Very fast electron transfer from cytochrome to the bacterial photosynthetic reaction center at low temperature

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Abstract Electron transfer from the proximal heme *c*-559 to the primary donor P has been studied in reaction centers of the photosynthetic bacterium *Rhodopseudomonas viridis* in which the tyrosine residue L162 was replaced by threonine. In the wild type, when the two high-potential hemes of the tetraheme cytochrome are reduced before flash excitation, a rapid electron transfer ($t_{1/2}$ = 190 ns) observed at ambient temperature disappears below 190 K. In the mutant, the reaction is partly maintained down to 8 K, leading to irreversible charge separation. The reaction rate is nearly temperature-independent between 294 K and 8 K ($t_{1/2} \approx 450$ ns). The different behavior of wild type and mutant reaction centers is attributed to differences in a network of water molecules, the freezing of which may block structural reorganizations associated with cytochrome oxidation, in the wild type but not in the mutant.

Key words: Electron transfer; Cytochrome; Reaction center; Low temperature; Water in proteins; *Rhodopseudomonas viridis*

1. Introduction

In purple photosynthetic bacteria, the reaction center (RC) is a large membrane protein complex which performs the primary steps of photo-induced electron transfer, starting at the level of a dimer of bacteriochlorophyll molecules, the primary electron donor P, which transfers an electron to an acceptor. The oxidized species P^+ is re-reduced by a *c*-type cytochrome. The best studied example is *Rhodopseudomonas* (*Rps.*) *viridis*, in which the RC structure has been solved [1] showing that the cytochrome has four hemes arranged 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 (see Fig. 1). The heme which donates an electron to P^+ is the proximal high-potential heme (*c*-559, E_m = +380 mV), which is itself re-reduced 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), according to the redox state of the system [2,3]. Electron transfer from cytochrome to P^+ is especially fast: it includes two phases with half-times of 190 ns and 1.5 μs (named very fast (VF) and fast (F) phases in [3,4]), the VF phase accounting for about 85% of P^+ re-reduction at room temperature.

The low temperature functional behavior of photosynthetic cytochromes has been at the forefront of research on electron transfer since the pioneering work of De Vault and Chance [5] who studied the rate of electron transfer from a bound tetraheme cytochrome to P^+ in the bacterium *Chromatium vinosum* poised at low redox potential. They found that the reaction is rather fast at ambient temperature ($t_{1/2}$ = 2 μs) and slows down considerably when temperature decreases, until it becomes temperature-independent ($t_{1/2}$ = 2.3 ms) below 120 K [5,6]. This observation led the authors to suggest the concept of electron tunnelling in biological systems, and strongly influenced the further development of electron transfer theories (e.g. [7,8]). We reinvestigated the same problem with the bacterium *Rps. viridis*, the RC of which has a known structure [1], and found a different behavior from that reported by De Vault and Chance: (i) at high temperature, the VF and F phases of cytochrome oxidation display Arrhenius behavior, i.e. they slow down slightly with decreasing temperature, with a small activation energy (about 6 kJ mol^{-1}); (ii) the amplitude of these phases, the sum of which amounts to nearly 100% of P^+ reduction at high temperature, decreases suddenly and becomes negligible at a temperature which varies with the redox state of the cytochrome: the temperatures of the half-decrease are 250, 210, 80 K if one, two or three hemes are reduced, respectively. This phenomenon precluded any detailed study of the effect of temperature on electron transfer per se. Below 80 K, the only remaining reaction is a rather slow ($t_{1/2}$ = 1.1 ms) electron transfer from a low-potential heme to P^+, like in *Chromatium vinosum* [5,6].

The 3-D structure of the *Rps. viridis* RC shows that a tyrosine (Y) residue (position 162 of the L subunit) is located between the donating heme *c*-559 and P (see Fig. 1). This residue is conserved in the RC of other purple bacteria. It has thus been proposed that tyrosine L162 plays a key role for function either by facilitating electron transfer [9] or for structural reasons [10]. We have recently investigated the rate of electron transfer from the proximal heme *c*-559 to P^+ not only in WT RC of *Rps. viridis* [3,4], but also in proteins where tyrosine (Y) 162 of the L subunit of the RC is replaced by other amino acids (T, L, G, W, M or F) [11]. These mutated proteins were prepared in order to investigate whether the L162Y residue is required for rapid electron transfer to P^+. We found that replacement of L162Y does not modify largely the kinetics of electron transfer at room temperature (the $t_{1/2}$ varies between 145 and 1000 ns in the whole series of mutants). Here we report the temperature dependence of the kinetics and extents of electron transfer from the proximal heme *c*-559 to P^+ in RC isolated from a strain of *Rps. viridis* where the residue L162 is T, instead of Y. The RC were poised at a redox potential where both high-potential hemes are reduced prior to the flash excitation and the kinetics of P^+ re-reduction studied by flash absorption spectroscopy at var-

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Abbreviations: E_h, redox potential of medium; E_m, midpoint redox potential; P, primary electron donor; Q_A, primary quinone acceptor; RC, reaction center; WT, wild type strain

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ious temperatures. The reactions display a few properties which are of general interest for inter-protein electron transfer.

2. Materials and methods

2.1. Cell growth, genetic methods and protein isolation

Growth conditions for WT and mutants strains of *Rps. viridis* (DSM 133) and methods for the construction of site-directed mutants are described in an earlier publication [11]. Harvesting of cells and RC isolation were described in the same reference.

2.2. Spectroscopic measurements

For spectroscopic measurements, samples were prepared with around 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). Glycerol was added to a final concentration of 60% (v/v). Static absorption spectra at 8 K were recorded in a Cary (Model 5E) spectrophotometer by inserting the cuvette in a cryostat cooled with helium gas. Flash absorption kinetics of electron transfer were measured by following P^+ formation and re-reduction at 1283 nm [3,4]. The cuvette was excited by short saturating flashes (9 ns, 595 nm) provided by a dye laser pumped by a YAG laser equipped with a light frequency doubler. The time resolution of the apparatus was 40 ns. 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 (260 to 8 K) or with a thermostated water-ethylene glycol mixture (300–250 K). Measurements below 243 K are the result of a single flash given to a dark-adapted sample cooled in darkness. At higher temperature the measurements are the average of four single flashes, with a time spacing sufficient to allow a return to equilibrium (1–5 min).

3. Results

3.1. Cytochrome oxidation at room temperature

In RC of *Rps. viridis*, the primary donor P is photooxidized by a short flash of light, and the oxidized species P^+ is re-reduced by electron transfer from the bound tetraheme cytochrome or by electron return from the electron acceptor Q_A. The kinetics of P^+ re-reduction after a laser flash are illustrated for RC of WT (Fig. 2A) and of L162T mutant strain (Fig. 2B) of *Rps. viridis*. These experiments were done at a redox potential where the two high-potential hemes (*c*-559 and *c*-556) were reduced prior to the flash ($E_h \approx +250$ mV). At 294 K, the decay curve in WT (Fig. 2A) includes three exponential components: a very fast (VF) phase ($t_{1/2} = 189$ ns), a fast (F) phase ($t_{1/2} = 1.5$ μs) and a slow (S) phase ($t_{1/2}$ of about 10 μs), accounting for 89% (VF), 8% (F) and 1% (S) of the total amplitude of P^+ re-reduction. These three kinetic phases have been clearly identified in a previous work, VF and F phases being assigned to electron transfer from the proximal heme *c*-559 to P^+ in two different RC conformational states [3,4]. The S phase has been attributed to the triplet state of P (^3P) formed in RC where Q_A is reduced before the flash. It is well resolved in our kinetic analysis and not further discussed in the analysis of the data reported here. The kinetics of absorption recovery in the L162T mutant strain at 294 K also include the three phases described above (Fig. 2B): a VF phase ($t_{1/2} = 430$ ns), a F phase ($t_{1/2} = 1.9$ μs) and a S phase ($t_{1/2}$ of about 10 μs), accounting for 70% (VF), 22% (F) and 5% (S). A very slow (VS) component ($t_{1/2} > 400$ μs), representing 2% in WT and 3% in the mutant, is also present. It was previously assigned in WT to the back-reaction between P^+ and Q_A^- in RC where cytochrome does not function [3].

3.2. Effect of temperature on the extent of cytochrome oxidation

We studied the effect of temperature between 300 K and 8 K on the amplitudes of the kinetic phases of P^+ re-reduction

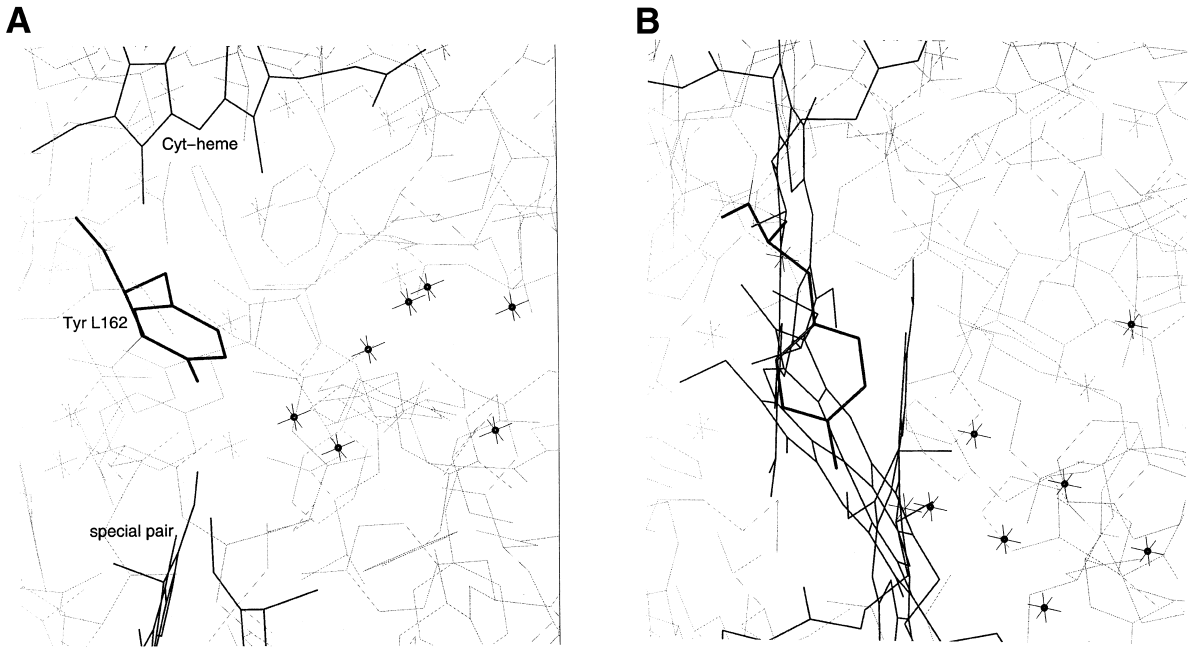

Fig. 1. Detail of the *Rps. viridis* reaction center structure. Left: Sectional view of the protein complex through a plane perpendicular to the membrane surface, showing the proximal heme of the cytochrome (cyt heme) and the special pair P, a dimer of bacteriochlorophyll. Right: View from the cytochrome along the two-fold axis of the RC. Water molecules of the structure are indicated by stars (oxygen). The seven water molecules in bold print are part of a hydrogen-bonded network which links tyrosine L162 (Tyr L162) with the heme-liganding histidine C253 on the one hand, and tyrosine M185 which is hydrogen-bridged to the special pair P on the other hand. (Figure based on unpublished crystallographic data of Dr. R. Lancaster and Dr. H. Michel).

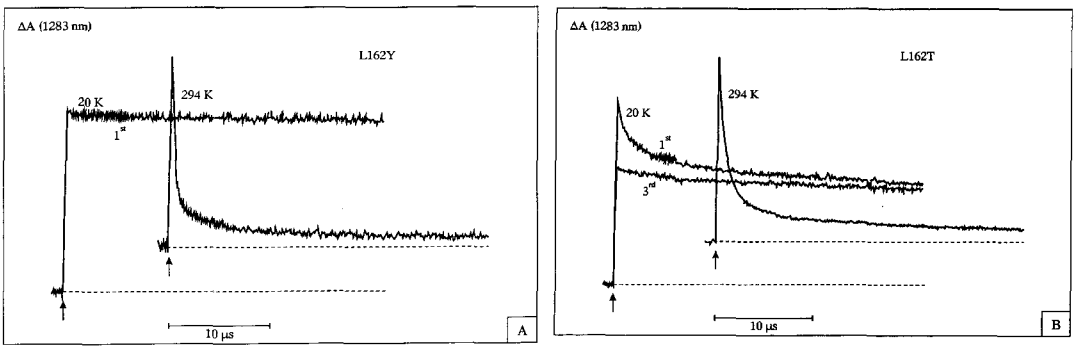

Fig. 2. Kinetics of flash-induced absorption changes at 1283 nm in RC of WT and the mutant strain L162T of *Rps. viridis*. The data at 294 K are the average of four single flashes separated by 2 min. At 20 K, the data are the effect of a single flash, the first or the third for L162T (panel B) (2 min between flashes). For WT (L162Y, panel A) the signal induced by the third flash (not shown) is not distinguishable from that induced by the first. The weights and kinetics of the kinetic phases of absorption recovery are given in Figs. 3 and 4, respectively.

after a laser flash. In WT RC at cryogenic temperatures the cytochrome appears not to be able to reduce P^+, which decays very slowly ($t_{1/2}$ about 2 ms) presumably by back-reaction with the reduced primary quinone Q_A (Fig. 2A, 20 K). Successive flashes induce the same signal as the first, showing that this reaction is reversible (data not shown). Fig. 3 shows the complete temperature dependence of the amplitudes of the fast (VF+F) and very slow (VS) components of P^+ re-reduction in WT RC. It is shown that the amplitude of the cytochrome oxidation (VF+F phases, full circles) abruptly diminishes with decreasing temperature from about 98% at 294 K to 10% of the total amplitude of P^+ re-reduction at 150 K. Below this temperature, the extent of this reaction progressively decreases with decreasing temperature, being about 0–5% at 20 K (see Fig. 2A). The weight of the VS component (filled triangles), attributed to the $P^+Q_A^-$ back-reaction, increases with decreasing temperature from a value of 2% at 294 K to a maximum value of nearly 90–95% of the total amplitude of P^+ re-reduction below 150 K.

The temperature dependence of cytochrome oxidation showed a rather different behavior in RC of the mutant strain in which an important proportion of fast phases remains at cryogenic temperatures (Fig. 2B). The first flash given at low temperature to RC cooled in darkness leads to kinetics which have the four phases observed at room temperature: at 20 K the VF phase ($t_{1/2}$ = 450 ns) makes up about 20% of the decay, the F phase ($t_{1/2}$ = 3.4 μs) 20%, and the VS phase ($t_{1/2}$ = 2.2 ms) 60%. The decay attributed to ^3P (S phase) has a $t_{1/2}$ of about 60 μs. The amplitudes of the two fast phases are much smaller at the second flash and they are negligible at subsequent flashes at 20 K (Fig. 2B). Fig. 3 shows the complete temperature dependence of the cytochrome oxidation by the first flash (filled symbols) and the third flash (open symbols) in the L162T mutant: decreasing temperature induces a decrease of cytochrome oxidation (circles) from about 98% at 294 K to a minimum value of about 40% at 8 K. The VS component (triangles) is very small in the high-temperature region, and it increases with decreasing temperature from a value of 2% at 294 K to a maximum value of about 60% of the total amplitude of P^+ re-reduction below 50 K. Cytochrome oxidation by the third flash becomes very small below 250 K.

These results show that fast electron transfer from *c*-559 to P^+ takes place down to 8 K in a fraction of RC in the L162T mutant strain investigated, producing a charge separated state which is nearly irreversible. We suppose that the electron which has left P in the primary photoreaction is located on the primary quinone Q_A. Absorption spectra of RC of the L162T mutant recorded at 8 K before and after a short illumination show that heme *c*-559 of the cytochrome is partly and irreversibly photooxidized (data not shown). At this temperature, the cytochrome does not work in about 60% of RC and P^+ decays presumably by back-reaction with Q_A^-, a reaction which has a $t_{1/2}$ of a few ms at low temperature [12]. These centers function identically at the first and subsequent flashes (Figs. 2B and 3).

3.3. *Effect of temperature on the kinetics of cytochrome oxidation*

The effect of temperature on the kinetics of P^+ re-reduction following a single flash have been studied between 300 K and 8 K. The WT (Fig. 4) showed Arrhenius behavior in the 300–200 K temperature region for the VF and F components of P^+ re-reduction. The rate of the VF component at 294 K is 3.7×10^6 s^{-1}. It diminishes with decreasing temperature to a rate of 1.4×10^6 s^{-1} at about 200 K. The rate of the F component is 4.6×10^5 s^{-1} at 294 K. It also decreases with decreasing temperature to a rate of 2.5×10^5 s^{-1} at 200 K. Below 150 K, it was not possible to distinguish between the VF and F phases because of their very small amplitudes.

The inhibition of cytochrome oxidation below 200 K thus precluded the investigation of the rates of the reaction at very low temperature in WT. However, the replacement of L162Y residue by threonine allows the reaction to continue in a fraction of the RC down to 8 K, making it possible to precisely measure the kinetics of cytochrome oxidation between 300 K and 8 K. In mutant T (Fig. 4), the temperature had no significant effect on the kinetics of the VF phase, the rate being almost temperature-independent between 294 K and 8 K (1.6×10^6 s^{-1} at 294 K and 1.5×10^6 s^{-1} at 8 K). Decreasing temperature induced a very slight slowing down of the kinetics of the F phase between 294 K and 8 K (3.6×10^5 s^{-1} at 294 K and 2.3×10^5 s^{-1} at 8 K). Inasmuch as it is significant, the activation energy for the component is 0.82 kJ mol^{-1}.

The rate of the VS phase versus temperature is nearly the same in WT and in the mutant, with $t_{1/2}$ of about 0.4 ms at room temperature and 2 ms below 100 K. These features are attributable to the back-reaction, electron return from Q_A^- to P^+ (see e.g. [12]), which is not modified by replacement of L162Y. The rate of the S phase, which has been attributed to ^3P in WT RC [3], is also nearly the same and it shows a

similar temperature dependence. The $t_{1/2}$ is about 60 μs below 100 K. These data are expected if the properties of P are not influenced by the mutations. The same conclusion has been reached earlier for absorption spectra and redox potentials of the P/P^+ couple [11]. In all cases, the total flash-induced absorbance increase changes little with temperature. An increase by 20% as observed is probably due to contraction of the sample.

4. Discussion

The results reported in Figs. 2–4 are interesting in several respects: (i) they are a first example of an efficient irreversible low-temperature oxidation of a cytochrome under conditions where only high-potential hemes are pre-reduced. In previous experiments, cytochrome oxidation below 100 K has been found to occur only when a low-potential heme was pre-reduced (literature on the question is discussed in [13]). (ii) The results also show that P^+ can be rapidly re-reduced by the cytochrome in a substantial fraction of reaction centers even

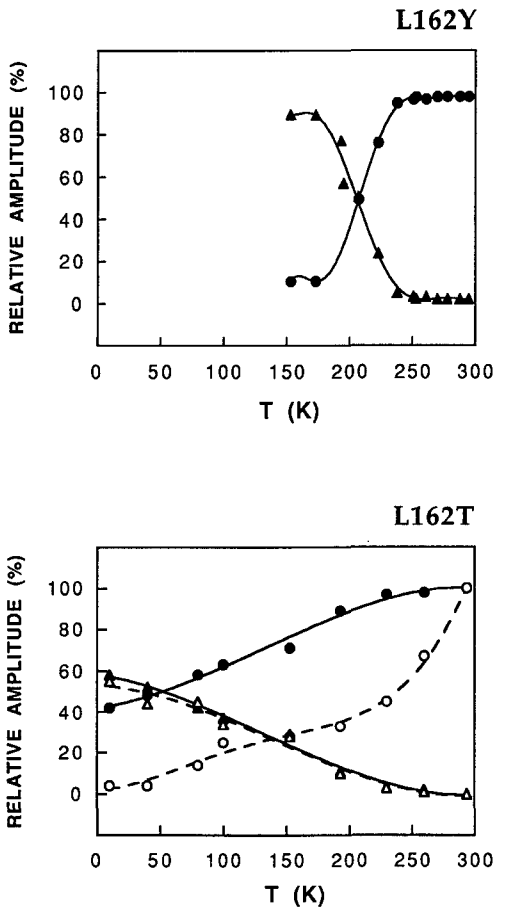

Fig. 3. Temperature dependence of the relative amplitudes of the different components of P^+ re-reduction in RC of WT (L162Y) and the mutant strain L162T of *Rps. viridis*. The data were obtained by analysis in exponential components of flash absorption experiments exemplified in Fig. 2. In these analyses, the contribution of the S phase (attributed to ^3P) has not been considered. 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. Filled symbols: first flash; open symbols: third flash; circles: sum of VF ($t_{1/2} < 1$ μs) and F phases ($t_{1/2} = 1$–4 μs); triangles: VS phase ($t_{1/2} > 400$ μs). The lines are drawn to guide the eye.

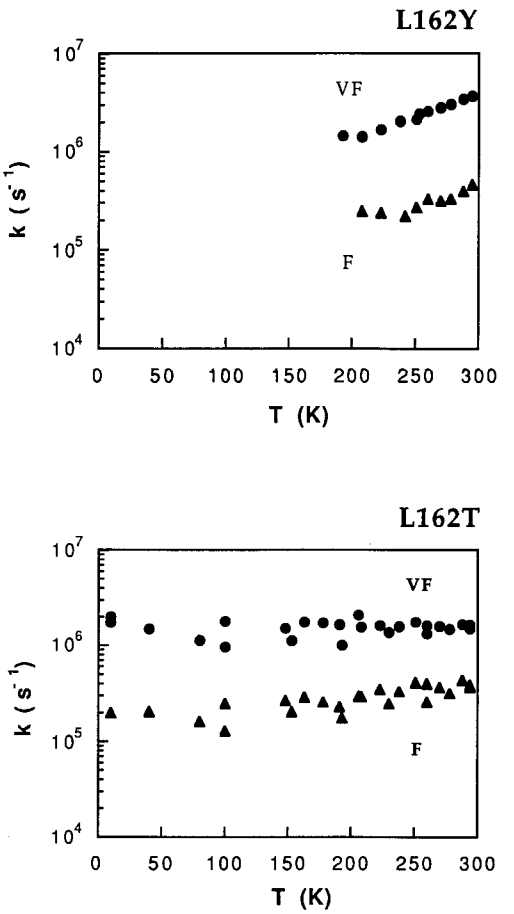

Fig. 4. Temperature dependence of the reaction rates ($k = (t_{1/2} \times 1.44)^{-1}$) of the kinetic components of P^+ re-reduction by the tetraheme cytochrome in RC of WT (L162Y) and L162T mutant strain of *Rps. viridis*. The data were obtained by analysis in exponential components of flash absorption experiments exemplified in Fig. 2. Filled circles: VF phase; filled triangles: F phase.

at 8 K. This is apparently the first clear example of such a rapid function of cytochromes at low temperature. (iii) The occurrence of a substantial fraction of rapid phases down to 8 K permits to determine rather accurately their rates at all temperatures (Fig. 4) and to show that they are practically temperature-independent.

To interpret the blocking of electron transfer in WT RC at low temperature, we proposed earlier [3,4] that freezing of networks of water molecules may hinder structural reorganizations associated with cytochrome oxidation. Several features plead in favor of this kind of interpretation. (i) A simple quantitative examination shows that electronic factors, i.e. a change in distance or in the exponential coefficient of decay with distance β, cannot explain the very large blocking, by 10^4 or more. The interpretation should then rely on relative values of driving force $-\Delta G°$ and reorganization energy λ. (ii) It is known that cytochrome *c* oxidation is accompanied by structural changes and displacement of water molecules [14–17]. These displacements obviously contribute to the reorganization energy λ. (iii) Water is considered as a protein plasticizer and its freezing might inhibit protein structural changes associated to function [18,19]. This is a common interpretation of low-temperature inhibition of protein activity (e.g. [20]), in addition to freezing out of internal motion of the backbone

and of sidechains of the protein. (iv) It has been shown that dehydration of RC-cytochrome complexes leads to inhibition of electron transfer [21]. We may wonder why electron transfer becomes blocked at low temperature in the L162Y protein and not (or at least only partly) in L162T. We have shown previously that the reaction center structure is nearly the same for the WT and the L162T mutant, with very limited structural differences restricted to the mutation site [11]. We hypothesize that water molecules involved in redox-associated structural changes are differently located in the mutant compared to the WT. Water molecules are present in the region between the proximal heme (*c*-559) and the special pair P. Seven of them, shown in Fig. 1, are organized in a network of hydrogen bonds which includes the tyrosine L162 residue. This network will obviously be modified when the tyrosine is replaced by a different amino acid.

The results reported here clearly show that the rate of electron transfer from the proximal heme to P^+ is essentially temperature-independent. In the classical Marcus theory [22,23], this property can result from two reasons. First, that the free energy variation ($-\Delta G°$) and the reorganization energy λ exactly cancel, $\Delta G°+\lambda = 0$. This situation is hard to envision since the free energy variation is quite small, about -130 meV, and the reorganization energy is certainly not below 500 meV [23,24]. A second possible reason is that electron transfer is coupled to high-energy vibrational modes and that its rate is controlled by nuclear tunnelling. This situation has been advocated in several cases, on the basis of measurements of rates versus $\Delta G°$ and temperature [23,25]. This is also what most probably happens in the proteins we studied. Partial aspects of similar behavior have been reported for high-potential cytochromes in WT *Rps. viridis* RC [3,4], for the complex between cytochrome c_2 and the RC of *Rhodobacter sphaeroides* [26] and for the complex between plastocyanin and the PS1 reaction center [27]. It is thus tempting to speculate that the behavior reported here for a mutant RC-cytochrome complex, i.e. temperature-independent rate and two states leading to yes or no in terms of electron transfer, with a temperature-dependent population of the two states, is rather general for interprotein electron transfer.

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