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Effect of temperature on the kinetics of electron transfer from the tetraheme cytochrome to the primary donor in *Rhodopseudomonas viridis*

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The kinetics of electron transfer from the third highest potential heme (c-552, E_m = +20 mV) to the primary donor (P-960) have been measured by flash absorption spectroscopy in isolated reaction centers of *Rhodopseudomonas viridis* between 300 K and 7 K. The data are analyzed on the basis of three exponential components with a very fast phase ($t_{1/2}$ = 120 ns) dominating at high temperature and a very slow one ($t_{1/2}$ = 1.2 ms) at low temperature. This multiphasic behavior is interpreted in terms of the existence of three states with a temperature-dependent population and a very limited effect of the temperature on the kinetics for each state.

Photosynthesis; Electron transfer; Cytochrome oxidation; Reaction center; Low temperature; Conformational state; *Rhodopseudomonas viridis*

1. INTRODUCTION

The reaction center of the purple bacterium *Rhodopseudomonas viridis* appears to be an ideal system for an in-depth study of biological electron transfer. Such a study indeed requires a good knowledge of functional and structural properties of the biological material. The 3D structure of the reaction center of this bacterium has been elucidated with high atomic resolution [1,2], and the functional properties can be studied by time-resolved absorption spectroscopy following excitation by a short laser flash. Electron transfer from the primary donor P-960 (hereafter named P) to electron acceptors has been resolved in great detail, down to the femtosecond time scale. When P has been oxidized as a consequence of the primary photochemical reaction (P^+), it is then re-reduced by a tightly bound tetraheme cytochrome; this reaction, although slower and of great potential interest, has not yet been studied in detail. It has been clearly established that the electron donation is quite fast ($t_{1/2} \approx 220$ ns) when only the high-potential hemes (c-559 and c-556, labelled according to the wavelength of the maximum of the α-band) are reduced, and that it becomes even faster ($t_{1/2}$ = 120 ns) when the low potential heme c-552 is also reduced [3,4]. In the latter case, it is c-552 which is the electron donor to P^+.

Temperature is an important parameter in studying the mechanism of electron transfer in biological systems, since it permits one to obtain information on the activation and reorganization energies, and to define conditions where various theoretical treatments can be applied. In this respect, DeVault and Chance [5] discovered in 1966 an unique temperature dependence of the rate constant for the reduction of P^+ by a tetraheme cytochrome c in the photosynthetic bacterium *Chromatium vinosum* poised at low redox potential. The reaction shows thermal activation above 120 K and near temperature independence below. This remarkable behavior has largely influenced the development of electron transfer theories over the last two decades, giving rise to a large number of theoretical treatments, which are still being continued (see e.g. refs. [6–11]). It is important to note, however, that the most recent quantitative analysis of kinetic data obtained with *Chromatium vinosum* has been made on the basis of the 3D structure of the *Rps. viridis* reaction center. Indeed, the effect of temperature on the kinetics of electron transfer from c-552 to P^+ has not been studied in *Rps. viridis*. In this work we have studied this effect, and found a behavior quite different from that previously reported.

2. MATERIALS AND METHODS

Reaction centers were isolated and purified from *Rps. viridis* according to [12]. The concentration was determined spectrophotometrically using the value $\Delta\varepsilon$ = 300 $mM^{-1} \cdot cm^{-1}$ at 830 nm [13].

For spectroscopic measurements at low redox potential, samples were prepared with 2 μM reaction centers in 40 mM Tris-buffer (pH 8.0) and 100 μM of the redox mediators diaminodurene, duroquinone, vitamine K3 and 2,5-dihydroxi-*p*-benzoquinone. After degassing the sample for 15 min with nitrogen, solid ascorbate was added to an approximate final concentration of 30 mM, prior to another degassing for 15 min. The reduction of the three highest potential hemes (c-559, E_m = +380 mV; c-556, E_m = +310 mV; c-552, E_m = +20 mV) was checked by the appearance of the α-peak of reduced heme c-552 in the

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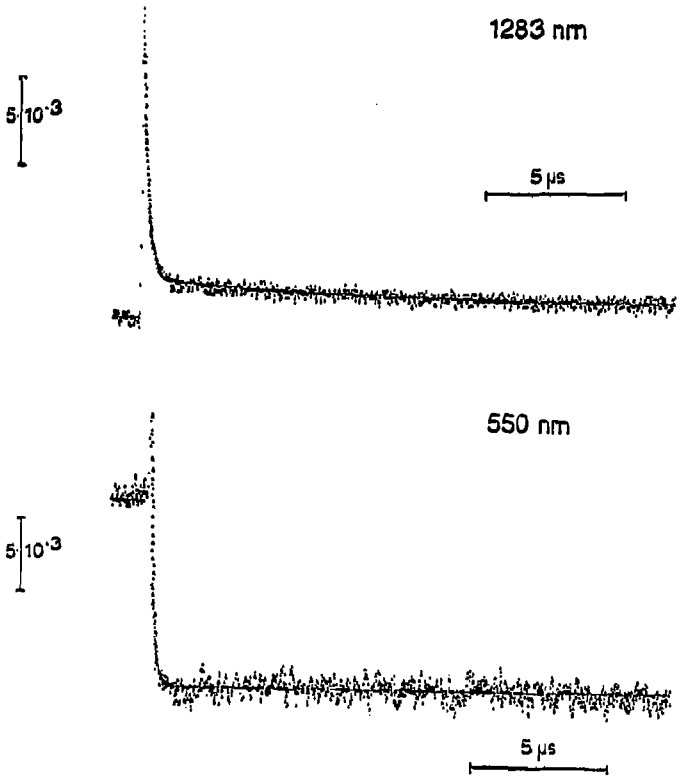

Fig. 1. Kinetics of flash-induced absorption changes at 1283 nm and at 550 nm at low redox potential with *Rps. viridis* reaction centers, at 293 K. Top: the curve is the effect of a single laser flash. Bottom: the trace has been obtained by averaging 16 individual records.

difference spectrum of the sample prepared as above minus a sample with only the high-potential hemes c-559 and c-556 reduced (only 100 µM ascorbate, sample not degassed). For low-temperature measurements, glycerol was added to the reaction mixture to a final concentration of 60% (v/v).

Flash absorption kinetics were measured in two different spectral regions. To measure P^+ formation and reduction we used a CW laser diode emitting at 1283 nm. The cuvette was excited by a short flash (10 ns, 694 nm) provided by a ruby laser. The measuring light was focussed through the cuvette onto a germanium photodiode (ϕ = 0.1 mm), the output of which was amplified and measured with a transient digitizer. The time resolution of the measurements was 40 ns. Cytochrome oxidation was measured in the α-band at 550 nm. The cuvette was excited by short flashes (9 ns, 595 nm) provided by a dye laser pumped by a YAG laser equipped with a light frequency doubler. The measuring light was provided by a xenon flash with a nearly rectangular profile for the light emission in the 20-µs time range. The measuring light was filtered before the cuvette and before the detector (photomultiplier, ITT type F4102) by band-pass interference filters ($\Delta\lambda$ = 3 nm). The photomultiplier output was amplified and digitized with a time resolution of 50 ns. The laser flash was synchronized so as to fire on the top of the xenon flash. The cuvette, with optical paths of 10 mm for the measuring light and 4 mm for excitation, was inserted in a cryostat cooled with helium gas (250 to 7 K) or with a thermostated water/ethylene glycol mixture (300–240 K).

Measurements below −30°C are the result of a single flash given to a dark-adapted (2 min) sample cooled in darkness. At higher temperature the measurements are the average of 4–32 flashes (at 550 nm), or of a single flash (at 1283 nm), with a time spacing sufficient to allow a return to equilibrium (1 to 5 min).

Exponential analyses of the kinetic data were performed using the Marquardt method with a software devised by Dr. P. Sétif.

3. RESULTS

We have studied electron transfer kinetics from the low potential heme c-552 to the primary donor P^+ by measuring both P^+ reduction and cytochrome oxidation after a laser flash. Fig. 1 shows typical kinetic traces of flash-induced absorption changes in the P^+ region (1283 nm) and in the α-band of cytochrome (550 nm) at room temperature when the three highest potential hemes are reduced prior to the flash. At 1283 nm (Fig. 1, top), the absorption rises immediately after the flash (P^+ formation) and the subsequent decay curve was well fitted by the sum of three exponentials: a very fast phase (VF) with $t_{1/2}$ = 115 ns, a fast phase (F) with $t_{1/2}$ = 665 ns and a slow phase (S) with $t_{1/2}$ = 12 µs. At room temperature, the very fast component (VF) was dominant, accounting for 85% of the total amplitude. Control experiments with Q_A reduced allow us to attribute the slow phase to the triplet state of P (data not shown). At 550 nm (Fig. 1, bottom), the initial absorption increase due to P^+ formation was followed by a rapid decrease of absorbance due to cytochrome oxidation well fit by a single exponential with $t_{1/2}$ = 120 ns. The same type of experiments carried out at different wavelengths in the α-band of the cytochrome showed a clear negative peak at 550 nm (data not shown) which is characteristic of the oxidation of heme c-552. We also measured the kinetics of

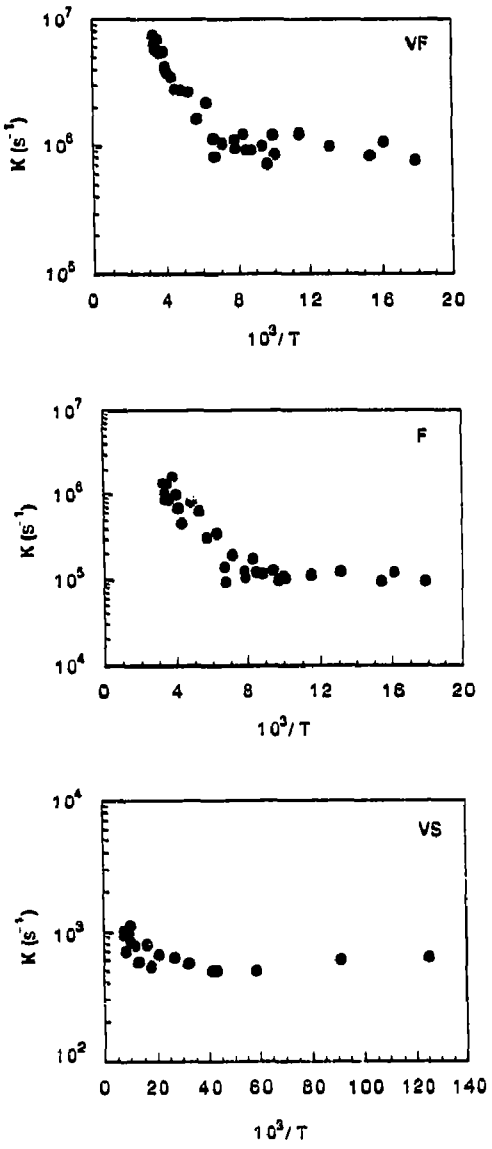

Fig. 2. Temperature dependence of the rates of the three different components of P^+ reduction measured at 1283 nm. Experiments analogous to those of Fig. 1 (top) were carried out at different temperatures. Top: very fast component (VF); middle: fast component (F); bottom: very slow component (VS).

P^+ reduction and cytochrome oxidation in the presence of glycerol (60%) as a control in view of low temperature measurements, and obtained identical results.

The temperature dependence of the kinetics of P^+ reduction following a single flash was measured between 300 K and 7 K (Fig. 2). The data were analyzed as a sum of exponential components. In the high-temperature range, three components were needed, in agreement with experiments at room temperature. The rate of the very fast component (Fig. 2, top) was 6.0×10^6 s^{-1} at 293 K and decreased with decreasing temperature to a rate of 1.1×10^6 s^{-1} at 150 K. The rate of the fast component (Fig. 2, middle) was 1.0×10^6 s^{-1} at 293 K and also decreased with decreasing temperature to a rate of 1.4×10^5 s^{-1} at 150 K. The rates of the two components became almost temperature-independent below 150 K. The activation energies for the very fast and fast components in the temperature-dependent region were 4.1 and 4.4 $kJ \cdot mol^{-1}$, respectively. Below 150 K, the absorption recovery at 1283 nm was well fitted by the sum of four exponentials: the very fast (VF) and fast (F) components, the slow component (S) and an additional very slow component (VS). The rate of this very slow component (Fig. 2, bottom) was 1.0×10^3 s^{-1} at 130 K and decreased slightly with decreasing temperature to a value of 5.8×10^2 s^{-1} at 77 K. Below this point, the rate became almost temperature-independent. Between 40 K and 7 K, it was not possible to distinguish between the two fastest phases because of their very small amplitudes. Below 150 K, a second flash (given 30 s after the first) induced a smaller absorbance change, and the recovery was well fitted by a single exponential with a $t_{1/2} = 59$ μs (1.2×10^4 s^{-1}) representing about 95% of the total amplitude and a very small constant (5%). This finding supports the proposal that the slow component (S) is due to the triplet state of P, ^3P. We have measured the kinetics of c-552 photo-oxidation at low temperature (data not shown). At 56 K, the decay curve was well fitted by a single exponential with a $t_{1/2}$ = 1.1 ms of about 90% of the total amplitude (spectrum with a clear negative peak at 550 nm) and small unresolved fast phases ($t_{1/2} < 70$ μs). From this result and those obtained at room temperature, it is possible to conclude that the very fast, fast and very slow phases are due to P^+ reduction by c-552 heme.

Fig. 3 shows how the amplitudes of the very fast, fast, and very slow components of the P^+ reduction vary with temperature. Above 150 K, the amplitudes of the very fast (VF) and fast (F) components exhibit antiparallel behavior: the amplitude of the VF component diminished with decreasing temperature (from 95% at 293 K to 50% at 150 K), while the amplitude of the F component increases with decreasing temperature (from 5% at 293 K to 50% at 150 K). In this temperature region the very slow component was not detectable. Below 150 K, both VF and F components decreased in parallel to a minimum value of 10%, while the slow component appeared and increased with decreasing temperature to a maximum value of 90% of the total P^+ reduction amplitude. Not taken into account in Fig. 3, the signal due to ^3P corresponded to 5–30% of the total amplitude at 1283 nm.

4. DISCUSSION

The results obtained in this work clearly show a large effect of temperature on the kinetics of electron transfer from the low-potential c-552 heme to the primary donor P-960. At room temperature, our results are in agreement with previous reports [3,4], but our improved signal-to-noise ratio provides a better accuracy on time

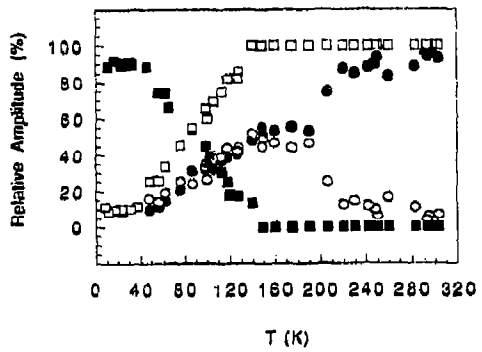

Fig. 3. Temperature dependence of the amplitudes of the different components of P^+ reduction. The data were taken from experiments used for Fig. 2. (●) very fast phase; (○) fast phase; (■) very slow phase; (○) sum of very fast and fast phases. For the calculation of the relative amplitude of each phase the sum of the amplitudes of very fast, fast and very slow phases was taken as the total P^+.

constants and evidence for several phases. The effect of temperature has not been studied in detail in *Rps. viridis*. Chance et al. reported a $t_{1/2}$ of 10 μs at 77 K [14], but this result was not included in a subsequent full paper [15]. Kaminskaya et al. [16] reported a $t_{1/2}$ of 20 μs at 77 K. Our results show that P^+ reduction obeys complex kinetics. The kinetics of c-552 oxidation could not be followed in as much detail because of a smaller signal-to-noise ratio. We have several reasons, however, to believe that P^+ is re-reduced by c-552: (i) there is a good fit of the kinetics in the temperature range from 300 K to 240 K; (ii) at 56 K, there is a good agreement between the very slow phases for P^+ reduction and for c-552 oxidation; (iii) the kinetics attributed to the reduction of P^+ by c-552 disappear after the first flash below 100 K, under conditions when c-552 is known to be a unique, efficient and irreversible donor [16,17]. In this discussion we do not consider the slow phase, the $t_{1/2}$ of which varies from about 10 μs at room temperature to 60 μs below 150 K, and which is due to ^3P (as will be discussed in detail in a separate paper).

Altogether the electron transfer kinetics from c-552 to P^+ vary considerably, from 120 ns at 293 K to 1.1 ms at 7 K. The detailed effect of temperature, however, is entirely different from that reported for *Chromatium vinosum* where a monoexponential reaction was assumed [5]. Our results are best interpreted by two effects of temperature. Firstly, there are three substates of the reaction center, which give rise to very fast, fast, and very slow electron transfer. Secondly, within each substate, the effect of temperature is rather weak as shown by the activation energy (4.1, 4.4 and below 1 kJ·mol^{-1}, respectively), and by the amplitude of variation (see Fig. 2). Temperature, however, changes dramatically the relative proportion of substates (Fig. 3). The concept of conformational substates in thermal equilibrium has been used [18] to describe the kinetics of CO rebinding after photodissociation.

We have no clue as to the difference between these putative conformational states. It is possible to hypothesize, following a discussion by Knapp et al. [19] that the state of water molecules located between the hemes and P may strongly influence electron transfer in a temperature-dependent manner. Conformational control has also been considered by Cartling [11]. It is clear that theoretical treatments of electron transfer from cytochrome to P^+ cannot be done in using the 3D structure of the *Rps. viridis* reaction center and the data obtained with *Chromatium vinosum* (experiments are now in progress with *Chromatium vinosum* chromatophores).

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