Low-Temperature Electron Transfer from Cytochrome to the Special Pair in *Rhodopseudomonas viridis*: Role of the L162 Residue

José M. Ortega,*§ Barbara Dohse,# Dieter Oesterhelt,# and Paul Mathis*
*Section de Bioénergétique, DBCM (CNRS, URA 2096), CEA-Saclay, 91191 Gif-sur-Yvette Cedex, France; #Max-Planck Institut für Biochemie, Abteilung Membranbiochemie, 82152 Martinsried, Germany; and §Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla y CSIC, Centro de Investigaciones Científicas "Isla de la Cartuja," E-41092 Seville, Spain

ABSTRACT Electron transfer from the tetraheme cytochrome *c* to the special pair of bacteriochlorophylls (P) has been studied by flash absorption spectroscopy in reaction centers isolated from seven strains of the photosynthetic purple bacterium *Rhodopseudomonas viridis*, where the residue L162, located between the proximal heme *c*-559 and P, is Y (wild type), F, W, G, M, T, or L. Measurements were performed between 294 K and 8 K, under redox conditions in which the two high-potential hemes of the cytochrome were chemically reduced. At room temperature, the kinetics of P^+ reduction include two phases in all of the strains: a dominant very fast phase (VF), and a minor fast phase (F). The VF phase has the following $t_{1/2}$: 90 ns (M), 130 ns (W), 135 ns (F), 189 ns (Y; wild type), 200 ns (G), 390 ns (L), and 430 ns (T). These data show that electron transfer is fast whatever the nature of the amino acid at position L162. The amplitudes of both phases decrease suddenly around 200 K in Y, F, and W. The effect of temperature on the extent of fast phases is different in mutants G, M, L, and T, in which electron transfer from *c*-559 to P^+ takes place at cryogenic temperatures in a substantial fraction of the reaction centers (T, 48%; G, 38%; L, 23%, at 40 K; and M, 28%, at 60 K), producing a stable charge separated state. In these nonaromatic mutants the rate of VF electron transfer from cytochrome to P^+ is nearly temperature-independent between 294 K and 8 K, remaining very fast at very low temperatures (123 ns at 60 K for M; 251 ns at 40 K for L; 190 ns at 8 K for G, and 458 ns at 8 K for T). In all cases, a decrease in amplitudes of the fast phases is paralleled by an increase in very slow reduction of P^+, presumably by back-reaction with Q_A^-. The significance of these results is discussed in relation to electron transfer theories and to freezing at low temperatures of cytochrome structural reorganization.

INTRODUCTION

In photosynthetic purple bacteria, the primary steps of light energy conversion take place in a large membrane protein complex named the reaction center (RC). After light excitation, a dimer of bacteriochlorophyll molecules, known as a special pair or primary electron donor P, transfers an electron to an acceptor, and the oxidized species P^+ is then rereduced by a secondary electron donor, a *c*-type cytochrome, and thus is reactivated. In some purple bacteria this secondary donor is a soluble c_2 cytochrome, but in most cases it is a RC-bound tetraheme cytochrome *c*. One of the most studied cases is *Rhodopseudomonas* (*Rps.*) *viridis*, in which the three-dimensional (3-D) structure of the RC has been solved to atomic resolution (Deisenhofer et al., 1985, 1995), and the functional properties can be studied by time-resolved absorption spectroscopy after excitation by a short laser flash.

The bound tetraheme cytochrome *c* is the largest subunit in the RC complex of *Rps. viridis* (Weyer et al., 1987). The 3-D structure shows that the four hemes of the cytochrome are arranged in a roughly linear sequence along the protein long axis, with a Fe-to-Fe separation distance of 14–16 Å, the proximal heme being within 21 Å of P. Individual spectral and redox characteristics of the four hemes have been resolved, showing different spectroscopic and thermodynamic properties (Dracheva et al., 1986, 1988; Shopes et al., 1987; Nitschke and Rutherford, 1989; Verméglio et al., 1989; Alegria and Dutton, 1991). A few functional properties of electron transfer from the tetraheme cytochrome to P^+ have been studied in isolated RC (Dracheva et al., 1988; Ortega and Mathis, 1992, 1993). It has clearly been established that after a short flash of light the proximal high-potential heme *c*-559 (E_m = +380 mV) donates an electron to P^+, and then is rereduced, either by the second high-potential heme *c*-556 (E_m = +310 mV) or by the first low-potential heme *c*-552 (E_m = +20 mV), depending on the redox state of the system. At room temperature, reduction of P^+ by the tetraheme cytochrome includes two phases: a largely dominant phase (accounting for ~85% of the total amplitude of P^+ rereduction) with a $t_{1/2}$ of 230 ns, and a minor phase with a $t_{1/2}$ of 1.5 μs (named very fast and fast phases, respectively, in Ortega and Mathis, 1992, 1993). The very fast phase (VF) is slightly accelerated ($t_{1/2}$ = 190 ns) when the second high-potential heme *c*-556 is reduced before the flash excitation.

The 3-D structure of the *Rps. viridis* RC showed that a tyrosine (Y) residue of the L subunit (L162Y) is located in a small pocket halfway between the proximal heme *c*-559 and the special pair P (see Fig. 1). A tyrosine residue is located similarly in the second RC structure known, which is *Rhodobacter sphaeroides*, and more generally a tyrosine residue at position 162 is found in the L-subunit of all RC

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Address reprint requests to Dr. Paul Mathis, DBCM-SBE, Bât. 532, CEA-Saclay, 91191 Gif-sur-Yvette Cedex, France. Tel.: 33-169083409; Fax: 33-169088717; E-mail: mathis@dsvidf.cea.fr.

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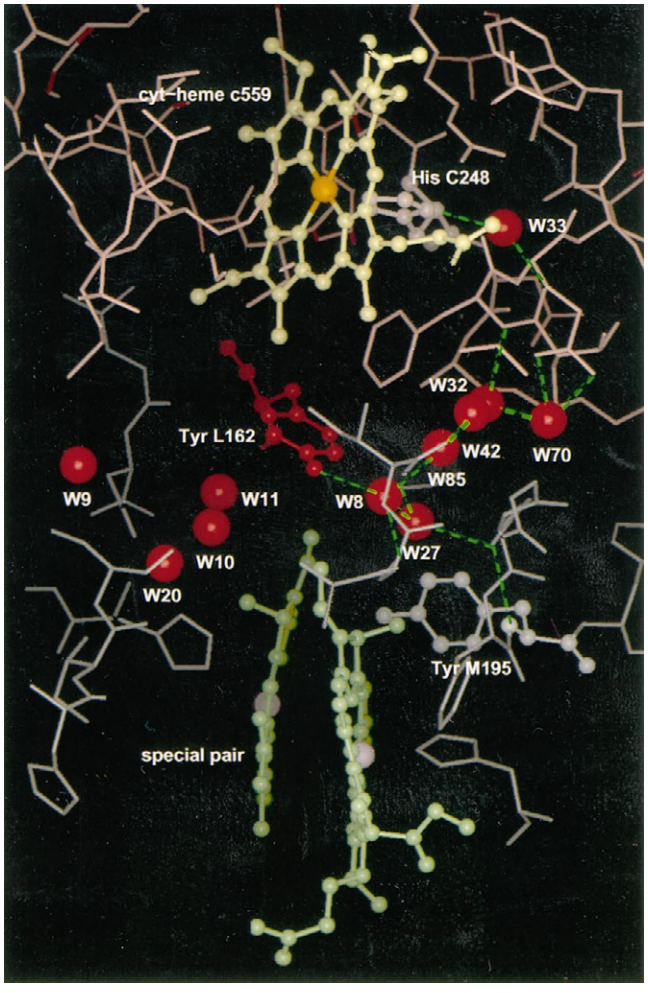


FIGURE 1 Details of the *Rps. viridis* RC structure, sketched as a sectional view of the protein complex through a plane perpendicular to the membrane surface. The cytochrome (cyt-heme 559, in *yellow*) faces the extracellular side, and the special pair P, a dimer of bacteriochlorophyll (in *green*), faces the cytoplasmic side. Water molecules of the structure are indicated by red spheres. The seven water molecules on the right side of the figure are part of a hydrogen-bonded network (*dashed green lines*) that links tyrosine L162 (Tyr L162) with the heme-liganding histidine C248 on the one hand and tyrosine M195, which is hydrogen-bridged, to the special pair P, on the other. This figure is based on unpublished crystallographic data of Dr. R. Lancaster and Dr. H. Michel.

in which this polypeptide has been sequenced (Williams et al., 1983; Youvan et al., 1984; Michel et al., 1986b; Shiozawa et al., 1989; Fathir et al., 1997). Because of their delocalized electronic structure, aromatic residues could play a role as intermediates in electron transfer reactions, especially tyrosine, which could form a mesomerically stabilized radical structure. Several authors have thus proposed that tyrosine L162 might have an important function in the RC, possibly by facilitating electron transfer between the tetraheme cytochrome and P (Michel et al., 1986a) or by playing a structural role (Wachtveitl et al., 1993). In an attempt to understand this function, this tyrosine has been replaced by other amino acids through site-directed mutagenesis in *Rb. sphaeroides* (Farchaus et al., 1993; Wachtveitl et al., 1993). From that work it was concluded that the tyrosine most probably plays a structural function in the docking of soluble cytochrome c_2 to the RC, although the complexity of the system did not allow a totally unambiguous answer. More recently, similar mutants were made for *Rps. viridis*, and a study of chromatophores showed that electron transfer from *c*-559 to P^+ remains fast in all of the mutants, the kinetics being not largely modified (the $t_{1/2}$ varies between 145 and 1000 ns in the whole series of mutants for the VF phase) (Dohse et al., 1995). The extent of the other kinetic phase, named fast (F), was found to vary significantly, being rather minor (less than 15%) for Y, F, M, W, and L, but fairly large (between 30% and 46%) for G, T, and H.

To understand some of these effects, we have studied the electron transfer kinetics in the same series of mutants in isolated RC at various temperatures. Tyrosine L162 (Y) has been replaced by phenylalanine (F) or tryptophan (W) as alternative aromatic residues, or also by glycine (G) as a control residue lacking any side chain. Further residues introduced were methionine (M) for its sulfur atom with d-orbitals, leucine (L) as a neutral hydrophobic residue, and threonine (T) as a typical hydrophilic residue. We anticipated that this study would also complement a previous study of the effect of temperature on electron transfer from the tetraheme cytochrome to P^+ in WT RC of *Rps. viridis*, a study that opened a new view on low-temperature electron transfer in these systems (Ortega and Mathis, 1992, 1993; Mathis et al., 1994). The whole kinetic behavior was fairly complex. A few key features are:

1. At high temperatures, the VF and F phases display an Arrhenius behavior with a small activation energy (~6 $kJ \cdot mol^{-1}$).

2. The amplitude of these phases, the sum of which amounts to nearly 100% of P^+ reduction at high temperatures, decreases suddenly and becomes negligible at a temperature that varies with the redox state of the cytochrome: the temperature of the half-decrease is 250 K, 210 K, or 80 K if one, two, or three hemes are reduced, respectively. The latter phenomenon precluded any complete study of the effect of temperature on electron transfer rates per se. At low temperatures, when no low-potential heme is reduced, P^+ is rereduced by electron return from Q_A^-, with half-times above 1 ms.

3. The molecular reason for the occurrence of two fast phases (VF and F) was not understood, but it was found that the extent of the F phase increased slightly at the expense of the VF phase at decreasing temperatures between 270 K and 240 K, a fact that could indicate a temperature-dependent interconversion, perhaps related to two conformations of the RC-cytochrome complex. The availability of the tyrosine L162 mutants provided an opportunity to check for the general character of these phenomena, specially because the F phase was found to be fairly large in several mutants at room temperature (Dohse et al., 1995).

Studies on tyrosine L162 are also related to previous works that attributed an important role to aromatic residues, and specially to tyrosine, in non-Arrhenius effects of low temperature on inter/intraprotein electron transfer. In 1966 DeVault and Chance discovered a unique temperature dependence of the rate of P^+ reduction by a tetraheme cytochrome in the photosynthetic bacterium *Chromatium vinosum* poised at a low redox potential. The rate constant, k, for electron transfer exhibited a sharp transition from a high-temperature activated region, where k drops by three orders of magnitude to a temperature-independent region at low temperatures ($T < 120$ K) where k remains constant (DeVault and Chance, 1966). The usual interpretation of these experiments has been based on the vibronic coupling mechanism, ascribing the temperature dependence of the rate at high temperatures to its nuclear factor, i.e., to the thermally averaged overlap of nuclear wave functions termed Franck-Condon factors (Hopfield, 1974; Jortner, 1976; Kuznetsov et al., 1978; Dogonadze and Zakaraya, 1987), whereas the reaction proceeds by temperature-independent nuclear tunneling at low temperatures. These properties have been reinterpreted theoretically by using the 3D structure of the *Rps. viridis* RC in terms of conformational control of electron transfer (Knapp and Fischer, 1987; Knapp and Nilsson, 1990; Cartling, 1991, 1992, 1993). The proposal was that the geometric fluctuations of the aromatic ring of the tyrosine L162 residue may be responsible for the temperature dependence of the electron transfer. These authors believed that the electronic factor for electron transfer is strongly influenced by the position of the aromatic ring of tyrosine L162, and that this position and its fluctuations are largely determined by the phenol group of tyrosine, by means of hydrogen bonding and proton transfer. The same authors suggested that the dramatic slowing down of electron transfer from cytochrome to P^+ in *Cr. vinosum* at low temperatures (DeVault and Chance, 1966; DeVault et al., 1967) could be associated with a loss of mobility of tyrosine L162 and with the need for its specific positioning for rapid electron transfer (Knapp and Fischer, 1987; Knapp and Nilsson, 1990; Cartling, 1991, 1992, 1993). It has also been proposed that low temperatures induce a gating of electron transfer reactions in many couples of proteins, an effect that has often been associated with the function of specific aromatic residues (Nocek et al., 1991). The L162 mutants of *Rps. viridis* thus seem to be good material for a detailed examination of these proposals.

In this work we examine the temperature dependence of the kinetics of electron transfer from the proximal heme *c*-559 to P^+ in RC isolated from seven strains of *Rps. viridis*, the only difference being that the residue L162 is Y (WT), F, W, G, T, M, or L. The RC were poised with a low concentration of ascorbate at a redox potential at which both high-potential hemes are reduced. The kinetics and amplitudes of P^+ rereduction were studied by flash absorption spectroscopy at various temperatures (between 294 K and 8 K), with P^+ being monitored at 1283 nm.

MATERIALS AND METHODS

Cell growth, genetic methods, and protein isolation

Growth conditions for WT and mutant strains of *Rps. viridis* (DSM 133) and methods for the construction of mutants are described in a preceding publication (Dohse et al., 1995). Harvesting of cells and RC isolation were described in the same reference. The concentration of isolated RC was determined spectrophotometrically, using the value $\Delta\epsilon = 300\ mM^{-1}\ cm^{-1}$ at 830 nm (Clayton and Clayton, 1978).

Spectroscopic measurements

For spectroscopic measurements, samples were prepared with ~3 μM RC in 50 mM Tris-buffer (pH 8.0) and the redox mediators diaminodurene (2,3,5,6-tetramethyl-*p*-phenylenediamine) (100 μM), 1,4-naphthoquinone (100 μM), and sodium ascorbate (100 μM). In all experiments, glycerol was added to the reaction mixture to a final concentration of 60% (v/v). Static absorption spectra at 8 K were recorded with a Cary (model 5E) spectrophotometer by inserting the cuvette in a cryostat cooled with helium gas (optical path: 10 mm for the measuring light, 4 mm for the actinic light). Flash absorption kinetics of electron transfer were measured by following P^+ formation and rereduction essentially as in Ortega and Mathis (1993). 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 temperatures the measurements are the average of four single flashes, with a time spacing sufficient to allow a return to equilibrium (1–5 min).

Data analysis

The traces obtained in spectrophotometric measurements were treated as sums of several exponential components to find $t_{1/2}$ values (or rates: $k = 0.693 \times t_{1/2}^{-1}$) and amplitudes. Exponential analyses were performed by the Marquardt method, with software devised by Dr. P. Sétif.

RESULTS

Electron transfer at room temperature

Electron transfer reactions at room temperature have been studied by measuring P^+ rereduction after a single laser flash in isolated RC of WT and of six different L162 mutant strains of *Rps. viridis*, poised at a moderate redox potential (E_h of ~ +250 mV), at which the two high-potential hemes (*c*-559 and *c*-556) were reduced before the flash excitation. Fig. 2 shows typical kinetic traces of flash-induced absorption changes in the near-infrared P^+ absorption band (1283 nm) for RC of L162F (Fig. 2 *A*) and of L162G (Fig. 2 *B*) mutant strains. The kinetics of absorption recovery in the L162F mutant at room temperature include three main exponential components: a very fast (VF) phase ($t_{1/2}$ = 135 ns), a fast (F) phase ($t_{1/2}$ = 2.1 μs), and a slow (S) phase ($t_{1/2}$ = 21 μs), accounting for 74% (VF), 10% (F), and 14% (S) of the total signal (see Table 1). The decay curves in the L162G mutant at 294 K also include the three above-described phases: a VF phase ($t_{1/2}$ = 200 ns), an F phase ($t_{1/2}$ = 1.0 μs), and a slow (S) phase ($t_{1/2}$ = 14 μs), accounting for 65% (VF), 25% (F), and 7% (S) (see Table 1). The kinetics of absorption changes after a laser flash similarly include these three exponential components in the

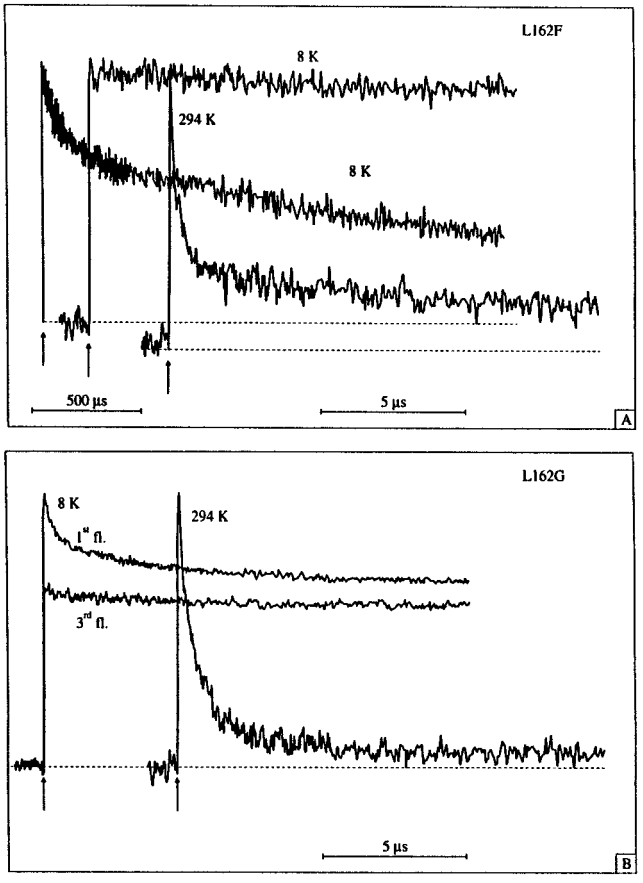


FIGURE 2 Kinetics of flash-induced absorption changes at 1283 nm in RC of the mutant strains L162F and L162G of *Rps. viridis*. The data at 294 K are the average of four single flashes separated by 2 min. At 8 K, the data are the effect of a single flash, the first or the third for L162G (*B*) (2 min between flashes). For L162F (*A*) the signal induced by the third flash is not distinguishable from that induced by the first. The decay curve at 8 K for this mutant is shown on two different time scales (the first trace on the left of the figure corresponds to the 500-μs time scale). The weights and kinetics of the two fast kinetic phases of absorption recovery are given in Figs. 3 and 5, respectively. For the very slow (VS) kinetic phase, weights and kinetics are reported in Figs. 3 and 6, respectively; $t_{1/2}$ is 2.0 ms at 8 K and above 0.4 ms at 294 K for mutant L162G (see Table 1).

other five strains investigated. Table 1 summarizes the kinetic and amplitude data at room temperature for all of the strains. The half-times and the amplitudes for VF and F phases have the following values: 189 ns (89%) for Y, 130 ns (83%) for W, 90 ns (84%) for M, 390 ns (63%) for L, and 430 ns (70%) for T, for the VF phase; and 1.5 μs (8%) for Y, 1.2 μs (11%) for W, 1.5 μs (8%) for M, 1.1 μs (28%) for L, and 1.9 μs (22%) for T, for the F phase. Half-times and amplitude values for the S phase are 12 μs (1%) for Y, 18 μs (4%) for W, 19 μs (5%) for M, 21 μs (6%) for L, and 10 μs (5%) for T (the $t_{1/2}$ are rather approximate, because the amplitudes are quite small).

In a previous work on WT RC of *Rps. viridis* (Ortega and Mathis, 1992, 1993), the existence of the three kinetic phases described above has been clearly identified, VF and F phases being assigned to electron transfer from the proximal heme *c*-559 to P^+ in two different RC conformational states. The slow phase (S), previously characterized as being due to the decay of the triplet state of P (^{3}P) (Ortega and Mathis, 1992, 1993), was quite small in all of the strains (less than 7% of the total absorption change), with the sole exception of mutant F (14%). It is well resolved in our kinetic analysis and not further discussed in the analysis of the data reported here, which deals only with electron transfer. The kinetic analysis also detected in all the strains a fourth component (named very slow, VS; assimilated to a constant in the analysis of three of the mutant strains) (Table 1). This kinetic component (3% of the signal and $t_{1/2}$ above 400 μs) was previously assigned in WT to the back-reaction between P^+ and Q_A^-, in RC where cytochrome does not function (Ortega and Mathis, 1993).

The data of kinetics and amplitudes of the different phases at 294 K (Fig. 2 and Table 1) for isolated RC display a few differences with those reported by Dohse et al. (1995) for the chromatophore membranes of the same L162 *Rps. viridis* mutants. These differences do not affect the essential conclusion reached with chromatophores: 1) electron transfer from cytochrome to P^+ is fast whatever the nature of the amino acid at position L162, and 2) tyrosine does not give rise to the fastest rereduction process. The differences are rather small for Y, F, and L, and are not large for M ($t_{1/2}$ of 147 ns instead of 90 ns, for the VF phase). For W the data on chromatophores were very noisy. The differences are specially large (faster in RC by a factor of ~2.5) for G and T, and we have no explanation for this discrepancy. It is worth noting that these are two of the mutants for which the F phase is larger. If this phase is attributed to modified docking of cytochrome to the RC core (see Discussion and Dohse et al., 1995), these differences observed in the T and G mutants may be explained if extraction of RC from the membrane modifies the structure slightly.

Effect of temperature on the extent of phases of P^+ reduction

Fig. 3 shows the temperature dependence of the amplitudes of the VF, F, and VS components of P^+ rereduction after a single laser flash. Data for the L162T strain have recently been published in a separate paper (Ortega et al., 1997). Table 2 summarizes the amplitude values of the sum of both fast components (VF and F) at seven different temperatures in the seven strains of *Rps. viridis* investigated. WT strain (L162Y) and the aromatic mutants L162F and L162W show very similar temperature behaviors for the three kinetic components of P^+ rereduction. The amplitudes of the VF component of P^+ rereduction abruptly diminish with decreasing temperature from ~85% at 294 K to 10% (18% for W) at 150 K. Below this temperature, the extent of this phase in mutants L162F and L162W progressively decreases with decreasing temperature. In the high temperature region (between 294 K and 200 K), the amplitude of the minor F component increases slightly with decreasing temperature in the three aromatic strains (from 10% at 294 K to

TABLE 1 Kinetics and relative amplitudes of the decay phases of flash-induced absorption changes at 1283 nm in RC of WT and of six tyrosine L162 mutant strains of *Rps. viridis* at room temperature and at a very low temperature*

Strain	T (K)	VF (ns)	F (μs)	S (μs)	VS (ms)
L162Y (WT)	294 K	189 (89%)[#]	1.5 (8%)	12 (1%)	0.4 (2%)
	20 K	(0%)	(0%)	82 (5%)	2.5 (95%)
L162F	294 K	135 (74%)	2.1 (10%)	21 (14%)	n.d. (2%)[§]
	8 K	(0%)	(0%)	55 (30%)	1.6 (70%)
L162W	294 K	130 (83%)	1.2 (11%)	18 (4%)	n.d. (2%)[§]
	80 K	514 (10%)	4.4 (6%)	75 (20%)	n.d. (64%)[§]
L162M	294 K	90 (84%)	1.5 (8%)	19 (5%)	n.d. (3%)[§]
	60 K	123 (10%)	0.8 (15%)	77 (11%)	2.1 (64%)
L162L	294 K	390 (63%)	1.1 (28%)	21 (6%)	0.4 (3%)
	40 K	251 (6%)	3.0 (13%)	74 (17%)	2.8 (64%)
L162G	294 K	200 (65%)	1.0 (25%)	14 (7%)	0.4 (3%)
	8 K	190 (20%)	2.2 (13%)	54 (16%)	2.0 (51%)
L162T	294 K	430 (70%)	1.9 (22%)	10 (5%)	0.4 (3%)
	8 K	458 (19%)	3.7 (17%)	54 (23%)	2.2 (41%)

*8–80 K. When several measurements were made in that range, it was found that all data have little temperature dependence.
Experimental conditions as described in Figs. 2 and 3. VF, very fast phase; F, fast phase; S, slow phase; VS, very slow phase; n.d., not determined.
[#]Kinetic data are given as $t_{1/2}$ values, and relative amplitudes as the percentage of the total extent of signal decay attributed to the phase.
[§]Assimilated to a constant in the exponential analysis (constant: cannot be distinguished from any $t_{1/2}$ above 150 μs).

~20% at ~200 K). Below this temperature, the amplitude of this phase abruptly decreases with decreasing temperature, reaching 0% below 150 K. Data of cytochrome oxidation below 150 K in WT strain are very similar, and have been reported previouly (Ortega and Mathis, 1993; Ortega et al., 1997). The weight of the VS component attributed to the $P^+Q_A^-$ back-reaction increases with decreasing temperature in L162Y, L162F, and L162W strains from a value of 2% at 294 K to a maximum value of nearly 90–97% of the total amplitude of P^+ rereduction below 150 K (85% for L162W at 150 K). The kinetic traces of absorption change after a single flash at 8 K are shown in Fig. 2 (panel *A*, 8 K) for the L162F mutant in two different time scales. The decay curve could be fitted by the sum of two exponential components (Table 1): a slow (S) phase (55 μs, attributed to ^3P) and a very slow (VS) phase (1.6 ms), accounting for 30% and 70% of the total signal, respectively. Successive flashes induce the same signal as the first one, showing that the reactions are reversible (data not shown). These data indicate that, in RC of WT and of the aromatic mutants L162F and L162W, at cryogenic temperatures, the cytochrome cannot reduce P^+, which decays very slowly (VS phase with $t_{1/2} \approx 2$ ms), presumably by reaction with Q_A^-. Mutant L162M shows a very similar effect of temperature on the amplitudes of the VF, F, and VS components in the temperature range between 294 K and 200 K, as described for the aromatic residues. However, a larger proportion of both fast phases (VF and F) remains below 150 K: 10% for VF and 15% for F, at 60 K (Fig. 3 and Table 2).

The effect of temperature on the amplitudes of P^+ reduction is rather different in RC of the nonaromatic mutant strains L162L, L162G, and L162T, in which an important proportion of fast phases remains at cryogenic temperatures (Fig. 3 and Table 2). In mutant L162L, the amplitudes of the VF and F components exhibit an antiparallel behavior above 250 K: the amplitude of the VF component decreases with decreasing temperature (from 65% at 294 K to ~35% at 250 K), whereas the amplitude of the F component increases with decreasing temperature (from 30% at 294 K to 55% at 250 K). Below 250 K, both VF and F components decrease in parallel to minimum values of 6% for VF and 13% for F phase at 40 K (see Table 1). In mutant L162G, both VF and F components decrease slightly with decreasing temperature from ~69% and 27%, respectively, at 294 K, to minimum values of ~20% for each phase below 100 K. At 8 K the extent of each phase is 20% for VF and 13% for the F phase (see Table 1). The temperature dependence of the amplitudes of kinetic phases of P^+ reduction was very similar for the mutant L162T (see Tables 1 and 2). The VS component is very small in the high-temperature region in both nonaromatic mutants L162L and L162G. It increases with decreasing temperature from a value of 3% at 294 K to a maximum value of ~60–75% of the total amplitude of P^+ rereduction below 50 K. The ^3P decay component is also present at cryogenic temperatures in all of the strains investigated, accounting for ~20% of the total decay signal (Table 1). It has not been considered in the analysis of the amplitudes of P^+ rereduction described above.

The surprising occurrence of fast phases of P^+ reduction at very low temperatures in several of the mutants led us to perform additional experiments to ascertain their interpretation. A first aspect to be considered is that fast reactions at low temperatures are associated with irreversible electron transfer. This is illustrated in Fig. 2 *B* for the L162G mutant. The kinetics after the first flash are analyzed in four phases, as described above (see Table 1). The amplitudes of the VF phase ($t_{1/2} = 190$ ns) and the F phase ($t_{1/2} = 2.2$ μs) make up altogether 39% of the decay of P^+. At the second flash, however, both phases have amplitudes reduced by a factor of ~3, and they are hardly measurable at the third flash. The total ΔA induced by the flash decreases accordingly, in agreement with the attribution of fast phases to an efficient, irreversible oxidation of a cytochrome heme accompanied by the stable reduction of Q_A. The remaining signal has

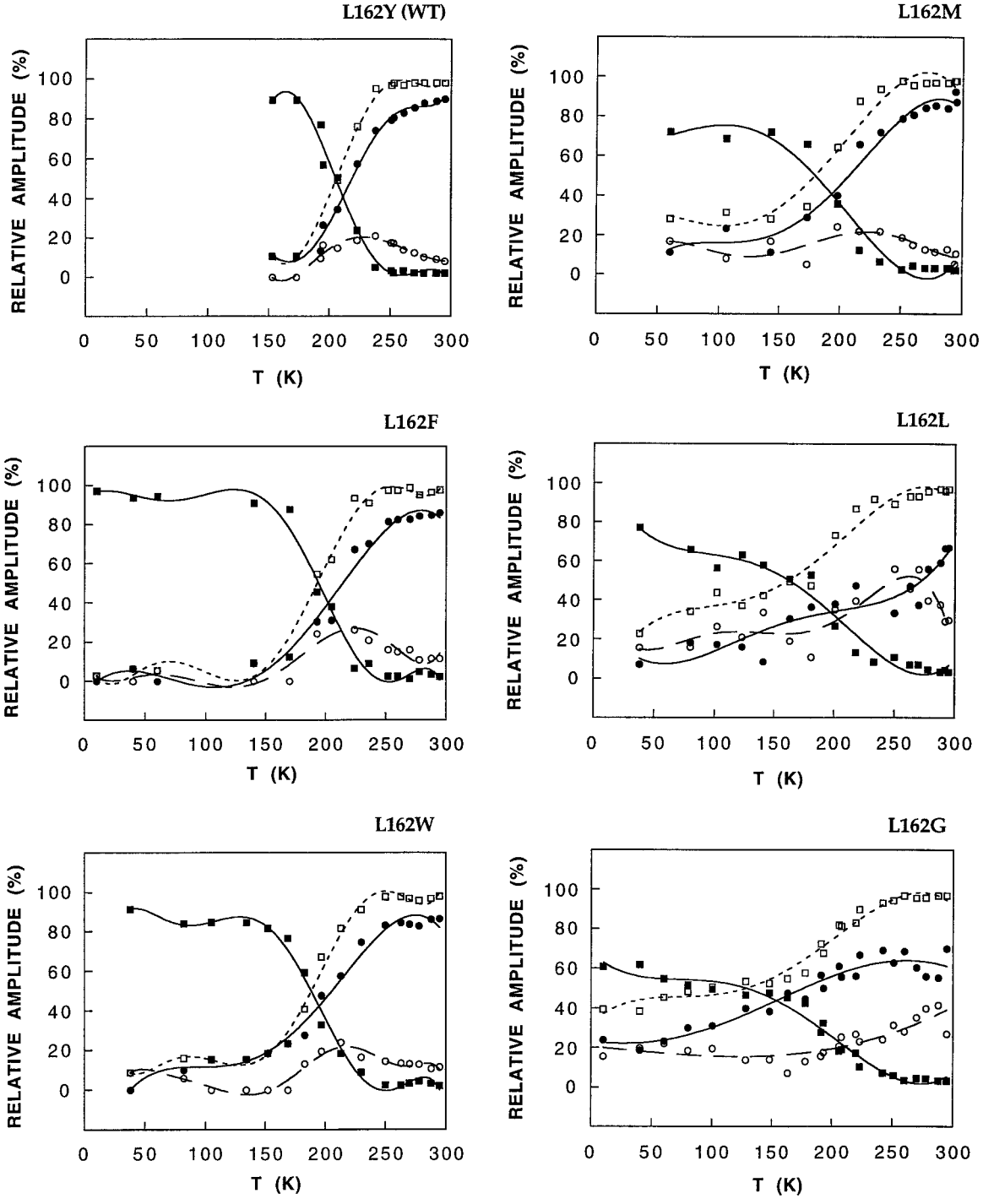


FIGURE 3 Temperature dependence of the relative amplitudes of the different components of P^+ rereduction in RC of WT and of five L162 mutant strains of *Rps. viridis*. The data were obtained by analysis in exponential components of flash absorption experiments exemplified in Fig. 2. The cuvettes were brought in darkness to the indicated temperature, after ~2 min of dark adaptation at room temperature. Measurements below 243 K are the result of a single flash given to the dark-adapted sample. At higher temperatures the measurements are the average of four single flashes, with a time spacing sufficient to allow a return to equilibrium (1–5 min). The S phase (attributed to ^3P) was considered in the analysis, but its weight is not included in the figure: for each temperature the contributions of the three kinetic phases (VF, F, and VS) are normalized to a total of 100 for the first flash. ●, ○, VF and F phases, respectively; □, sum of VF and F phases; ■, VS phase. The total amplitude of the first flash signal does not vary significantly with temperature, and the observed 20% increase is probably due to contraction of the sample. The lines are drawn to guide the eye.

kinetic phases attributable to ^3P and to the $P^+Q_A^-$ back-reaction. This behavior was clearly found in G, L, M, and T mutants, and not in Y and F (for Y and T strains, see Ortega et al., 1997). In the W mutant, this effect was observable but small, in agreement with the small size of fast phases in this mutant at low temperatures.

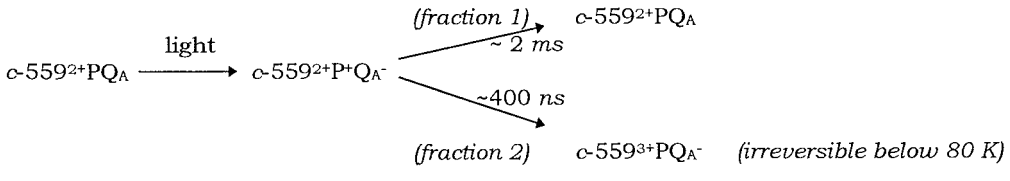
TABLE 2 Effect of temperature on the relative amplitude (in percentage) of the fast kinetic components of electron transfer between the tetraheme cytochrome and P^+ in RC of WT and of six tyrosine L162 mutant strains of *Rps. viridis*

Strain	294 K	260 K	200 K	150 K	100 K	40 K	8 K
L162Y (WT)	98*	97	49	11	4[#]	0[§]	n.d.
L162F	98	98	55	10[¶]	n.d.	6	3
L162W	98	98	67	18	15	9	n.d.
L162M	98	97	64	28[¶]	31	28[‖]	n.d.
L162L	97	90	73	45**	44	23	n.d.
L162G	97	94	82[##]	53	51	38	40
L162T	98	95	90	73	66	48	43

Experimental conditions as described in Figs. 2 and 3. n.d., not measured.
*Fast phases represent the sum of the VF and F kinetic components of P^+ absorption recovery after a laser flash. The S phase of absorption recovery has not been taken into consideration in the amplitudes analysis (% fast phases + % VS = 100).
[#]80 K, from Ortega and Mathis (1993).
[§]20 K.
[¶]140 K.
[‖]60 K.
**163 K.
[##]208 K.

Difference spectra were also measured, to determine which heme becomes oxidized. With the T mutant, absorption spectra were recorded at 8 K before and after a progressive illumination (Fig. 4). The α band of heme *c*-559, which is located at 556 nm at low temperatures (Verméglio et al, 1989), bleaches with a very weak illumination, up to a maximum of ~30%, which is reached by 10 s of illumination (*trace 2* in Fig. 4). Bleaching of the band of *c*-556 (double peak at 554.5 and 551 nm; see Verméglio et al., 1989) also takes place, although with a much lower efficiency. In Fig. 4 the difference spectrum 1 is attributable essentially to heme *c*-559. Spectrum 2 is due mainly to *c*-559, with some admixture of *c*-556, the contribution of which still increases in spectrum 3. Spectrum 4 is the difference between spectra 2 and 3; it reflects the effect of an additional illumination given after 10 s of light, and it looks like a pure spectrum of heme *c*-556 bleaching. In agreement with kinetic studies of P^+, bleaching of *c*-559 is absent in WT RC (L162Y), but the bleaching of heme *c*-556 is identical. A detailed study showed that the latter effect is not due to the oxidation of heme *c*-556, but to the photodissociation of its methionine ligand upon direct excitation of the cytochrome (Ortega et al., 1998).

The results described above show that, after light excitation, fast electron transfer from the proximal *c*-559 heme to P^+ takes place down to 8 K in a fraction (20–40%) of RC (fraction 2 of Eq. 1) in some of the L162 mutant strains investigated, producing a charge separated state that is very stable, as shown by the scheme below:



At this temperature, the cytochrome does not transfer an electron in ~60–80% of RC (fraction 1) in which P^+ decays, presumably by electron return from Q_A^-, a reaction that has a $t_{1/2}$ of a few milliseconds at low temperature (Shopes and Wraight, 1987). This fraction of RC exhibits identical kinetics upon excitation with the first or subsequent flashes, if applied with an appropriate time delay (Fig. 2 *B*). These results demonstrate an efficient (although partial) irreversible low-temperature oxidation of heme *c*-559 under conditions where only high-potential hemes are prereduced, in several mutant strains. In previous works, efficient photooxidation of cytochrome in *Rps. viridis* at low temperature has been reported only at low potential, when heme *c*-552 is prereduced (for a review, see Nitschke and Dracheva, 1995).

Effect of temperature on reaction rates

We have also analyzed the effect of temperature on the kinetics of P^+ absorption recovery after a single flash in RC of WT and of the six L162 mutant strains of *Rps. viridis* (Fig. 5 and Table 3). Table 3 summarizes the kinetic values ($t_{1/2}$) of the VF component at seven different temperatures in all of the strains investigated. The WT and the two aromatic mutants L162F and L162W show a very similar Arrhenius behavior for the VF and F components of P^+ rereduction in the 294–200 K temperature region (Fig. 5). The rates of the VF component at 294 K are 3.7×10^6 s^{-1} (Y), 5.1×10^6 s^{-1} (F), and 5.3×10^6 s^{-1} (W); they diminish slightly with

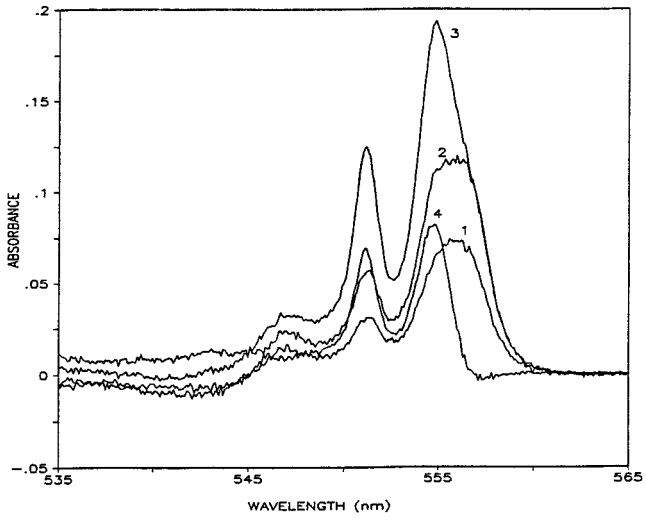


FIGURE 4 Difference absorption spectra in the α-band region of cytochrome hemes in RC of mutant L162T of *Rps. viridis* resulting from illumination at 8 K. The same cuvette was illuminated for different time periods with a 1.5-W tungsten lamp placed ~10 cm from the cuvette. Other experimental conditions were as described in Materials and Methods. Trace 1: dark minus light (2 s); trace 2: dark minus light (10 s); trace 3: dark minus light (3 min); trace 4: light (10 s) minus light (3 min).

decreasing temperature to rates of $1.4 \times 10^6\ s^{-1}$ (Y), $1.5 \times 10^6\ s^{-1}$ (F), and $1.4 \times 10^6\ s^{-1}$ (W) at ~200 K. The rates of the F component are $4.6 \times 10^5\ s^{-1}$ (Y), $3.3 \times 10^5\ s^{-1}$ (F), and $5.8 \times 10^5\ s^{-1}$ (W) at 294 K; they also decrease with decreasing temperature to $2.5 \times 10^5\ s^{-1}$ (Y), $2.4 \times 10^5\ s^{-1}$ (F), and $1.0 \times 10^5\ s^{-1}$ (W) at ~200 K. Below 150 K, it was not possible to distinguish between the VF and F phases in strains Y and F because of their very small amplitudes. In mutant L162W, however, the good signal-to-noise ratio made it possible to obtain the kinetics of VF and F phases below this temperature. The rates of both phases were almost temperature-independent between 200 K and 80 K ($1.4 \times 10^6\ s^{-1}$ at 200 K, and $1.3 \times 10^6\ s^{-1}$ at 80 K, for the VF phase; $1.0 \times 10^5\ s^{-1}$ at 200 K and $1.5 \times 10^5\ s^{-1}$ at 80 K, for the F phase). The activation energies for the VF and F components in the high-temperature region are, respectively, 5.5 and 5.4 $kJ \cdot mol^{-1}$ (Y), 5.6 and 1.6 $kJ \cdot mol^{-1}$ (F), and 5.5 and 6.7 $kJ \cdot mol^{-1}$ (W).

Replacement of the tyrosine L162 residue by nonaromatic residues such as methionine, threonine, glycine, or leucine permits electron transfer from cytochrome to P^+ in a fraction of the reaction centers down to 8 K, allowing the measurement of the kinetics of cytochrome oxidation between 294 K and 8 K. In mutants L162G and L162L, the temperature has no significant effect on the kinetics of the VF phase (Fig. 5 and Table 3), the rates being almost temperature-independent ($2.0 \times 10^6\ s^{-1}$ at 294 K and $1.7 \times 10^6\ s^{-1}$ at 8 K for G; $1.8 \times 10^6\ s^{-1}$ at 294 K and $2.8 \times 10^6\ s^{-1}$ at 40 K for L). Decreasing the temperature, however, induces a slight slowing down of the kinetics of the F phase in the high-temperature region in both strains; the rates are $8.7 \times 10^5\ s^{-1}$ (G) and $6.3 \times 10^5\ s^{-1}$ (L) at 294 K, and $4.3 \times 10^5\ s^{-1}$ (G) and $4.6 \times 10^5\ s^{-1}$ (L) at ~200 K. Below this temperature, the rates are almost temperature-independent for mutant L162G ($4.3 \times 10^5\ s^{-1}$ at 40 K), whereas they decrease slightly for mutant L162L ($2.4 \times 10^5\ s^{-1}$ at 80 K). The activation energies for the VF and F components are, respectively, 0.04 and 0.35 $kJ \cdot mol^{-1}$ (G), and -0.14 and 0.65 $kJ \cdot mol^{-1}$ (L) (these values are probably not significantly different from zero). A similar temperature behavior of the kinetics has recently been described for the mutant L162T (Ortega et al., 1997).

Mutant L162M displays a more complex temperature dependence for the kinetics of VF and F phases (Fig. 5 and Table 3). The rates of these phases decrease with decreasing temperature in the high-temperature region (between 294 K and 230 K), as for the aromatic residues. The VF phase rates are $7.5 \times 10^6\ s^{-1}$ at 294 K, and $2.6 \times 10^6\ s^{-1}$ at 230 K; the F phase rates are $6.9 \times 10^5\ s^{-1}$ at 294 K, and $0.9 \times 10^5\ s^{-1}$ at 230 K. Below 200 K, however, the rate of the VF component increases slightly, reaching a value of $5.6 \times 10^6\ s^{-1}$ at ~140 K. Between 140 K and 60 K, the rate of the VF component remains almost temperature-independent. The F phase showed a similar behavior in the temperature range between 200 K and 60 K. The rate increases with decreasing temperature between 230 K and 140 K ($8.7 \times 10^5\ s^{-1}$ at ~140 K) and remains almost temperature-independent below 100 K ($8.7 \times 10^5\ s^{-1}$ at 60 K). A similar acceleration induced by decreasing temperature has been reported for the $P^+Q_A^-$ back-reaction in RC of *Rb. sphaeroides* and attributed to a decrease in the reorganization energy (Ortega et al., 1996).

Fig. 6 shows the effect of temperature on the rates of the S and VS phases of P^+ reduction in RC of some of the mutant strains studied. The rates of the S phase, which has been attributed to ^3P in WT RC (Ortega and Mathis, 1993), are nearly the same for all strains, with a $t_{1/2}$ of ~15 μs at 294 K and 60 μs below 100 K (Fig. 6, S; see also Table 1). The kinetics of the VS phase are also nearly the same for the mutants investigated (Y, M, G, and L), with a $t_{1/2}$ of ~0.4 ms at room temperature and 2 ms below 100 K (Fig. 6, VS; see also Table 1). This kinetic phase is attributed to the electron return from Q_A^- to P^+ (see, e.g., Shopes and Wraight, 1987), which is not modified by replacement of L162Y. These data seem to indicate that the properties of P are not much influenced by the mutations. The same conclusion had been reached earlier from absorption spectra and redox potentials of the P/P^+ couple (Dohse et al., 1995).

DISCUSSION

An evolutionarily conserved tyrosine residue is placed at position 162 of the L subunit of all RC of photosynthetic bacteria in which this polypeptide has been sequenced (Williams et al., 1983; Youvan et al., 1984; Michel et al., 1986b; Shiozawa et al., 1989; Fathir et al., 1997). The location of this residue, halfway between the proximal heme of the tetraheme cytochrome and the bacteriochlorophyll special

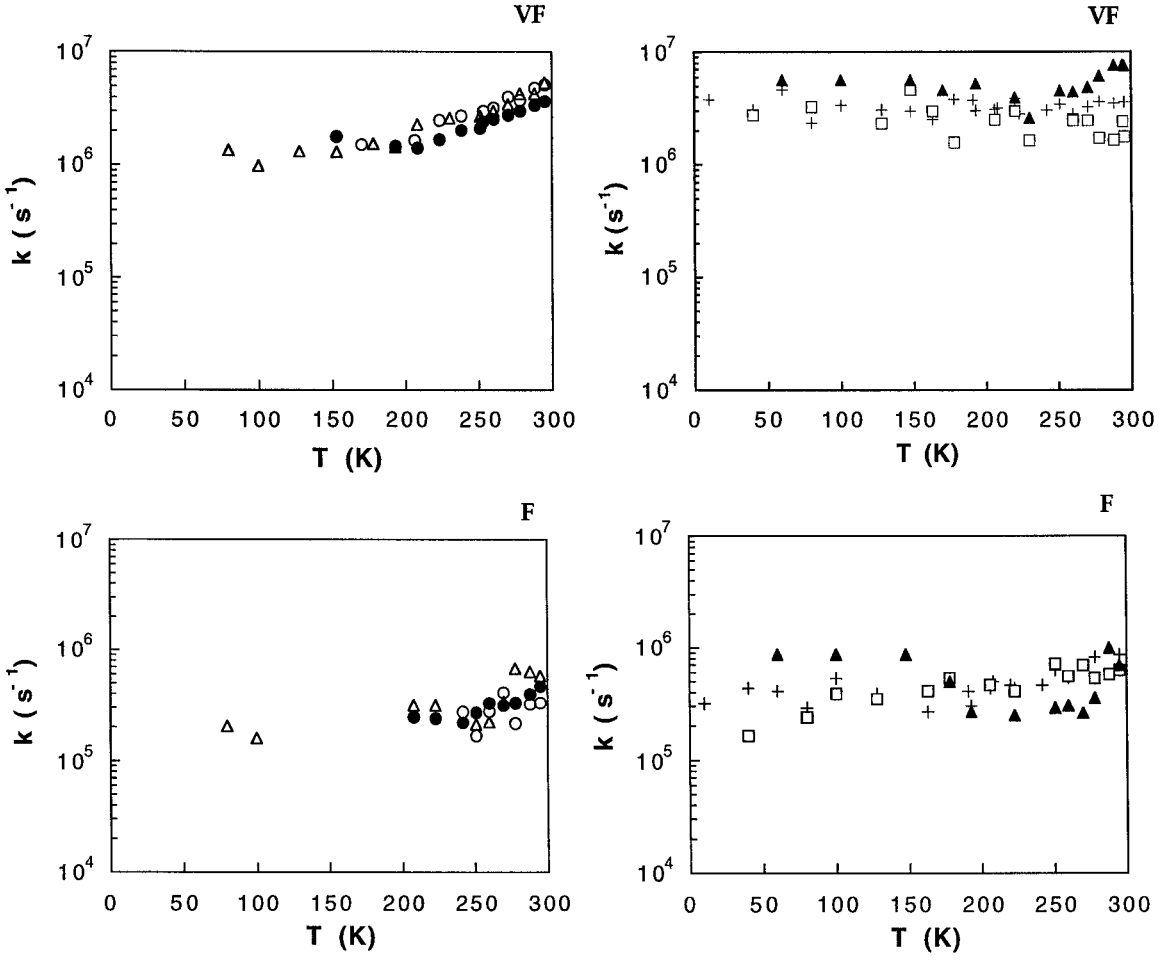


FIGURE 5 Temperature dependence of the rates ($k = (t_{1/2} \times 1.44)^{-1}$) of the VF and F kinetic components of P^+ rereduction by the tetraheme cytochrome in RC of WT and of five L162 mutant strains of *Rps. viridis*. The data were obtained by analysis in exponential components of flash absorption experiments exemplified in Fig. 2. All experimental conditions were as in Fig. 3. ●, L162Y (WT); ○, L162F; △, L162W; ▲, L162M; □, L162L; +, L162G.

pair, and its conservative character led several authors to postulate that it might play a key role in facilitating electron transfer between these two cofactors (Michel et al., 1986a; Knapp and Fischer, 1987; Cartling, 1991). This tyrosine residue has also been implicated in the effects of low temperature on electron transfer between the cytochrome

TABLE 3 Effect of temperature on the half-time (in ns) of the VF phase of electron transfer between the tetraheme cytochrome and P^+ in RC of WT and of six tyrosine L162 mutant strains of *Rps. viridis*

Strain	294 K	260 K	200 K	150 K	100 K	40 K	8 K
L162Y (WT)	189	271	470	400	n.d.*[#]	n.d.[§#]	n.d.[¶]
L162F	135	217	420	460[‖]	n.d.[¶]	n.d.[#]	n.d.[#]
L162W	130	230	482	534	520	n.d.[#]	n.d.[¶]
L162M	90	150	132	123[‖]	120	123**	n.d.[¶]
L162L	390	280	275	231[##]	220	251	n.d.[¶]
L162G	200	245	230[§§]	230	210	230	190
L162T	430	440	420	463	391	470	458

Experimental conditions as described in Figs. 2 and 4. n.d., not determined.
*80 K, from Ortega and Mathis (1993).
[#]It was not possible to obtain the kinetic value due to its very small amplitude.
[§]20 K.
[¶]Data not measured.
[‖]140 K.
**60 K.
[##]163 K.
[§§]208 K.

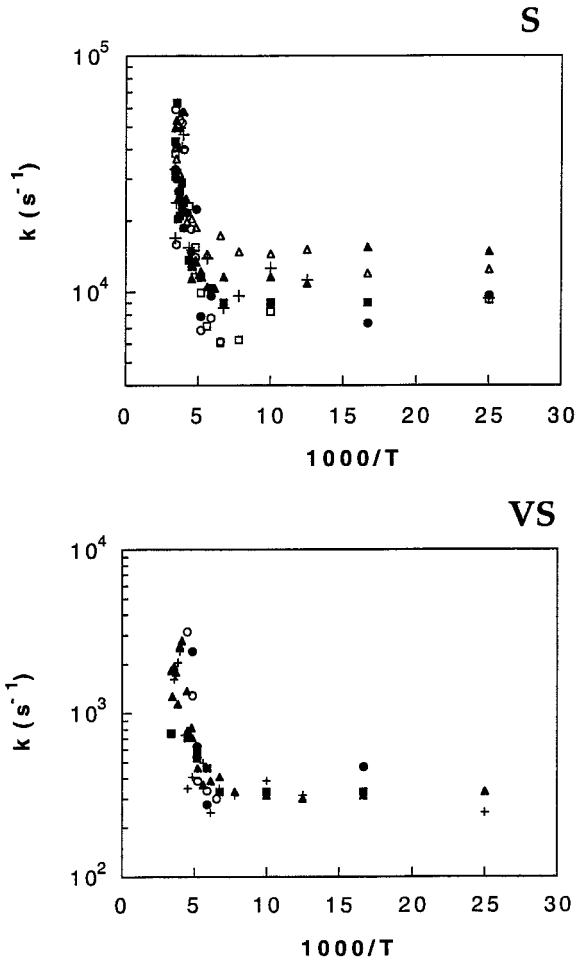


FIGURE 6 Temperature dependence of the rates of the slow (S) and very slow (VS) kinetic components of P^+ absorption recovery measured at 1283 nm in RC of WT and of L162 mutant strains of *Rps. viridis*. All experimental conditions were as in Fig. 3. ○, ●, L162Y and L162F, respectively; □, ■, L162W and L162M, respectively; △, ▲, L162T and L162G, respectively; +, L162L.

and the special pair (Knapp and Fischer, 1987; Cartling, 1993), but no experimental confirmation has yet been obtained. To examine these hypotheses, we have studied in this work the effect of substitution of the tyrosine residue L162 of the *Rps. viridis* RC on electron transfer from tetraheme cytochrome to P^+, between 294 K and 8 K.

Heterogeneity of cytochrome oxidation

The physical basis of the two fast components of P^+ rereduction by the tetraheme cytochrome (VF and F phases) observed in chromatophores (Dohse et al., 1995) and RC of *Rps. viridis* (Ortega and Mathis, 1993) is not clear. In WT RC, the small microsecond phase (F phase; $t_{1/2} = 1.5$ μs; extent of 10%) was attributed to electron transfer from *c*-559 heme to P^+ in a different conformation of the RC (Ortega and Mathis, 1993). The results obtained with chromatophore membranes of the same *Rps. viridis* mutant strains used in this work led authors to propose that the physical basis of the F phase is a substate in which some variability in the binding of the cytochrome to the RC exists (Dohse et al., 1995). The results of the present work confirm these results. As shown in Figs. 3 and 5, the kinetic properties that we attribute to various substates of the cytochrome-RC complex are significantly different among the series of mutants. Thus they cannot be directly related to macroscopic properties, due, e.g., to the water-glycerol solvent. The present work also extends our previous observation of a temperature-dependent equilibrium of VF and F components (Ortega and Mathis, 1992, 1993). This phenomenon is even rather pronounced in strains L162T (data described in Ortega et al., 1997) and L162L (Fig. 3), so that there is no doubt about its occurrence. However, we obtain no precise clue to interpret the phenomenon. Perhaps it could be stated that it is more prominent in strains T, G, and L, which also have a large extent of fast phases (VF and F) remaining at low temperature, a possible indication of structural heterogeneity. Structural analysis (Dohse et al., 1995), however, has still not revealed any large difference between the WT and the T mutant, except that heme *c*-559 is moved 0.3 Å toward P, a property that cannot explain a larger extent of slower phase of electron transfer.

Blocking of cytochrome oxidation at low temperature

An essential feature appeared in the early studies on the effects of low temperature on electron transfer from tetraheme cytochromes to P^+ following illumination: under redox conditions where one or both of the high potential hemes are reduced but the low potential hemes are oxidized, lowering the temperature results in a drastic decrease of the extent of heme oxidation by P^+, from close to 100% at room temperature to relatively low values below 100 K. In a previous study of the effect of temperature on electron transfer from the tetraheme cytochrome to P^+ in WT RC of *Rps. viridis* (Ortega and Mathis, 1992, 1993), we have observed the same phenomenon: the amplitude of the fast phases of this reaction, the sum of which amounts to nearly 100% of P^+ rereduction at high temperature, decreases suddenly around 210 K and becomes negligible at low temperatures. As shown in Fig. 3 and in Table 2, this behavior is clearer and sharper in WT RC (L162Y), but it also takes place, and is more or less well defined, with all of the strains studied. Tyrosine, or even an aromatic amino acid, is not required at L162 for the inhibition of cytochrome oxidation at low temperature to take place, although the nature of the amino acid residue at this position clearly influences the behavior. In nonaromatic mutants (G, T, M, and L), an important proportion of cytochrome oxidation remains at low temperature.

It thus appears that the most remarkable effect of lowering the temperature is a transition from a high-temperature state of the RC (S_{HT}), characterized by a fast electron transfer, to a low-temperature state (S_{LT}) where the reaction

is slower or unmeasurable. The results of Fig. 3 and Table 2 can be read as indicating that the low temperature population of these two states is influenced by the amino acid at position L162. What is the physical origin of this transition? Nocek and co-workers have studied the effect of low temperature on the quenching by cytochrome *c* of the triplet excited state of Zn-substituted cytochrome *c* peroxidase (Nocek et al., 1991). They observed a transition (220 K $< T <$ 250 K) between a low-temperature state that does not exhibit triplet quenching and a high-temperature state that does. They proposed that the protein-protein complex adopts several conformations, some of them allowing electron transfer and others not, and that it is locked in an inactive conformation at low temperature. We found, however, that the effect of temperature is about the same for the complex between cytochrome c_2 and the RC of *Rb. sphaeroides* (Venturoli et al., 1993) and for *Rps. viridis* with a firmly bound tetraheme cytochrome (Ortega and Mathis, 1992, 1993; and this work). It is also the same when cytochrome c_2 is cross-linked to the RC of *Rb. sphaeroides* (Drepper et al., 1997). We thus conclude that the proposal of Nocek and co-workers is not applicable to our system. It is much more probable that low temperatures block electron transfer by rendering impossible structural reorganizations associated with cytochrome oxidation (Ortega and Mathis, 1993; Mathis et al., 1994; Ortega et al., 1997). A model system for a similar phenomenon has been studied by Gaines et al. (1991).

Our hypothesis for interpreting the blocking of electron transfer at low temperature is that lowering the temperature induces a stepwise freezing of the protein, which mostly involves the water molecules included in the cytochrome structure. The inhibition is attributed to a freezing-like transition of networks of water molecules that blocks structural changes of the protein that are normally associated with cytochrome oxidation. This is the hypothesis we consider the most likely to account for the transition from S_{HT} to S_{LT}. Several features plead in favor of an interpretation of this phenomenon, which relies on changes of relative values of driving force $-\Delta G°$ and reorganization energy λ: 1) The very large blocking of electron transfer in WT, by 10^4 or more, cannot be explained by electronic factors, i.e., a change in distance or in the exponential coefficient of decay with distance β. 2) Several techniques have demonstrated the occurrence of a large number of structural changes in cytochrome *c* associated with heme oxidation, including displacement of water molecules (Williams et al., 1985; Berghuis and Brayer, 1992; Berghuis et al., 1994; Qi et al., 1994). 3) The role of water as plasticizer for proteins has been recognized, and its freezing might inhibit protein structural changes associated with function (Doster et al., 1986; Pethig, 1992). This is a common interpretation of low temperature inhibition of protein activity (e.g., Rasmussen et al., 1992). 4) The effect of temperature lowering on inhibition of electron transfer has been mimicked at room temperature by partial dehydration of RC-tetraheme cytochrome complexes (Kihara and McCray, 1973; Chamarovsky et al., 1986) or by the addition of cryoprotectants (Kihara and McCray, 1973). Frolov and co-workers have recently proposed that the low-temperature restriction of electron transfer between heme *c*-559 and P^+ in *Rps. viridis* is caused by an energetic inhibition that originates in the opposite shifting of the energy levels of heme *c*-559 and P^+ due to the freezing of protein dynamics and protein-bound water mobility (Frolov et al., 1996). On the other hand, this phenomenon can also be interpreted in terms of adiabatic electron transfer occurring under conditions of very low protein mobility (A. I. Kotelnikov et al., personal communication).

Why does electron transfer become blocked at low temperatures in the L162Y protein and not (or at least only partly) in L162T, L162L, and L162G? Data of the temperature dependence of the $P^+Q_A^-$ back-reaction and of the decay of ^3P in these mutants show that the properties of P are not much influenced by the mutation. In a previous work we have shown that the RC structure is nearly the same for the WT and the L162T mutant, with very limited structural differences restricted to the mutation site (Dohse et al., 1995). In that work it was also shown that the redox potentials of the high-potential hemes are not much modified in the mutants compared to the WT. We thus conclude that our results cannot be interpreted in terms of a change in driving force for electron transfer, unless unmeasurable effects develop at low temperature. The results of the present work lead us to propose that the substitution of aromatic residues by nonaromatics modifies the water molecule network in the region between *c*-559 and P. One suggestion is that smaller residues than aromatics at position L162, such as M, T, G or L, allow the existence of a slightly larger pool of water molecules in the region close to heme *c*-559 (mutations would allow for one or two additional water molecules at most), with the consequence that electron transfer will remain more efficient at cryogenic temperatures. In support of this hypothesis, it is with the hydrophilic residue T that fast phases remain most abundant at low temperatures. As a second possibility we hypothesize that water molecules involved in redox-associated structural changes are differently located in the mutants compared to the WT. The 3-D structure of *Rps. viridis* RC shows the presence of seven water molecules in the region between the proximal heme *c*-559 and the special pair P (Fig. 1), which are organized in a network of hydrogen bonds, including the tyrosine L162 residue. Both possibilities, different number or different location of water molecules, might lead to the same consequence, which is a disturbance of the network of hydrogen bonds between the heme *c*-559 and P.

Temperature dependence of the kinetics of cytochrome oxidation

DeVault and Chance discovered in 1966 a unique temperature dependence for the rate of P^+ rereduction by a tetraheme cytochrome in the photosynthetic bacterium *Cr. vino-*

sum with low-potential hemes reduced. The rate constant, k, exhibited a sharp transition from a high-temperature activated region, where k drops by three orders of magnitude to a temperature-independent region at low temperatures ($T <$ 120 K), where k remains constant (DeVault and Chance, 1966; DeVault et al., 1967). In a previous work we studied the same reaction in a related species of purple bacteria, *Rps. viridis*. When low-potential hemes are reduced, we found that the fast electron transfer reaction from heme *c*-559 to P^+ has a rate that is only weakly temperature-dependent. It becomes temperature-independent below 200 K. When low-potential hemes are oxidized and one or both high-potential hemes are reduced, electron transfer from *c*-559 to P^+ has a slightly lower rate, which is also only weakly temperature-dependent (Ortega and Mathis, 1992, 1993). The results obtained in the present work indicate that when low-potential hemes are oxidized, the temperature dependence of the rate of electron transfer between cytochrome *c* and P^+ cannot be related to the presence of the aromatic tyrosine residue at position L162 between the proximal heme *c*-559 and the special pair P, as has been proposed by several authors to interpret the data obtained by DeVault and Chance with *Cr. vinosum* (Knapp and Fischer, 1987; Knapp and Nilsson, 1990; Cartling, 1991, 1992, 1993). The similar temperature behavior found in this work for mutants in which the tyrosine L162 residue has been replaced by other aromatic residues does not support this hypothesis, because neither phenylalanine nor tryptophan has the hydroxyl OH group required for the existence of the high-energy activated state proposed by Cartling or for the fluctuations of the dielectric constant (Knapp and co-workers). Substitution of Y by G, T, or L leads to a temperature-independent rate for the VF phase of cytochrome oxidation; this is an extreme case of a weak activation energy.

One important aspect of the results obtained with non-aromatic mutants is that fast phases of cytochrome oxidation remain rather abundant at low temperatures, allowing us to determine their kinetics rather precisely. Moreover, the S phase due to ^3P is rather slow at low temperatures, and it does not interfere in the exponential analysis. This accuracy was not possible with WT because the fast phases "disappear" below 150 K. Thus we can nicely demonstrate that, in the VF conformational state, the rate varies by at most a factor of 5 when temperature is lowered, and that it remains temperature-independent below ~100 K (see Fig. 5, VF). This behavior is only superficially similar to that reported by DeVault and Chance (1966) and is not compatible with the model proposed by Bixon and Jortner (1988), in which the oxidation of two different cytochromes has been postulated to explain the experiment by DeVault and Chance. Their model concerned *Chromatium*, but we consider it highly probable that the tetraheme cytochromes of different purple bacteria behave similarly. The results obtained in this work clearly show that the rate of electron transfer from the proximal heme *c*-559 to P^+ is essentially temperature-independent. The Marcus theory (Marcus and Suttin, 1985; Moser et al., 1992) predicts the temperature independence of electron transfer for a reaction when the free energy variation ($-\Delta G°$) and the reorganization energy λ exactly cancel, $\Delta G° + \lambda = 0$. This explanation is not compatible with our system because the free energy variation is quite small (about -130 meV) and the reorganization energy is certainly not below 500 meV (Moser et al., 1992; Lin et al., 1994). It is now well established that several electron transfer steps within photosynthetic RC are nearly temperature-independent (P^+ to H, H^- to Q_A, Q_A^- to P^+) (Gunner and Dutton, 1989) under conditions where $\Delta G° + \lambda \neq 0$. These authors proposed that in RC, electron transfer is coupled to high-energy vibrational modes and that its rate is controlled by nuclear tunneling (Gunner and Dutton, 1989; Moser et al., 1992). This is also what most likely happens in the system we studied, i.e., electron transfer between tetraheme cytochrome and P^+ in mutated RC.

CONCLUSIONS

In this work we have investigated the effects of substitution of tyrosine L162 of the *Rps. viridis* RC by other amino acids (F, W, G, T, M, and L) on the kinetics and extents of electron transfer from the tetraheme cytochrome to the special pair, between 294 K and 8 K. The results obtained are interesting in several respects: 1) Confirming the results of a previous work using chromatophores of the same mutant strains at room temperature (Dohse et al., 1995), we have found that neither tyrosine nor aromaticity is required for fast electron transfer between cytochrome and P. 2) Tyrosine L162 seems not to be responsible either for the Arrhenius behavior of this reaction in the high temperature region, or for its blocking at low temperature. 3) Our data demonstrate an efficient irreversible low-temperature oxidation of a cytochrome under conditions where only high-potential hemes are prereduced. 4) The results also show that P^+ can be rapidly rereduced by the cytochrome ($t_{1/2}$ in the submicrosecond range) in a substantial fraction of RC even at 8 K. This is apparently a first example of such rapid function of cytochromes at low temperature. 5) The occurrence of a substantial fraction of rapid phases down to 8 K has permitted us to determine rather accurately their rates at all temperatures and to show that they are practically temperature-independent. Further studies are required to know whether the behavior reported here for a mutant RC-cytochrome complex, i.e., temperature-independent rate and two states (leading to electron transfer or to no electron transfer), with a temperature-dependent population of the two states, could be rather general for interprotein electron transfer reactions.

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