Electron Transfer from the Tetraheme Cytochrome to the Special Pair in Isolated Reaction Centers of *Rhodopseudomonas viridis*†

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Received September 8, 1992; Revised Manuscript Received November 10, 1992

ABSTRACT: Kinetics of electron transfer from the bound tetraheme cytochrome *c* to the primary donor (P) have been measured in isolated reaction centers of the purple bacterium *Rhodopseudomonas viridis* by time-resolved flash absorption spectroscopy. The influence of two major parameters has been studied: temperature (7–305 K) and the redox state of the cytochrome. Most experiments were done with one heme (*c*–559), two hemes (*c*-559 and *c*-556), or three hemes (*c*-559, *c*-556, and *c*-552) poised in a reduced state before the flash. Measurements were done at 1283 nm in the absorption band of P^+, and in the region of cytochrome α-bands. At room temperature, *c*-559 donates an electron to P^+ with a half-time of 115, 190, or 230 ns (with three, two, or one heme reduced, respectively) and is then eventually rereduced by *c*-556 ($t_{1/2}$ = 1.7 μs) or by *c*-552 (in less than 40 ns). The kinetics also include a minor microsecond phase of P^+ reduction. At decreasing temperatures, the polyphasic character of P^+ rereduction is accentuated. Fast phases (115 ns–10 μs) are slightly slowed down, following Arrhenius behavior with a weak activation energy (3.6–8.6 kJ·mol^{-1}), until they become temperature-independent. Their extent decreases rather sharply, at temperatures which vary according to the redox poising: 250, 210, or 80 K when one, two, or three hemes are reduced, respectively. In the last case, P^+ can still be reduced at low temperature, apparently directly by *c*-552 ($t_{1/2}$ = 1.1 ms, nearly temperature-independent). The effects of temperature on electron transfer from *c*-556 to *c*-559 and on the triplet-state ^3P have also been measured. The results are discussed with respect to the thermodynamic and spatial properties of cytochrome hemes. They indicate that the reaction center has several substates, the relative population of which is temperature-dependent. Within each substate, the kinetics have a weak temperature dependence in the high-temperature region, and they become temperature-independent at low temperature.

The photosynthetic reaction center (RC)[1] of the purple bacterium *Rhodopseudomonas viridis* appears to be an ideal model for studying the mechanism of electron transfer in biological systems. Its 3-D structure has indeed been solved with high atomic resolution [Deisenhofer et al., 1984, 1985; refined coordinates deposited in the Brookhaven Protein Data Bank, entry 1PRC, version of April (1989)], and its functional properties can be studied down to the subpicosecond time range by time-resolved absorption spectroscopy following excitation by a short laser flash. The RC from this bacterium contains 4 polypeptides (L, M, H, and cytochrome) and 14 major cofactors. Four heme groups are covalently attached to the cytochrome subunit. Four bacteriochlorophylls *b*, two bacteriopheophytins *b*, one menaquinone 9 (Q_A), one ubiquinone 9 (Q_B), one non-heme Fe, and one carotenoid molecule are associated with the subunits L and M. After excitation of the primary donor, a special pair of bacteriochlorophylls (P-960, hereafter named P), an electron is transferred to a bacteriopheophytin in about 1 ps, and then to the primary quinone acceptor Q_A in about 230 ps (Holten et al., 1978). Secondary electron transfer includes the transfer of an electron from Q_A to the secondary quinone acceptor Q_B in the microsecond domain and the rereduction of P^+ by the cytochrome.

Subunits L, M, and H are common to RCs from all purple photosynthetic bacteria, but the *Rps. viridis* RC also contains a tightly bound, four-heme cytochrome *c* subunit (Deisenhofer et al., 1984, 1985). The hemes of this bound cytochrome act as electron donors to the photooxidized special pair (Case et al., 1970; Prince et al., 1976). The oxidized hemes are themselves probably reduced by soluble cytochrome c_2 located in the periplasm (Shill & Wood, 1984). A similar tetraheme cytochrome *c* is likely to be present also in the RC from many purple bacteria. The bound cytochrome *c* is the largest subunit in the RC complex of *Rps. viridis* (Weyer et al., 1987). The three-dimensional structure of the RC shows that the four hemes of the cytochrome are arranged in a roughly linear sequence along the protein long axis with an Fe–to–Fe separation distance of 14–16 Å, the proximal heme being within 21 Å of P (Deisenhofer et al., 1984, 1985).

Individual spectral and redox characteristics of all four hemes of the *Rps. viridis* RC cytochrome have recently been resolved (Dracheva et al., 1986, 1988; Shopes et al., 1987; Fritzsch et al., 1989; Nitschke & Rutherford, 1989; Vermeglio et al., 1989; Alegria & Dutton, 1991). The E_m values for the four hemes are the following: *c*-554, –60 mV; *c*-556, +310 mV; *c*-552, +20 mV; *c*-559, +380 mV, where the different heme groups are denoted according to their α-band absorption maxima in room temperature difference spectra (Dracheva et al., 1986, 1988). Nitschke and Rutherford (1989) identified and characterized EPR signals from the hemes in terms of their redox, relaxation, orientation, and electron-transfer properties.

† J.M.O. was partly supported by the Spanish Government and the European Communities (Science Programme).

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[1] Abbreviations: RC, reaction center; P, primary electron donor; Q_A and Q_B, primary and secondary quinone acceptors, respectively; diaminodurene, 2,3,5,6-tetramethyl-*p*-phenylenediamine; duroquinone, tetramethyl-*p*-benzoquinone; E_m, midpoint redox potential; E_h, redox potential of medium; ΔA, absorbance change.

0006-2960/93/0432-1141$04.00/0

The cytochrome has recently been partly characterized with respect to electron-transfer kinetics at room temperature. It has been clearly established that after a short flash the electron donation from heme *c*-559 to P^+ is quite fast ($t_{1/2}$ about 220 ns) when only the high-potential hemes (*c*-559 and *c*-556) are reduced; P^+ reduction becomes still faster ($t_{1/2}$ around 120 ns) when the low-potential heme *c*-552 is also reduced (Holten et al., 1978; Shopes et al., 1987; Dracheva et al., 1988). In the latter case, it is *c*-552 which appears to be the electron donor. Photooxidation of *c*-556 via *c*-559 ($t_{1/2}$ about 2 μs) has also been demonstrated by room temperature kinetic studies (Shopes et al., 1987; Dracheva et al., 1988). A faster electron transfer to P^+ from the low-potential hemes than from high-potential ones has also been reported in other purple bacteria (Case et al., 1970; Parson & Case, 1970; Seibert & De Vault, 1970; Kononenko et al., 1974).

On the basis of structural, physicochemical, and rapid kinetics data, the following sequence of cytochrome hemes has been suggested by most of the groups working in this subject (Shopes et al., 1987; Dracheva et al., 1988; Nitschke & Rutherford, 1989; Fritzsch et al., 1989; Vermeglio et al., 1989): *c*-554 *c*-556 *c*-552 *c*-559 P. According to this model, the high-potential heme *c*-559 is the only redox center capable of direct electron donation to P^+, and all other hemes can be photooxidized only indirectly via *c*-559. However, this proposal is questioned by several experimental observations. First, electron transfer to P^+ is faster from heme *c*-552 than from heme *c*-559, as mentioned above: this difference does not agree with the proposed arrangement unless *c*-559 acts as an intermediate in the oxidation of *c*-552, a property that has not yet been evidenced. Second, according to the classical data of several laboratories obtained on a number of bacterial species, one low-potential heme remains fully photooxidizable at low temperatures (Vredenberg & Duysens, 1964; De Vault & Chance, 1966; De Vault et al., 1967; Kihara & Chance, 1969; Kihara & Dutton, 1970; Dutton et al., 1970, 1971; Dutton, 1971; Tiede et al., 1976; Nitschke & Rutherford, 1989) whereas a major part of the high-potential hemes is no longer capable of efficient electron donation to P^+ (Vredenberg & Duysens, 1964; Dutton et al., 1970, 1971; Dutton, 1971; Rubin et al., 1989; Nitschke & Rutherford, 1989). This fact has long served as an argument for the concept of independent and parallel pathways for low- and high-potential heme oxidation by P^+, a concept which could also account for room temperature results showing that a faster electron donation from low-potential hemes than from high-potential hemes takes place in many bacterial species (Parson & Case, 1970; Seibert & De Vault, 1970; Kononenko et al., 1974; Tiede et al., 1976; Dutton & Prince, 1978; Bixon & Jortner, 1986a,b, 1989).

De Vault and Chance (1966) discovered a unique temperature dependence for the rate constant of P^+ reduction by a tetraheme cytochrome *c* in the photosynthetic bacterium *Chromatium vinosum* poised at low redox potential. The rate constant for electron transfer exhibits a sharp transition from a high-temperature activated region, where it drops by 3 orders of magnitude, to a temperature-independent region at low temperatures ($T < 120$ K), where it remains nearly constant (De Vault & Chance, 1966; Dutton et al., 1971; Hales, 1976). The characteristic features of this reaction were attributed to the transition from temperature-independent nuclear tunnelling at low temperatures to an activated process at high temperatures (Hopfield, 1974; Jortner, 1976; Kuznetsov et al., 1978; Dogonadze & Zakaraya, 1987). This remarkable behavior has greatly influenced the development of electron-transfer theories over the last 2 decades, giving rise to a large number of theoretical treatments, which are still being continued (see, e.g., Bixon & Jortner, 1986a,b, 1988, 1989; Kuhn, 1986; Knapp & Fischer, 1987; Knapp & Nilsson, 1990; Cartling, 1991).

It is important to notice, however, that most of the data on the low-temperature cytochrome *c* photochemistry have been obtained on bacteria other than *Rps. viridis* for which the three-dimensional structure and the most detailed characterization of the individual heme's redox and spectral properties are available. Quantitative and theoretical analyses of kinetic data obtained with *Chromatium vinosum* have also been made on the basis of the 3-D structure of the *Rps. viridis* RC. The effect of temperature on the kinetics of electron transfer from cytochrome to P^+ has not been studied in detail in *Rps. viridis*. In this respect, it is possible to find in the literature only fragmentary and rather controversial data. Shopes et al. (1987) have reported a $t_{1/2}$ for the cytochrome oxidation of 1.94 ms at 125 K when the two high-potential hemes are reduced. Nitschke and Rutherford (1989) found, however, that high-potential hemes are not stably photooxidized at low temperatures (4 K). Kaminskaya et al. (1990) recently reported a rapid cytochrome photooxidation at 77 K when only *c*-559 heme was reduced before the flash (in fact, it seems highly probable that *c*-556 was also partially reduced in their experiments) but with a small extent (20% of the total P^+ rereduction). That reaction took place within the apparatus rise-time limited by the 6-μs half-width of the exciting flash. Chance et al. (1969) and Kaminskaya et al. (1990) have reported $t_{1/2}$ values of 10 and 20 μs for the photooxidation of cytochrome at low redox potential (with the three highest potential hemes reduced) at 77 K.

In the present work, we have investigated in more detail the kinetic and amplitude behavior of electron transfer from cytochrome hemes to P^+ at room temperature and the temperature dependence of these reactions using RC preparations from *Rps. viridis* poised under different redox conditions. We have measured cytochrome photooxidation with only the *c*-559 heme reduced, with the two high-potential hemes *c*-556 and *c*-559 reduced, and with the three highest potential hemes, *c*-552, *c*-556, and *c*-559, reduced before the flash. We have not measured the kinetics of cytochrome photooxidation under the conditions where all four hemes are reduced because these are conditions where it is very difficult to avoid the accumulation of reduced Q_A. The kinetic behavior observed when three hemes are reduced has been partly published earlier (Ortega & Mathis, 1992). In this report, we provide a full account of our results.

EXPERIMENTAL PROCEDURES

Cultures of *Rps. viridis* cells were grown according to Cohen-Bazire et al. (1957). Chromatophore membranes were prepared by passing the cells through a French press, followed by centrifugation steps to remove soluble components and cell debris. RCs were prepared according to Breton (1985). The RC concentration was determined spectrophotometrically using the value $\epsilon = 300\ mM^{-1}\ cm^{-1}$ at 830 nm (Clayton & Clayton, 1978). It is of note that nearly all experiments were done with LDAO (0.005%), which precluded addition of dithionite. Only one experiment, on the triplet state of P, was done after exchange of LDAO with Triton X-100.

The redox potential was measured with a platinum electrode versus a calomel reference electrode. The solution potential was varied by additions of ferricyanide and ascorbate. The redox titrations of the high-potential cytochrome *c* hemes were carried out aerobically and those of the low-potential hemes anaerobically under a stream of nitrogen gas.

For spectroscopic measurements at high redox potential, samples were prepared with around 2 μM RCs in 40 mM Tris

buffer (pH 8.0) and the redox mediators diaminodurene (100 μM), vitamin K_3 (100 μM), *N,N'*-phenylenediamine (50 μM), and ferricyanide (approximately 100 μM). At moderate redox potential, samples were prepared with 2 μM RCs in 40 mM Tris buffer (pH 8.0) and 100 μM of the redox mediators diaminodurene, vitamin K_3, and ascorbate. For spectroscopic measurements at low redox potential, samples were prepared with around 2 μM RCs in 40 mM Tris buffer (pH 8.0) and 100 μM of the redox mediators diaminodurene, duroquinone, vitamin K_3, and 2.5-dihydroxy-*p*-benzoquinone. After the sample was degassed for 15 min, solid ascorbate was added to an approximate final concentration of 30 mM prior to another degassing for the same time period. 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 difference spectrum of the sample above minus a sample with only the high-potential hemes *c*-559 and *c*-556 reduced (only 100 μM ascorbate). The reduction of heme *c*-552 is clearly shown by the difference spectra and agrees with previous results from Nabredryk et al. (1991). These authors obtained a *c*-552 reduction with 30 mM ascorbate; we used the same concentration of reductant, and furthermore took care of removing oxygen from our sample. For low-temperature measurements in the three redox conditions described above, glycerol was added to the reaction mixture to a final concentration of 60% (v/v).

Static absorption spectra of RCs at room temperature were recorded in a Shimadzu (Model UV 160) spectrophotometer. Flash absorption kinetics of electron transfer from cytochrome to P^+ were measured in two different spectral regions. To measure P^+ formation and rereduction, we used a CW laser diode emitting at 1283 nm where P^+ has a broad absorption band peaking around 1320 nm [see, e.g., Olson et al. (1985) or Breton et al. (1992)] and where P has no absorption, a feature which avoids any actinic effect of the measuring light. The cuvette was excited by short flashes (10 ns, 694 nm) provided by a ruby laser. The measuring light was focused through the cuvette onto a germanium photodiode (ϕ = 0.1 mm), the output of which was amplified and measured with a transient digitizer. When needed (i.e., for measurements at 1283 nm at low temperature), the 2048 channels of the memory were grouped with different times/channel (e.g., 10 ns, 100 ns, 1 μs, 10 μs) in order to cover an appropriate time span with enough short time resolution. The time resolution of the measurements was 40 ns. The good signal-to-noise ratio allowed us to obtain single-turnover traces of sufficient quality without averaging. Cytochrome oxidation was measured in the 540–570-nm region. 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 (from 250 to 7 K) or with a thermostated water–ethylene glycol mixture (305–240 K). Measurements below 243 K are the result of a single flash given to a dark-adapted (2 min at room temperature) sample cooled in darkness. At higher temperature, the measurements are the average of 4–32 flashes (540–570-nm region), or of a single flash (at 1283 nm), with a time spacing sufficient to allow a return to equilibrium (1–5 min).

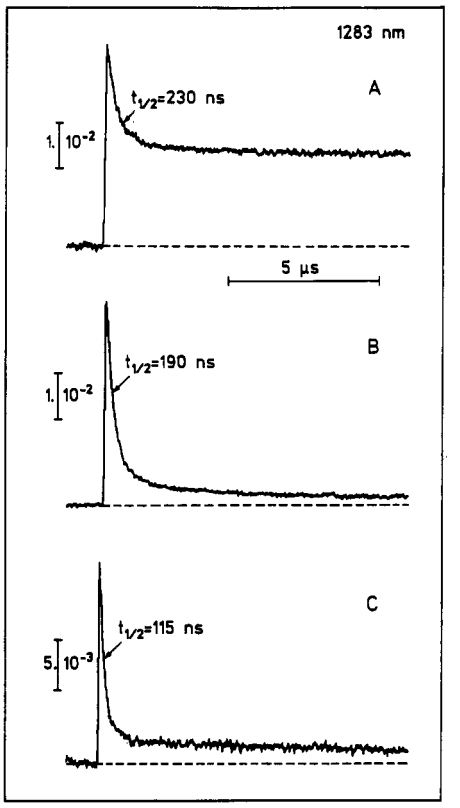

FIGURE 1: Kinetics of flash-induced absorption changes at 1283 nm in *Rps. viridis* RC under three different redox conditions, at 293 K. The basic medium for the three redox conditions is described under Experimental Procedures. (A) E_h = +380 mV; (B) E_h = +250 mV; (C) E_h = −20 mV. The curves are the effect of a single laser flash. The solid lines result from the computer exponential analysis; the $t_{1/2}$ values are for the dominant fast phase.

The traces obtained in spectrophotometric measurements were treated as sums of several exponential components to find $t_{1/2}$ values (or rates: $k = 0.69 \times t_{1/2}^{-1}$) and amplitudes. Exponential analyses were performed using the Marquardt method with software devised by Dr. P. Sétif.

RESULTS

Electron Transfer at Physiological Temperatures: Spectra and Kinetics. We have studied at room temperature electron-transfer kinetics from cytochrome *c* to the primary donor P^+ by measuring both P^+ rereduction and cytochrome *c* oxidation after a laser flash. Figure 1 shows typical kinetic traces of flash-induced absorption changes in the P^+ region (1283 nm) under three different redox conditions. At a redox potential where only the high-potential heme *c*-559 was reduced (and only partly—about 50%—implying that *c*-556 is at least 95% oxidized) prior to the flash (Figure 1A; E_h = +380 mV), the absorption rises immediately after the flash (P^+ formation), and the subsequent decay curve (P^+ rereduction) was well fitted by the sum of three exponential components: a very fast phase (VF) with $t_{1/2}$ = 230 ns, a fast phase (F) with $t_{1/2}$ = 2.1 μs, and a slow phase ($t_{1/2}$ above 0.5 ms). When the two high-potential hemes (*c*-559 and *c*-556) were reduced prior to the flash (Figure 1B; E_h = +250 mV), the decay curve of P^+ rereduction was well fitted by the sum of the three exponential components: a very fast phase (VF) with $t_{1/2}$ = 190 ns, a fast phase (F) with $t_{1/2}$ = 1.5 μs, and a slow phase (S) with $t_{1/2}$ = 20 μs. At low redox potential where the three highest potential hemes were reduced prior to the flash (Figure 1C; E_h = −20 mV), the decay curve of P^+ rereduction was also

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 at moderate and low redox potential, accounting for about 85% of the total amplitude of P^+ rereduction. At high redox potential (Figure 1A), only about half of the *c*-559 heme was reduced (E_m = +380 mV), and the very fast phase represented 45% of the total amplitude. The very slow phase occurring in that case is probably due to the back-reaction between P^+ and Q_A^-. We did not study its kinetic properties [see, however, Shopes and Wraight (1987) or Sebban and Wraight (1989)]. It is appropriate to point out that the initial ΔA is smaller in Figure 1C than in Figure 1A or Figure 1B (25–50%). This decrease is presumably due to the partial (25–50%) prereduction of Q_A. It is associated with a flash-induced formation of ^3P, as it will be shown more clearly in low-temperature experiments.

Figure 2 shows kinetic traces of cytochrome *c* oxidation by photogenerated P^+ in RCs under the three redox conditions described above, and the difference spectra of this reaction at two times after the flash. Figure 2A reports data obtained at E_h = +380 mV, where only the high-potential heme (*c*-559) was partly reduced prior to the flash. At 559 nm, which is the absorption maximum of the high-potential heme *c*-559, the initial absorption increase due to P^+ formation was followed by a decrease of absorbance well fitted by the sum of two exponential components: a fast phase with $t_{1/2}$ = 275 ns and a slow phase too slow to be resolved on the time scale used. The same type of experiments carried out at different wavelengths in the α-band of the cytochrome (Figure 2A) showed a clear negative peak at 560 nm in difference spectra measured either 1 μs or 19 μs after the flash. This bleaching, which is characteristic of the oxidation of heme *c*-559, is surperimposed on a positive background due to P^+ (this is a reasonable assumption since only about 50% of heme *c*-559 is prereduced).

At moderate redox potential (E_h = +240 mV), where the two high-potential hemes *c*-559 and *c*-556 were reduced prior to the flash, the kinetics of the photoinduced absorbance changes following the initial absorbance increase were clearly biphasic when measured at the maximum of the high-potential hemes *c*-559 and *c*-556 (Figure 2B). At 559 nm, a rapid absorbance decrease (cytochrome oxidation) with $t_{1/2}$ = 220 ns was followed by a slow absorbance increase with $t_{1/2}$ = 1.7 μs. At 556 nm, a slower phase of heme oxidation with a $t_{1/2}$ = 1.7 μs was observed following the initial rapid absorbance decrease. Figure 2B shows the spectrum of absorption changes measured at two time intervals. The spectrum measured 1 μs after the flash showed a minimum at 560 nm (with a shoulder on the short-wavelength side) which is characteristic of heme *c*-559 oxidation. Under the same conditions, the spectrum observed 19 μs after the flash showed a minimum at 556 nm, characteristic of heme *c*-556 oxidation. There was no significant net increase of cytochrome oxidation amplitude within the 1–19-μs interval, which indicates that the slow *c*-556 oxidation does not merely add to the *c*-559 oxidation but rather is accompanied by a stoichiometric reduction of the latter. These results allow us to conclude that when both high-potential hemes are reduced before the flash, it is heme *c*-559 which serves as an immediate electron donor to P^+ with a $t_{1/2}$ = 190 ns (we give more confidence to the times obtained from P^+ measurements at 1283 nm, with a better signal-to-noise ratio than in the cytochrome region). The secondary reaction with $t_{1/2}$ = 1.7 μs then likely involves *c*-556 oxidation with a concomitant reduction of *c*-559.

Figure 2C shows the kinetic measurements of cytochrome photooxidation by P^+ at a redox potential (E_h = −20 mV) at which also the third heme *c*-552 (E_m = +20 mV) was reduced before the flash, and the spectrum of this reaction at two different time intervals. At 552 nm (Figure 2C), the initial rapid absorbance increase due to P^+ formation was followed by an absorbance decrease well fitted by one single exponential with $t_{1/2}$ = 126 ns. At 559 nm (Figure 2C), the absorbance decrease was well fitted by the sum of two exponential components: a fast phase ($t_{1/2}$ = 125 ns) and a slow phase not very well resolved on the time scale used ($t_{1/2}$ = 2–15 μs). The spectrum of the flash-induced cytochrome photooxidation under these conditions showed a minimum at 552 nm when measured 1 or 19 μs after the flash. In some experiments at low redox potential, at 552 nm a small absorbance increase with a $t_{1/2}$ = 2 μs has been observed after the rapid cytochrome photooxidation (see Figure 2C, 552 nm). This is likely due to the low-potential interheme electron transfer (*c*-554 to *c*-552). We have not measured the kinetics of cytochrome photooxidation under the conditions where all four hemes were fully reduced: at redox potentials below the E_m of *c*-554 (−60 mV), there is an important attenuation of the photoreactions because Q_A is significantly reduced before the flash and an increasing spectral contribution of the triplet state of P (data not shown, obtained after exchange of LDAO with Triton X-100).

We have measured the kinetics of P^+ reduction and cytochrome oxidation in the presence of glycerol (60%) as a control in view of low-temperature experiments, and obtained identical results (data not shown; the trace in Figure 7A, was obtained with glycerol, however).

Temperature Dependence of Electron Transfer between Cytochrome and P$^+$. We have studied the effects of temperature on the kinetics of P^+ rereduction and cytochrome oxidation following a single flash between 300 and 8 K under the three different redox conditions described in the room temperature experiments.

(*i*) *Effect of Temperature at High Redox Potential.* The temperature dependence of the kinetics of P^+ rereduction following a single flash was measured between 300 and 205 K at a redox potential (E_h = +380 mV) where only the high-potential heme *c*-559 is only about 50% reduced prior to the flash (the redox conditions were chosen in order to have a negligible proportion of RCs with two hemes reduced). The data were analyzed as a sum of exponential components (Figure 3A). In the measured temperature range, three components were needed, in continuity with experiments at room temperature. The rate of the very fast component (Figure 3A, VF) was 3.0×10^6 s^{-1} ($t_{1/2}$ = 230 ns) at 293 K and decreased with decreasing temperature to a rate of 1.4×10^6 s^{-1} at 205 K. The rate of the small fast component (Figure 3A, F) was 3.3×10^5 s^{-1} ($t_{1/2}$ = 2.1 μs) a 293 K and also decreased with decreasing temperature to a rate of 1.4×10^5 s^{-1} at 245 K. In this series of experiments, the rate of the very slow phase could not be measured correctly because we did not use breakpoints on the digitizer. The data in Figure 3A correspond to upper limits for the rate of that very slow phase. Below 200 K, it was not possible to measure significantly the two fastest phases, since the very slow component makes up almost 100% of the total amplitude of the P^+ rereduction [in another series of measurements, using a slower time scale for the digitizer, we found that its half-time is temperature-independent, at 4.8 ms, in good agreement with the $P^+Q_A^-$ back-reaction studied by Shopes and Wraight (1987) and by Sebban and Wraight (1989)]. The activation energies for the very fast and fast components were 6.7 and 8.6 kJ·mol^{-1}, respectively (Figure 3B).

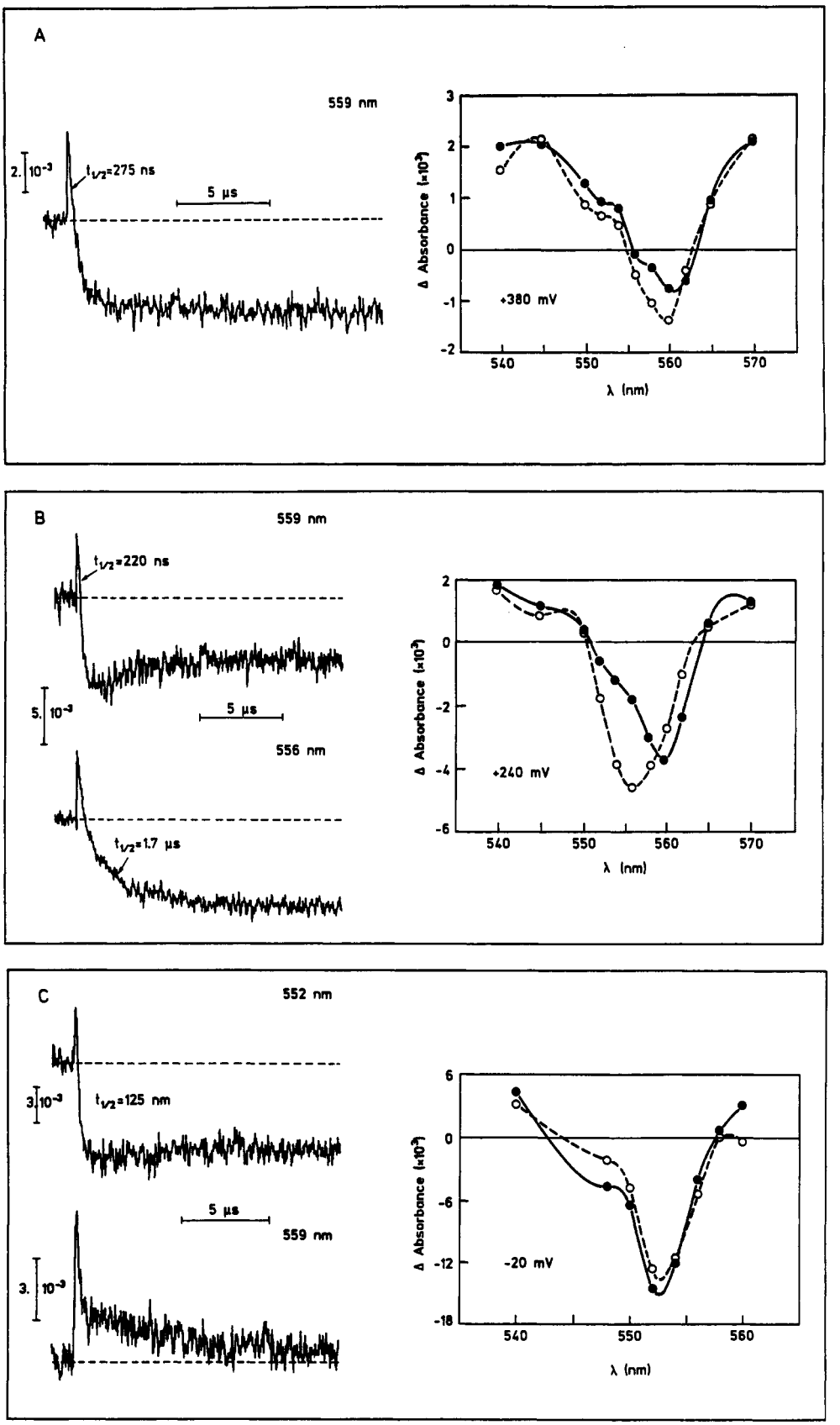

FIGURE 2: Kinetics and spectra of flash-induced absorption changes at 293 K in the α-band of cytochrome c under three different redox conditions: (A) E_h = +380 mV; (B) E_h = +240 mV; (C) E_h = −20 mV. The basic medium is described under Experimental Procedures. Left: the traces have been obtained by averaging 16 individual records. Solid lines and $t_{1/2}$ values are the results of the computer exponential analysis. Right: spectra of the absorption changes measured 1 μs (filled circles) and 19 μs (open circles) after the flash (flash energy was different from that used for the kinetic traces).

Figure 3C shows how the amplitudes of the very fast, fast, and very slow components of the P^+ reduction vary with temperature. The amplitude of the very fast component diminished with decreasing temperature (from 45% at 293 K to 4% at 205 K), and the amplitude of the fast component also decreased with decreasing temperature (from 8% at 293 K to 0% at 230 K). The amplitude of the very slow component increased with decreasing temperature (from 47% at 293 K

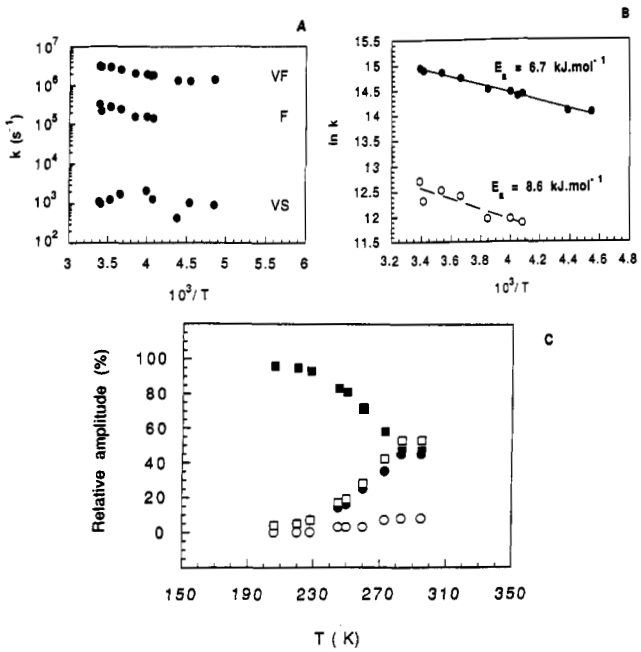

FIGURE 3: Temperature dependence of the rates and amplitudes of the three different components of P^+ rereduction measured at 1283 nm at high redox potential (+380 mV). Experiments analogous to those of Figure 1A were carried out at different temperatures. The data are extracted from the effect of a single flash, the sample being dark-adapted before being cooled to the indicated temperature. (A) Top, very fast component (VF); middle, fast component (F); bottom, very slow component (VS). (B) Arrhenius plots and activation energies of very fast (filled circles) and fast (open circles) components. (C) Temperature dependence of the amplitudes of the different components of P^+ rereduction at high redox potential. Filled circles, very fast phase; open circles, fast phase; filled squares, very slow phase; open squares, sum of very fast and fast phases.

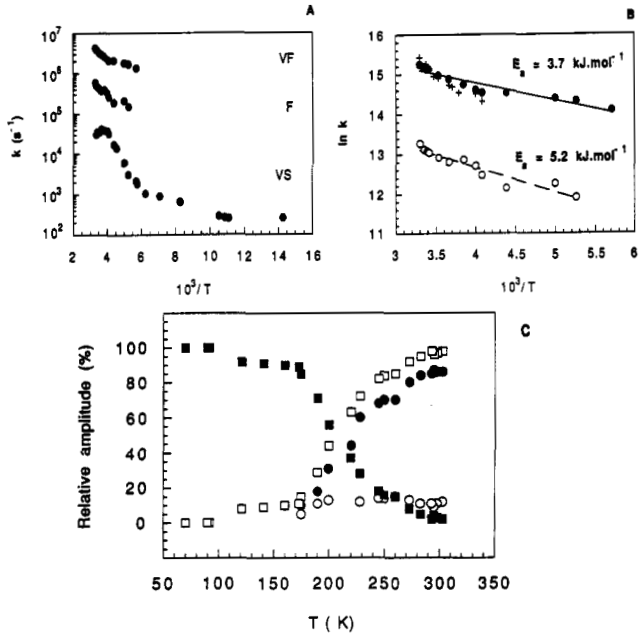

FIGURE 4: Temperature dependence of the rates and amplitudes of the different kinetic components of electron transfer between cytochrome *c* and P^+ at moderate redox potential (+250 mV). Experiments analogous to those of Figure 1B for P^+ reduction and Figure 2B for cytochrome oxidation were carried out at different temperatures. (A) Different components of P^+ reduction measured at 1283 nm: very fast phase (VF); fast phase (F); very slow phase (VS). (B) Arrhenius plot of very fast (filled circles) and fast (open circles) components of P^+ reduction, and cytochrome oxidation (crosses) measured at 560 nm. (C) Temperature dependence of the amplitudes of the different components of P^+ rereduction at high redox potential. Filled circles, very fast phase; open circles, fast phase; filled squares, very slow phase; open squares, sum of very fast and fast phases.

to 96% at 205 K) in the same proportion that the sum of both very fast and fast components decreased. To be clear about the meaning of these results, we feel it is useful to emphasize that the very slow phase of P^+ reduction at 280–300 K takes place in reaction centers where heme *c*-559 is oxidized before the flash whereas the increase in the extent of that slow phase appearing at low temperature indicates an inability of reduced heme *c*-559 to reduce P^+ in a fraction of reaction centers. Under the redox conditions of Figure 3, we observed that the first flash and subsequent flashes induce the same signals, indicating that all reactions are fully reversible within the 30-s interval between flashes. The same reversibility also takes place in experiments (not shown) done between 7 and 200 K under these redox conditions.

(*ii*) *Effect of Temperature at Moderate Redox Potential.* The temperature dependence of the kinetics of electron transfer between cytochrome *c* and P^+ was also measured at a redox potential (E_h = +250 mV) where the two high-potential hemes *c*-559 and *c*-556 were reduced prior to the flash excitation. In the high-temperature region (between 300 and 175 K), the decay curves of P^+ rereduction (Figure 4A, see also Figure 1B) were well fitted by the sum of three exponential components. The rate of the very fast component (Figure 4A, VF) was 3.7×10^6 s^{-1} ($t_{1/2}$ = 190 ns) at 293 K and diminished with decreasing temperature to a rate of 1.3×10^6 s^{-1} at 175 K. The rate of the fast component (Figure 4A, F) was 4.6×10^5 s^{-1} ($t_{1/2}$ = 1.5 μs) at 293 K and also decreased with decreasing temperature to a rate of 1.5×10^5 s^{-1} at 190 K. The activation energies for the very fast and fast components of P^+ reduction were 3.7 and 5.2 kJ·mol^{-1}, respectively (Figure 4B).

Figure 4C shows the temperature dependence of the amplitudes of the very fast, fast, and very slow components of the P^+ rereduction at moderate redox potential. The amplitude of the very fast component diminished with decreasing temperature (from 85% at 293 K to 10% at 175 K), and the amplitude of the minor fast component also decreased with decreasing temperature (from 10% at 293 K to 5% at 175 K). Below 175 K, it was not possible to distinguish between the two fastest components because of their very small amplitudes. The weight of the very slow component increased with decreasing temperature from a value of 5% at 293 K to a maximum value of nearly 95–99% of the total amplitude of P^+ rereduction below 90 K.

We have also measured the effect of temperature on the kinetics of cytochrome photooxidation at moderate redox potential (Figure 4B and 5). The data were analyzed as a sum of two exponential components, in continuity with experiments at room temperature: a fast component (heme *c*-559 oxidation) and a slow component (interheme electron transfer). There is a rather good fit between the kinetics of the very fast component of P^+ rereduction and the kinetics of cytochrome oxidation (Figure 4B). The rate of cytochrome photooxidation was 3.1×10^6 s^{-1} ($t_{1/2}$ = 220 ns) at 290 K and decreased with decreasing temperature to a rate of 1.7×10^6 s^{-1} ($t_{1/2}$ = 410 ns) at 240 K. The kinetics of the slow component (heme *c*-559 rereduction) were also temperature-dependent (Figure 5). The rate was 4.1×10^5 s^{-1} ($t_{1/2}$ = 1.7 μs) at 290 K and decreased with decreasing temperature to a rate of 1.1×10^5 s^{-1} ($t_{1/2}$ = 6.3 μs) at 240 K. The activation energies for the rapid oxidation and the slow rereduction of the high-potential heme were 10.8 and 20.9 kJ·mol^{-1}, respectively.

Figure 6 shows in two different time scales the kinetic traces of flash-induced absorbance changes in the P^+ region (1283 nm) at 77 K when the two high-potential hemes *c*-559 and

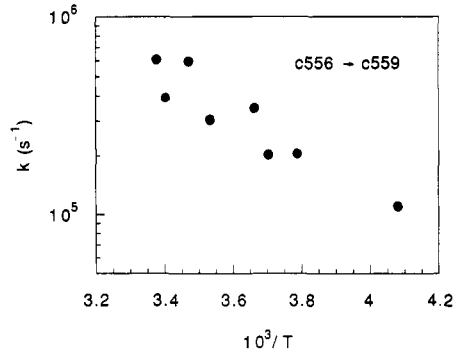

FIGURE 5: Temperture dependence of the rate of electron transfer between the two high-potential hemes of cytochrome *c* at moderate redox potential. Experiments analogous to those of Figure 2B were carried out at different temperatures. Data shown are the rates of the slow cytochrome oxidation component ($t_{1/2}$ = 1.7 μs at room temperature) measured at 560 nm.

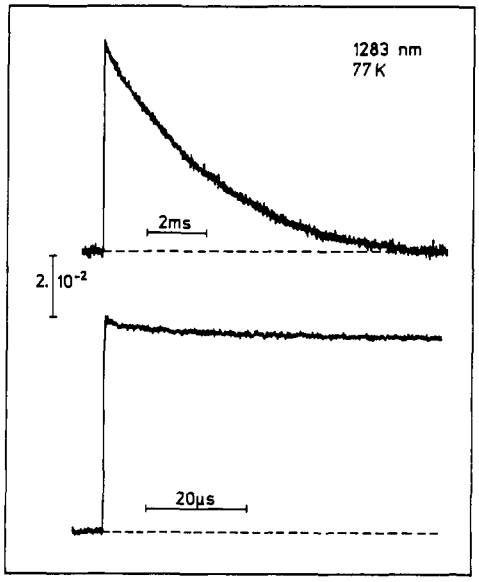

FIGURE 6: Flash-induced absorption change measured at 1283 nm in *Rps. viridis* RC at moderate redox potential, at 77 K. Conditions as for Figure 4. Effect of a single flash. Upper and lower traces are the same experiment with different time scales.

c-556 were reduced prior to the flash. After the rapid absorption increase (P^+ formation), the subsequent decay curve (P^+ rereduction) was well fitted by a single-exponential component with $t_{1/2}$ = 2.7 ms (Figure 6, top). In a shorter time scale (Figure 6, bottom), it was possible to distinguish a very small fast component with $t_{1/2}$ = 2–10 μs, accounting for only about 4% of the total amplitude of P^+ rereduction. A detailed examination showed that the slow phase is in fact complex and is probably made of three components: a small component due to ^3P (see below) and the two components of the $P^+Q_A^-$ back-reaction which, below 200 K, have half-times of 5.8 ms (85%) and 1.0 ms (15%). These times are in fair agreement with those reported by Sebban and Wraight (1989), and like these authors, we find that the slow back-reaction is dominant. We cannot eliminate, however, that, following the analysis of Shopes et al. (1987), the P^+ rereduction includes a significant contribution of slow electron donation from heme c-559.

(iii) Effect of Temperature at Low Redox Potential. We have studied the electron-transfer kinetics from the low-potential heme *c*-552 to the primary donor P^+ by measuring both P^+ rereduction and cytochrome oxidation after a laser flash. Some of the data concerning the P^+ reduction have been recently reported (Ortega & Mathis, 1992). Figure 7 shows the kinetic traces of flash-induced absorbance changes in the P^+ region (1283 nm) at a redox potential where the three highest potential hemes *c*-559, *c*-556, and *c*-552 were reduced prior to the flash (E_h = −20 mV) and at three different temperatures. The kinetic behavior at room temperature (Figure 7A) has been described in Figure 1. At 100 K, the curve of absorption recovery after a single flash (Figure 7B, upper trace) is well fitted by the sum of four exponential components: a very fast phase with a rate of 1.2×10^6 s^{-1} ($t_{1/2}$ = 570 ns), a fast phase with a rate of 1.1×10^5 s^{-1} ($t_{1/2}$ = 6.4 μs), a slow phase with $t_{1/2}$ = 56 μs, and a very slow phase with $t_{1/2}$ = 0.83 ms. The percentages of the total amplitude of the signal for the four phases are 22, 23, 30, and 25, respectively. The averaged effect of four flashes given after the first with a spacing of 30 s is shown in Figure 7C, lower trace. The absorbance change is much smaller, and the recovery can be well fitted by a major exponential with a $t_{1/2}$ = 59 μs ($k = 1.2 \times 10^4$ s^{-1}), accounting for about 76% of the total amplitude of the signal and a smaller constant (24%). The same experiment is shown in Figure 7C with a shorter time scale. It clearly demonstrates that the fast phase of P^+ rereduction is seen only after the first flash. At very low temperature (8K), the curve of absorption recovery following a single flash is well fitted by the sum of three exponential components: a small fast phase with $t_{1/2}$ = 4 μs, an intermediate component with $t_{1/2}$ = 60 μs, and a very slow phase with $t_{1/2}$ = 1.1 ms (Figure 7D, upper trace). At this temperature, it is not possible to distinguish between the two fastest phases because of their very small amplitudes. The very slow component is predominant, accounting for 70% of the total amplitude. The percentages for the fast and intermediate phases are 5 and 25, respectively. The average absorption change induced by a series of four flashes following the first one is shown in Figure 7D (lower trace). The decay can be well fitted by a single-exponential component with $t_{1/2}$ = 60 μs, representing 80–85% of the total amplitude.

Several features lead us to think that the kinetic component with an intermediate half-time (about 10 μs at ambient temperatures, 60 μs at low temperature) is due to the triplet-state ^3P which could be formed in reaction centers with reduced Q_A, by charge recombination in the primary radical pair: (i) this phase was observed only at low redox potential; (ii) its amplitude after the first flash was rather variable in amplitude (5–30% of the total ΔA); (iii) at low temperature, it represents the essential part of the decay kinetics after all flashes except for first one; (iv) similar $t_{1/2}$ values have been reported in the literature for ^3P, which was also shown to have a rather large absorption around 1300 nm (Shuvalov & Parson, 1981). The intermediate phase found in the four-exponential analysis of ΔA induced by the first flash has a rate of 5.2×10^4 s^{-1} ($t_{1/2}$ = 13 μs) at 293 K. It decreases with decreasing temperature down to a rate of 1.3×10^4 s^{-1} ($t_{1/2}$ = 53 μs) at 150 K. Between 150 and 8 K, the rate is almost temperature-independent. The activation energy in the temperature-dependent region is 8.9 kJ·mol^{-1}. Similar properties were reported for ^3P in *Rb. sphaeroides* by Shuvalov and Parson (1981), who found a nearly identical activation energy of 9.2 kJ·mol^{-1} and a breakpoint around 160 K. This similarity gives us additional confidence that this phase is due to the triplet-state ^3P. We also found similar temperature-dependent kinetics under two conditions: (i) for the dominant phase induced by all flashes after the first one; (ii) with reaction centers in which LDAO had been exchanged with Triton X-100 and which were then supplemented with sodium dithionite (0.3 mg·mL^{-1}).

Figure 8 shows Arrhenius plots of the kinetics of the very fast and fast components of P^+ rereduction (at 1283 nm) and cytochrome *c* oxidation (at 552 nm) at low redox potential.

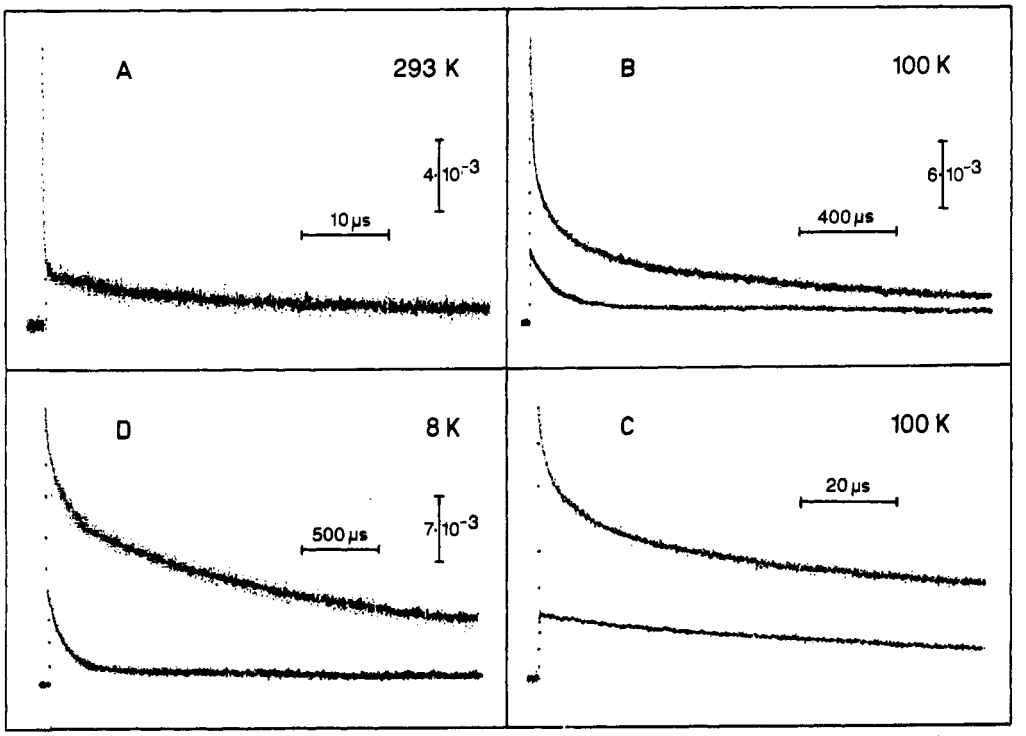

FIGURE 7: Flash-induced absorption changes measured at 1283 nm at different temperatures in *Rps. viridis* RC at low redox potential. Experiments analogous to those of Figure 1C were carried out at different temperatures. (A) 293 K; (B) 100 K; the upper trace is the result of a single flash, and the lower trace is the averaged effect of four flashes given afterward (30 s between flashes). (C) The same experiment shown in (B) displayed on an expanded time scale. (D) 8 K; the upper trace is the result of a single flash, and the lower trace is the averaged effect of four flashes, as in (B).

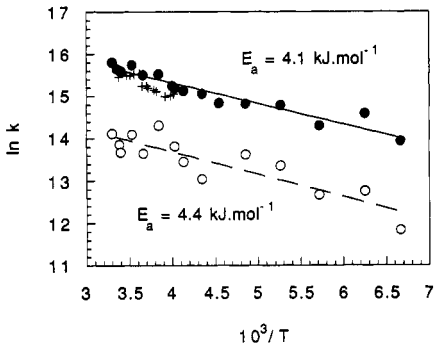

FIGURE 8: Arrhenius plots of the very fast (filled circles) and fast (open circles) components of P^+ reduction measured at 1283 nm and of cytochrome oxidation measured at 550 nm (cross symbols) at low redox potential. Conditions as for Figure 7.

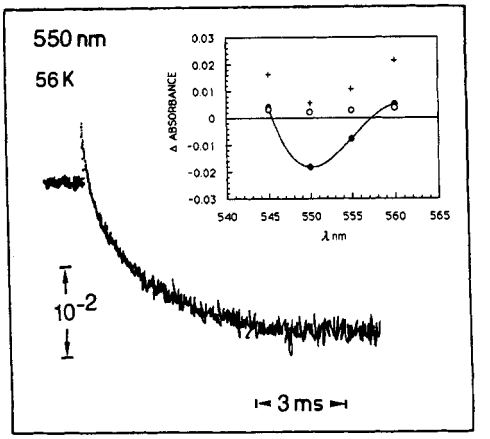

FIGURE 9: Flash-induced absorption changes of *Rps. viridis* RC at low redox potential measured at 550 nm, at 56 K. Conditions as for Figure 7. The curve is the effect of a single laser flash. The inset shows the spectrum of these changes in the α-band of cytochrome *c*. Filled circles, absorption change measured 8.5 ms after a single flash; crosses, initial absorption increase measured after a single flash; open circles, absorption change measured 8.5 ms after a flash given after a series of 10 flashes.

The temperature dependence of the rates of both components of P^+ rereduction has been extensively described in a previous paper (Ortega & Mathis, 1992). There is a good fit for the kinetics of the very fast component of P^+ rereduction and the kinetics of cytochrome oxidation. The rate of cytochrome photooxidation is 6×10^6 s^{-1} ($t_{1/2}$ = 115 ns) at 293 K and decreases with deceasing temperature to a rate of 3.3×10^6 s^{-1} ($t_{1/2}$ = 210 ns) at 250 K. The activation energies for the very fast and fast phases of P^+ rereduction and for cytochrome oxidation are 4.1, 4.4, and 8 kJ·mol^{-1}, respectively. The cytochrome kinetics were not accurate enough to allow for an analysis into multiexponential components. Moreover, the measurements in the microsecond range required signal-averaging: this explains why the data points for cytochrome in Figure 8 do not extend below 250 K.

We have been able, however, to measure the kinetics of *c*-552 photooxidation at low temperature after a single flash, albeit with a low time resolution. Figure 9 shows a kinetic trace of the flash-induced absorbance change in the α-band region of cytochrome *c* at 56 K. At 550 nm, an initial absorbance increase attributed to P^+ formation is followed by a decay curve well fitted by a single-exponential component with a $t_{1/2}$ = 1.1 ms accounting for 90% of the total amplitude and small unresolved fast phases ($t_{1/2}$ < 70 µs). The same type of experiments carried out at different wavelengths in the α-band of the cytochrome shows a clear negative peak at 550 nm, which is characteristic of the oxidation of heme *c*-552 (Figure 9, inset, closed circles). A flash given after a series of 10 flashes induced only very small positive absorbance changes attributed to a small fraction of centers where the reactions are reversible, perhaps because *c*-552 was not initially fully reduced (Figure 9, inset, open circles). The initial difference spectrum in these experiments (Figure 9, inset, crosses) already includes, in addition to partial P oxidation, the *c*-552 oxidation associated with the fast phases of P^+

reduction. From these results and from those obtained at room temperature, it is possible to conclude that the very fast, fast, and very slow phases of P^+ rereduction at low redox potential are due to reduction of P^+ by cytochrome *c*-552.

DISCUSSION

The reaction center of the purple bacterium *Rhodopseudomonas viridis* appears to be an ideal system to confront current theories of electron transfer with experimental results. For the first time, its 3-D structure shows with enough details how redox centers are spatially organized in a complex biological system in which reactions can be precisely triggered by a short flash of light. This paper reports our functional study of the electron transfer between the tetraheme cytochrome *c* and the photooxidized primary donor P in the *Rps. viridis* reaction center. The results clearly show a large effect of temperature on the reaction kinetics which reveal several components over a wide range of temperature. In our opinion, this multiphasic behavior supports the proposal of kinetically distinct, but interconvertible, states of the reaction center, as discussed below.

Our experiments at room temperature are essentially confirmatory. With better accuracy and an improved time resolution than in previous reports (Dracheva et al., 1986, 1988; Shopes et al., 1987), we find the following: (i) when only one heme is reduced, *c*-559 reduces P^+ with a half-time of 230 ns; (ii) when two hemes are reduced, *c*-559 reduces P^+ with a half-time of 190 ns (the slight acceleration has not been previously reported; it was quite reproducible in our experiments), and *c*-556 then reduces oxidized *c*-559 with a half-time of 1.7 μs; (iii) when three hemes are reduced, P^+ is reduced with a half-time of 115 ns, apparently directly by heme *c*-552. In that case, contrary to our expectations, we have not been able to detect a transitory oxidation of *c*-559. Our data, however, do not exclude that P^+ is reduced by *c*-559 with $t_{1/2}$ = 115 ns and *c*-552 then reduces oxidized heme *c*-559 much more rapidly (an upper limit of 40 ns sounds reasonable). We consider that kinetic sequence as the most probable one since edge-to-edge distances are about 12 and 24 Å between P and hemes *c*-559 or *c*-552, respectively, and since it is established that distance is the dominant factor for the rate of electron transfer (Moser et al., 1992): thus, it is hardly possible to envision that direct electron transfer to P^+ is faster for heme *c*-552 than for heme *c*-559. A very fast transfer from *c*-552 to *c*-559 is perfectly expectable since their distance is as short as about 5 Å (edge-to-edge) or 14 Å (center-to-center). At 5-Å edge-to-edge distance, the electron-transfer rate may be as high as 10^{12} s^{-1} ($t_{1/2}$ = 0.7 ps), and at 12 Å (as for heme *c*-559 and P^+), it could be 6.7×10^7 s^{-1} ($t_{1/2}$ = 10 ns) (Moser et al., 1992). Electron transfer from heme *c*-559 to P^+ appears to be slightly accelerated (20%) by the reduction of *c*-556, and much more (2 times) by the reduction of *c*-552. These accelerations can probably be accounted for by interheme electrostatic interactions. It has indeed been recently proposed (Gunner & Honig, 1991) that the free energy of cytochrome oxidation in *Rps. viridis* is dependent on several intramolecular parameters. From calculations based on the properties of model systems, these authors concluded that neighboring hemes, 14–16 Å apart, have an interaction energy of 50–77 meV, while between the two high-potential hemes, 28 Å apart, the electrostatic interaction is 10–14 meV. The free energy change ($\Delta G°$) associated with electron transfer from heme *c*-559 to P^+ would thus be increased by 14 meV when heme *c*-556 is also reduced and by 77 meV more when both hemes *c*-556 and *c*-552 are reduced. The $\Delta G°$ for the reaction is rather small [–120 mV if we accept an E_m of + 500 mV for the P/P^+ couple; see Gao et al. (1990)], and a putative increase in $\Delta G°$ could well explain the observed increased rates.

Temperature is an important parameter for understanding the mechanism of electron transfer in biological systems. We studied the effect of temperature on electron transfer P^+ with reaction centers in three redox conditions and always found a behavior fairly different from that reported in the historical experiments on *Chromatium* (De Vault & Chance, 1966). A common feature to all three redox conditions is that P^+ reduction includes two fast phases with half-time ranging from 110 ns to 10 μs, which have a rather weak temperature coefficient. Their activation energy lies between 3.7 and 8.6 kJ·mol^{-1}, and their range of variation is not more than a factor of 10. Although these values may seem to be rather low, they may correspond to general properties of preformed donor–acceptor complexes as already shown for many electron-transfer steps in reaction centers (Gunner & Dutton, 1989). In *Chromatium*, De Vault and Chance (1966) reported an activation energy of 13.8 kJ·mol^{-1} in the high-temperature region.

Our analysis of P^+ reduction kinetics in exponential components always gives two fast phases which differ approximately by a factor of 10. We raise the question of the genuine significance of these two phases. We can practically exclude an electronic artifact because both phases are slowed down when the temperature is lowered and because their relative amplitudes can vary in rather large proportions [see Figure 3 in Ortega and Mathis (1992)]. The microsecond phase is also present at low potential and thus it cannot be due to electron transfer from *c*-556 to *c*-559 in reaction centers where *c*-559 might have been oxidized before the flash. Heterogeneities are fairly common in photosynthetic reaction centers [for *Rps. viridis*, see, e.g., Shopes and Wraight (1987) or Sebban and Wraight, (1989)]. This may be one more case of kinetic heterogeneity. When three hemes are reduced, the rate of the fastest phase becomes temperature-independent below 150 K (Figure 10, left). The behavior of that phase is thus rather "classical" in that it comprises a high-temperature region where the Arrhenius law is obeyed and a low-temperature region when the rate is constant. These properties could not be studied when only one or two hemes are reduced, essentially because fast phases practically vanish at a fairly high temperature (Figure 10, right).

A common feature in all three redox states of the reaction center is indeed that the amplitudes of the fast phases abruptly decrease at lowered temperatures. The temperature of half-decrease is 250, 210, and 80 K, respectively, when one, two, or three hemes are reduced before the flash (Figure 10, right). When one heme is reduced, the fast phase makes up about 4% of the signal at 205 K (Figure 3C). It decreases to 1–3% below 180 K (not shown). These results significantly differ from those reported by Kaminskaya et al. (1990), who concluded that about 20% of P^+ was reduced by *c*-559 in less than 6 μs at 77 K. These authors also reported a sharp decrease in a rapid (but not directly measured) reduction of P^+ around 170 K (instead of 250 K in our work). In their experiments, however, a large fraction of reaction centers could have had two hemes reduced since the redox potential was fixed at +320 mV. With two hemes reduced, we find a rather abrupt decrease in the extent of fast phases around 210 K (Figures 4C and 10, right). What can be the reason for the inability of heme *c*-559 to reduce P^+ at low temperature? Obviously, an important and progressive slowing down of the electron-transfer reaction cannot be invoked. A structural change leading to an increased distance between *c*-559 and P would

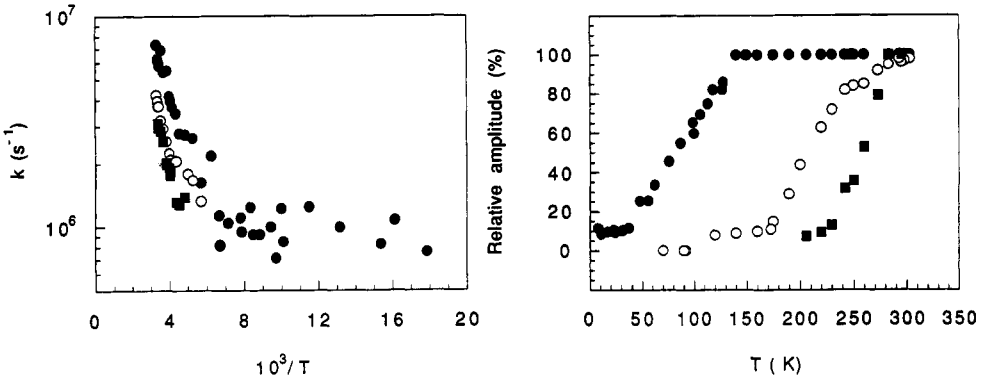

FIGURE 10: Effect of temperature on the rates of the very fast component (left) and on the amplitudes of the fast components (right) of P^+ rereduction under three redox conditions. Filled squares, high redox potential; data were taken from experiments used for Figure 3. Open circles, moderate redox potential; data were taken from experiments used for Figure 4. Filled circles, low redox potential; data were taken from experiments published in a previous paper (Ortega & Mathis, 1992). For the calculation of the amplitudes at E_h = +380 mV, 50% of P^+ rereduction was considered as total P^+. In all cases, data are the sum of the amplitudes of the very fast and fast phases of P^+ rereduction.

have to be rather dramatic to induce a thousandfold slower rate (although we will not consider it in the following discussion, we recognize that that hypothesis cannot be fully discarded). A clue to the interpretation may reside in the fact that a similar inability for *c*-559 to reduce P^+ happens under all studied redox conditions, at a break temperature which decreases when the cytochrome becomes more reduced (Figure 10, right). Therefore, we think that a unique mechanism is involved, with the redox state (i.e., perhaps the electrostatic properties) as a modulating parameter. The most straightforward explanation is a change in the relative E_m of the P/P^+ and *c*-559/*c*-559$^+$ couples, as already proposed by Kaminskaya et al. (1990). This proposal would be in agreement with the effect of the oxidation state of hemes *c*-556 and *c*-552 which very probably changes the E_m of *c*-559, as discussed before on the basis of the work by Gunner and Honig (1991). However, Gao et al. did not find any change of the relative E_m of P and *c*-559 between 300 and 275 K (Gao et al., 1990). The effect of temperature on the relative E_m's has to be rather steep. It could be related to the freezing of water molecules within the cytochrome subunit. An effect of local freezing has already be proposed by Knapp and Fisher (1987) and by Knapp and Nilsson (1990). Their proposals are largely based on the possible role of a conserved tyrosine residue (Tyr-L162) which was supposed to facilitate electron transfer from the cytochrome to P^+. Their interpretations relied on the requirement for a precise positioning of the tyrosine, which could have a low probability at low temperature because of the freezing of neighbor water molecules or of a decreased population of a thermally excited favorable configuration. This kind of argument may keep its interest since the debate on the role of the protein medium is not settled [see Wuttke et al. (1992) and references cited therein] in spite of the recent conclusion from Moser et al. (1992) against electron-gating mechanisms. The electron-switch model involving Tyr-L162 has been further elaborated recently by Cartling (1992). We consider more probable that freezing induces an electrostatic reorganization around *c*-559 which substantially increases its E_m. This may mean that oxidation of *c*-559 requires a fairly large structural change which would be hindered at low temperature [energetic aspects of geometry changes in cytochrome oxidation have been analyzed by Churg et al. (1983)]. As far as we can tell [see Figure 2 in Ortega and Mathis (1992)], the slow rate of direct electron donation from *c*-552 to P^+ is not modified by temperature.

Our interpretation should be discussed a little bit further for the case where three hemes are reduced. In that case heme *c*-552 remains fully oxidizable by a single flash at low temperature [Figure 7 and Ortega and Mathis (1992)]. According to the previous discussion, it can be proposed that electron transfer from *c*-559 to P^+ is blocked and that the 1.1-ms phase observed under these conditions corresponds to direct electron transfer from *c*-552 to P^+ without *c*-559 being a redox intermediate (not considering the small amount of fast phases which remain at low temperature under these conditions). In some respects, this model would resemble the last version of the models proposed by Bixon and Jortner (1988), in which two parallel electron paths were proposed for P^+ reduction in *Rps. viridis* when three hemes are reduced. The main difference lies in the temperature dependence of electron transfer from *c*-559 to P^+: relying on the data published for *Chromatium*, they proposed a thermally activated transfer becoming slower and slower as the temperature is lowered, until the direct transfer from *c*-552 becomes dominant. Our results show a fairly different (and more complex) situation. The edge-to-edge distance between *c*-552 and P is about 24 Å, leading to a possible rate of about 4 s^{-1} ($t_{1/2}$ = 170 ms) according to Moser et al. (1992). This rate is not compatible with our observations ($k = 6 \times 10^2$ s^{-1}; $t_{1/2}$ = 1.1 ms). The interpretation advocated by Kaminskaya et al. (1990) seems to be more appropriate. At very low temperature, the extent of fast reduction of P^+ is larger at low potential (about 10%) than at moderate or high potential (below 3%). We interpret that fast phase as an electron donation from *c*-559 to P^+. If the low-temperature blockage of fast electron transfer from *c*-559 to P^+ is effectively due to a change in their respective E_m, it can be expected that the electrostatic effect discussed above maintains *c*-559 as a more efficient electron donor to P^+ when three hemes are reduced. The reaction would thus consist of microsecond electron donation from *c*-559 to P^+, followed by rapid and irreversible reduction of *c*-559 by *c*-552; the reaction with $t_{1/2}$ = 1.1 ms would be rate-limited by establishment of the redox equilibrium between P and *c*-559.

This study provides a more complete account of electron-transfer kinetics on the donor side of the reaction center of *Rps. viridis*. In conclusion, we would like to state the following: (i) Our kinetic data are in general agreement with heme attribution with respect to the 3-D structure of the reaction center. (ii) The cytochrome–P ensemble exists under several substates which give rise to complex kinetics, in particular a biphasic fast phase of donation from *c*-559 to P^+ and a fast-or-slow behavior at low temperature. (iii) Temperature changes the relative proportion of substates, but it has a rather weak effect on the kinetics of electron transfer within each substate. (iv) The redox state of the three highest

potential hemes has a complex but rationalizable influence on the electron-transfer kinetics. A more complete interpretation will require additional structural data, for instance on the structure of the cytochrome in its various redox states and on eventual structural changes taking place at low temperature.

ACKNOWLEDGMENTS

We thank J. Breton, N. Foloppe, W. Nitschke, B. Robert, A. W. Rutherford, and A. Vermeglio for their help and for fruitful discussions. We especially thank P. Sétif for his help in data analysis.

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