midpoint potential of P/P^+ shifts significantly in the presence and absence of cytochrome c_2 [19]. It would seem, therefore, that the binding of oxidized cytochorme c_2 is independent of the redox state of the reaction center. The relative midpoint potentials of the primary donor and cytochrome c_2 preclude the possibility of an analogous experiment with reduced cytochrome c_2. Nevertheless, if we suppose that the same is true for reduced cytochrome c_2, then the binding constant for P^+ and c_2 will also be about 3×10^5 M^{-1}. Since the association rate constant was directly determined as 8×10^8 M^{-1} $.s^{-1}$, we obtain $k_{21} \simeq 10^3$ s^{-1}. This is in good agreement with the value recently obtained for reaction centers and cytochrome c_2 from *Rhodospirillum rubrum* [10].

The estimates for the association and dissociation rate constants in the dark (k'_{12}, k'_{21}) and immediately following the flash (k_{12}, k_{21}) differ by ~2 orders of magnitude, while maintaining the binding constant roughly the same. This is a rather larger difference than can be readily accommodated by electrostatic considerations, although such effects do tend to change the on and off rates similarly [20]. We have recently found that the higher values of the association and dissociation rate constants are observed for reaction centers in negatively charged phospholipid vesicles and are a sensitive function of ionic strength and temperature ([3], unpublished observations). Thus, we are led to suggest that this effect is real and is due, in part, to changes in the ionic interactions between the reaction center and the cytochrome.

* For the linear scheme:

$$RC + c \underset{}{\overset{K_1}{\rightleftharpoons}} RC \cdot\cdot c \overset{K_2}{\rightleftharpoons} RC \cdot c$$

the net binding of c;

$$\frac{[RC \cdot\cdot c) + (RC \cdot c)]}{[RC] \cdot [c]}$$

is given by:

$$K_{net} = K_1 (1 + K_2)$$

4.2. *Relationship to the in vivo mechanism*

Due to the lack of data on the concentration dependence of the in vivo cytochrome c_2 oxidation, it has not been possible to describe the mechanism of interaction with the reaction center in vivo. This study has established the properties of the in vitro reaction and we have been able to describe the origin of the various kinetic phases in terms of plausible mechanisms consistent with known structural and ionic properties of the reactants. In view of the close similarity of the in vivo kinetics, it seems to us likely that the same properties play a role in the in vivo oxidation. It is noteworthy that the characteristics of the in vivo oxidation that have led to the suggestion of a tight association [4,12] are common to the solutional interaction where a second order mechanism prevails. The upper limit to the binding constant for cytochrome c_2 to the reaction center, which we find to be 10^5 M^{-1}, is weaker than that which we have found for the binding to phosphatidylserine vesicles ($K \simeq 10^7$ M^{-1}, data not shown). It is also weaker than the value found ($K \sim 10^6$ M^{-1}) for the binding of cytochrome c to the stripped mitochondrial membrane [18]. As the binding of cytochrome c has been shown to be electrostatic, we may consider that binding to the membrane itself does occur.

The rereduction kinetics of cytochrome c_2 in vivo have been shown to be second order [12], establishing the reaction as collisional. However, the occurrence of a collisional mechanism in the oxidation kinetics may be obscured as, for example, in solution where the kinetics of the oxidation at high concentration are apparently first order. It is not quite clear whether the limit to the second order phase is due to binding of the cytochrome to the reaction center or to a rate limiting step in their approach. The sharpness of the break suggests that the latter may also be occurring. Furthermore, the apparent lack of 'mobility' of the cytochrome c_2 in vivo does not provide evidence for a tight complex, as we have found the same result

in vitro under conditions when tight binding was demonstrably absent and the reaction was proceeding via a collisional mechanism. Thus the evidence for the in vivo function of cytochrome c_2 as a mobile electron carrier is increasing.

The scheme we have presented describes the cytochrome c_2 and reaction center interaction both in solution and in vivo. It is not yet complete due, especially, to the difficulty in directly measuring the dissociation constant k_{21} and to the use of net rate constants for k_{23} and k_{34}. However, the dark dissociation constant k'_{21}, which applies to the non-oxidized reaction center and the reduced cytochrome, is an accessible parameter and we have shown that the slowness of this dissociation is not an indication of binding. In a forthcoming paper, we will show that a negatively charged membrane can influence this parameter and the resulting mobility of the cytochrome.

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References

[1] Dutton, P. L. and Prince, R. C. (1978) in: The Photosynthetic Bacteria (Clayton, R. K. and Sistrom, W. R. eds) ch. 28, Plenum Press, New York.
[2] Dutton, P. L., Petty, K. M. and Prince, R. C. (1976) Fed. Proc. Exp. Biol. 35, 1597. (abstr. no. 1214).
[3] Overfield, R. E. and Wraight, C. A. (1978) Biophys. J. 21, 9a; (1979) 25, 62a.
[4] Dutton, P. L., Petty, K. M., Bonner, H. S. and Morse, S. D. (1975) Biochim. Biophys. Acta 387, 536–556.
[5] Ke, B., Chaney, T. H. and Reed, D. W. (1970) Biochim. Biophys. Acta 216, 373–383.
[6] Wraight, C. A. (1979) Biochim. Biophys. Acta in press.
[7] Bartsch, R. G. (1971) Methods Enzymol. 23, 344–383.
[8] Meyer, T. E. (1970) PhD Thesis, University of California at San Diego.
[9] Taylor, W. B., LeBlanc, J. C., Whillans, D. W., Herbert, M. A. and Jones, H. S. (1972) Rev. Sci. Inst. 43, 1797–1799.
[10] Rickle, G. K. and Cusanovich, M. (1979) Arch. Biochem. Biophys. in press.
[11] Prince, R. C., Cogdell, R. J. and Crofts, A. R. (1974) Biochim. Biophys. Acta 347, 1–13.
[12] Prince, R. C., Bashford, C. L., Takamiya, K., Van den Berg, W. H. and Dutton, P. L. (1978) J. Biol. Chem. 253, 4137–4142.
[13] Van Grondelle, R., Duysens, L. N. M. and Van der Wal, H. N. (1976) Biochim. Biophys. Acta 449, 169–187.
[14] Rosen, D., Okamura, M. Y. and Feher, G. (1979) Biophys. J. 25, 204a.
[15] Pettigrew, G. W., Meyer, T. E., Bartsch, R. G. and Kamen, M. D. (1975) Biochim. Biophys. Acta 430, 197–208.
[16] Prince, R. G. and Dutton, P. L. (1977) Biochim. Biophys. Acta 459, 573–577.
[17] Dutton, P. L., Wilson, D. F. and Lee, C. P. (1970) Biochemistry 9, 5077–5082.
[18] Vanderkooi, J., Erecinska, M. and Chance, B. (1973) Arch. Biochem. Biophys. 154, 219–229.
[19] Prince, R. C. and Dutton, P. L. (1978) in: The Photosynthetic Bacteria (Clayton, R. K. and Sistron, W. R. eds) ch. 24, Plenum Press, New York.
[20] Eigen, M. (1974) in: Quantum Statistical Mechanism in the Natural Sciences, Plenum, Press, New York.
[21] DeVault, D. (1978) Methods Enzymol. 54E, 32–46.