Electron Transfer from the Tetraheme Cytochrome to the Special Pair in the *Rhodopseudomonas viridis* Reaction Center: Effect of Mutations of Tyrosine L162†

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ABSTRACT: The structure of the photosynthetic reaction center (RC) from *Rhodopseudomonas viridis* is known to high resolution. It contains a firmly bound tetraheme cytochrome from which electrons are donated to a special pair (P) of bacteriochlorophylls, which is photooxidized upon absorption of light. Tyrosine at position 162 of the L-subunit of the reaction center (L 162 Y) is a highly conserved residue positioned halfway between P and the proximal heme group (*c*-559) of the cytochrome. By specific mutagenesis this residue was exchanged against the amino acids phenylalanine (F), glycine (G), methionine (M), leucine (L), tryptophan (W), threonine (T), and histidine (H). All mutants were expressed in *Rps. viridis* using a recently established transformation system [Laussermair & Oesterhelt (1992) *EMBO J. 11*, 777−783]. They were shown biochemically to synthesize all four subunits of the RC (cytochrome, subunits L, M, and H) and to assemble them correctly into the membrane. The structures of two mutants (L 162 F and L 162 T) were determined and found not to differ significantly from the wild-type structure. All mutants grew photosynthetically. The absorption spectrum of all the mutants is the same as in WT, but the redox potential of P and of *c*-559 was changed by the mutations. The kinetics of electron transfer from the heme group to the special pair were measured in chromatophores by flash absorption. As found earlier in the wild type (Y) several exponential components were needed to fit the data. For the dominant fastest phase, the half-time varies from 147 to 1000 ns, in the order M, F, Y, W, H, L, G, T. We conclude that the tyrosine residue at position L 162 is not required for fast electron transfer from *c*-559 to P^+.

Bacterial photosynthesis is functionally determined by the interplay of the reaction center (RC)[1] and the cytochrome bc_1 complex cooperating in light-driven cyclic electron flow. Electron exchange on the acceptor side is mediated by diffusible ubiquinones and on the donor side by a soluble cytochrome of the *c*-type. Of the three species mainly studied in bacterial photosynthesis, *Rhodobacter* (*Rb.*) *sphaeroides*, *Rhodopseudomonas* (*Rps.*) *viridis*, and *Rhodobacter* (*Rb.*) *capsulatus*, the structures of the former two have been elucidated to atomic detail (Deisenhofer *et al.*, 1985; Allen *et al.*, 1987; Ermler *et al.*, 1994). Similarities but also differences have been found between these structures with respect to the L, M, and H subunits, and a fundamental difference is the lack of a firmly bound cytochrome in *Rb. sphaeroides*. In *Rps. viridis* such a cytochrome carries four heme groups (Deisenhofer *et al.*, 1985), and the proximal one (*c*-559) donates the electron to the photooxidized special pair (P^+) during cyclic electron flow (Dracheva *et al.*, 1988).

The reaction centers of all known photosynthetic bacteria contain a highly conserved tyrosine which is located between the special pair and the heme group of the reducing cytochrome *c* (Figure 1A) (Youvan *et al.*, 1984; Williams *et al.*, 1986; Michel *et al.*, 1986; Shiozawa *et al.*, 1989). In the reaction centers of *Rb. sphaeroides* and *Rps. viridis* this tyrosine occupies position 162 of the L-subunit (L 162 Y). It is the only conspicuous amino acid residue on the potential electron path between the heme group and the special pair and therefore was investigated in detail for a functional role in the reaction center of *Rb. sphaeroides* (Farchaus *et al.*, 1993; Wachtveitl *et al.*, 1993). This analysis revealed that the tyrosine residue clearly plays a critical role in the interaction between the electron-donating soluble cytochrome and the reaction center. Upon mutation of this residue the incorrect interaction (docking) of the macromolecules prevents efficient electron transfer and slows eventually this process to the millisecond range, finally retarding photosynthetic growth. Due to the intrinsic kinetics of the docking and the electron-transfer process, no statement could be made about the role of this tyrosine residue in the electron-transfer process itself (Wachtveitl *et al.*, 1993). This is of interest because one of the highly topical questions in biological electron transfer concerns the question whether only the distance of electron donor−acceptor groups (Moser *et al.*, 1992) or, in addition, backbone or amino acid side chains (Beratan *et al.*, 1991; Wuttke *et al.*, 1992) contribute to the

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[1] Abbreviations: CD, circular dichroism; *c*-559, the reducing heme of tetraheme cytochrome; LDAO, *N,N*-dimethyldodecylamine *N*-oxide; OD, optical density; P, primary donor (bacteriochlorophyll dimer); *Rb.*, *Rhodobacter*; RC, reaction center; *Rps.*, *Rhodopseudomonas*; WT, wild type. For amino acids the one-letter code is used.

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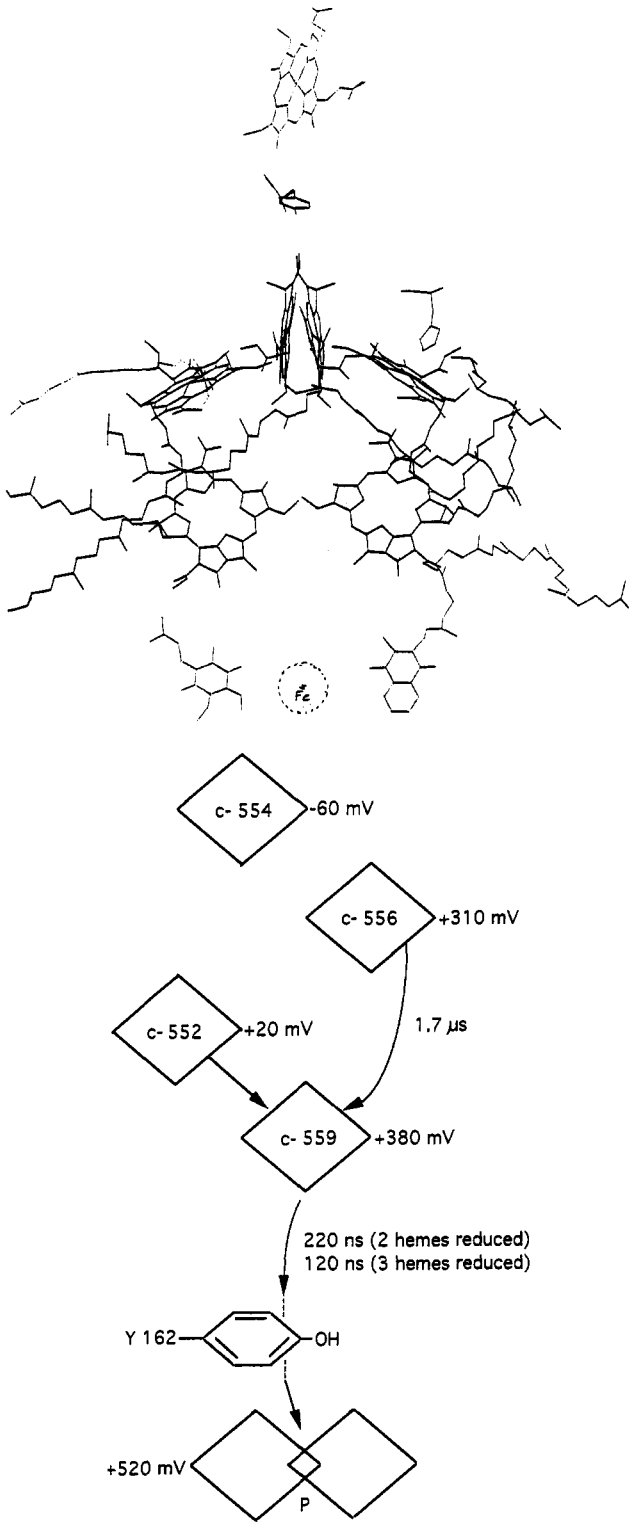

FIGURE 1: (A, top) Arrangement of the cofactors in the RC of *Rps. viridis*. From the tetraheme cytochrome only the heme *c*-559, closest to the special pair P, is shown. The structure shows the location of tyrosine L 162, halfway between the heme *c*-559 and P. (B, bottom) Scheme of the midpoint potentials and the kinetics of electron transfer concerning the tetraheme cytochrome and the special pair. The kinetic data are taken from Ortega and Mathis (1993). Midpoint potentials are taken from Nitschke and Dracheva (1994).

rate of electron transfer (not considering nuclear factors). To address this question, the reaction center of *Rps. viridis* is an ideal system. It contains a firmly bound cytochrome, and therefore the structural coordinates of the heme groups, the tyrosine residue, and the special pair P are known to a resolution of 2.3 Å (Deisenhofer *et al.*, 1985). A specific role has been attributed to L 162 Y on the basis of theoretical arguments (Knapp *et al.*, 1987; Cartling, 1991). In recent years we developed a system for microaerophilic growth (Lang & Oesterhelt, 1989) and a transformation system as a basis for producing site-specific mutants of the *Rps. viridis* reaction center and their homologous expression (Laussermair & Oesterhelt, 1992). This allowed the present investigation of effects of site-specific mutations of L 162 Y on the kinetics of rereduction of the special pair. Because of their delocalized electronic structure, aromatic residues in general could play a role in electron-transfer reactions as intermediates, especially tyrosine which could form a mesomerically stabilized radical structure. Indeed, a tyrosine radical forms as an intermediate in electron-transfer reactions in photosystem II of higher plants. Therefore, we exchanged L 162 Y against phenylalanine, tryptophan, or histidine as alternative aromatic residues and also glycine as a control residue lacking any side chain. Further residues introduced were methionine for its sulfur atom with d orbitals, leucine as a neutral hydrophobic residue, and threonine as a typical hydrophilic residue. The kinetics of the rereduction of the special pair in the RC were determined by absorption measurements at 1283 nm. Chromatophores were used to guarantee the native environment of the complex. The results obtained and reported here do not indicate any specific influence of the tyrosine residue on the electron-transfer rate beyond the variation allowed by the Marcus theory of electron transfer.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. Rps. viridis cells (DSM 133) were grown photoheterotrophically in N-medium (Lang & Oesterhelt, 1989). The mutants were cultivated on plates with 20 μg/mL kanamycin and 10 μg/mL tetracycline and grown in liquid culture with only 20 μg/mL kanamycin in N-medium. The mutant strains contain plasmid pRK 404 (Ditta *et al.*, 1985) with the RC genes and the appropriate mutation. The mutation introduced was confirmed for all mutants by sequence analysis of the DNA isolated from the cell batch used for isolation of the RC.

Construction of Strains. The construction of a *puf* operon deletion strain and the complementation to site-specific mutants have been described previously (Laussermair & Oesterhelt, 1992). Site-specific mutagenesis was performed following the instruction manual of the Muta-Gene *in vitro* mutagenesis kit (Bio-Rad).

The mismatch oligonucleotides were synthesized by the phosphoamidite method on an Applied Biosystems DNA synthesizer (model 381A). The wild-type tyrosine L 162 codon TAT is replaced in the mutants by the following base triplets: L 162 F, TTT; L 162 G, GGT; L 162 M, ATG; L 162 L, CTT; L 162 W, TGG; L 162 T, ACT; L 162 H, CAC.

Materials. All antibiotics were purchased from Sigma (Munich, Germany). The Gas-Pak-BBL Microbiology System was obtained from Becton Dickinson & Co. (Paramus, NJ), the ionic exchange material DE52 was from Whatman Biosystems, and the detergent *N,N*-dimethyldodecylamine *N*-oxide (LDAO) (30% solution) was from Fluka-Chemie (Neu-Ulm, Germany). Enzymes were from Boehringer Mannheim (Mannheim, Germany); all other chemicals were of analytical grade and from Merck.

Protein Isolation. Rps. viridis was grown phototrophically in 25 L Nalgene bottles completely filled with N-medium containing 20 μg/mL kanamycin. The RC was isolated using a modified method as described for *Rb. sphaeroides* (Farchaus *et al.*, 1993). The cells were harvested after 1 week of growth by centrifugation in a Stock centrifuge for 30 min at 4000*g* and washed in 20 mM Tris-HCl, pH 8.0. The yield of cells was typically 30−40 g wet weight/25 L. The cell pellet could be frozen in liquid nitrogen and stored at −20 °C. A sample was taken out of each culture, and from these the DNA was isolated and sequenced. The processing of the cells was only continued in those cases when the sequence of the introduced mutation proved to be obviously right. For protein isolation the pellet was resuspended in 3 mL/g cells of 20 mM Tris-HCl, pH 8.0, and stirred for 30 min at room temperature in the presence of a spatula tip of DNase I. The cells were broken by treatment in a French pressure cell (Aminco, Lorch, Germany). Chromatophores were isolated as described (Farchaus *et al.*, 1993) and suspended in 20 mM Tris-HCl, pH 8. To isolate reaction centers, chromatophore suspensions with an OD of 50 at 1020 nm were solubilized by addition of LDAO to a final concentration of 5% (w/v). Light-harvesting complexes (LHC) are destroyed at this LDAO concentration. The solution was centrifuged for 60 min at 200000*g* and the supernatant dialyzed against 20 mM Tris-HCl, pH 8, and 0.08% LDAO ("TL" buffer). Reaction centers were then purified on a DEAE ion-exchange column. When the column was rinsed with 20 mM Tris-HCl buffer, pH 8, containing 1.2% LDAO, free pigments from the LHC and contaminating membrane proteins were eluted. Before the reaction center fraction was eluted with 150 mM NaCl in 20 mM Tris-HCl, pH 8, the column was washed with 3 column volumes of TL buffer. Fractions of the eluate were analyzed spectroscopically, and only fractions not exhibiting a 680 nm peak (indicating contamination by free pigments) were combined and dialyzed against TL buffer. If necessary, reaction center solutions were concentrated by centrifugation in Centricon-30 tubes, frozen in liquid nitrogen, and stored at −20 °C. Prior to any experiment the material was assayed by SDS gel electrophoresis to check whether the RC isolated contained all four subunits.

Isolation of the DNA. A sample of the cells was resuspended in 100 μL of proteinase K (10 mg/mL) solution, incubated for 30 min at 37 °C, and afterward blended with 30% sodium sarcosinate. The proteins were separated by adding 400 μL of phenol. The phenol was removed from the aqueous phase containing the DNA by extraction with $CHCl_3$; then the DNA was precipitated with ethanol and redissolved in H_2O.

Sequencing. With the aid of two primers a fragment of 800 bases in length was amplified from the *pufL* gene of the chromosomal DNA. This product was sequenced by means of the Taq-Dye-Deoxy-Terminator-Cycle-Sequencing Kit (Applied Biosystems, Germany) exactly according to the instructions of the manufacturing firm.

CD Spectra. CD spectra were recorded with a photomultiplier R316 from Hamamatsu in the range from 300 to 1100 nm. Samples of the isolated RC had an OD_{830} of 0.5, as prepared by dilution with 20 mM Tris-HCl, pH 8, and 0.08% LDAO. Spectra were recorded twice and averaged to enhance the signal-to-noise ratio. Measurements were done with the water-cooled instrument CD6 of Jobin Yvon (Longiumeau, France). A piezo crystal served as a modulator of the light coming from a deuterium lamp. Samples in a 1 cm quartz cuvette were kept in the measuring compartment under nitrogen.

Table 1: X-ray Data Collection and Refinement Statistics

	mutant Y → F	mutant Y → T
X-ray data		
resolution range (Å)	100−2.5	40−3.2
total observations	163375	184469
unique reflections	71719	38481
completeness (%)	71.8	78.4
R_{merge}[a] (%)	7.6	13.6
refinement statistics		
resolution (Å)	10−2.5	6−3.2
reflections ($F > 2F(\sigma)$)	69682	30649
no. of atoms	12640	12001
R-factor[b] (%)	19.1	18.7
rms deviations from ideality		
bond lengths (Å)	0.012	0.012
bond angles (deg)	1.8	1.9
side-chain torsion angles (deg)	23.0	23.8
improper torsion angles (deg)	2.6	3.0
peptide ω angles (deg)	6.7	7.9
av *B*-factors (Å^2)		
all atoms	23.8	24.3
protein atoms	23.8	24.7
cofactors	20.5	18.1
water molecules	26.9	

[a] $R_{merge} = \sum_h\sum_i|I_i(h) - \langle I(h)\rangle|/\sum_h\sum_i I_i(h)$, where $I_i(h)$ is the *i*th measurement and $\langle I(h)\rangle$ is the mean of all measurements of $I(h)$. [b] *R*-factor $= \sum_h||F(h)_{obs}| - |F(h)_{calc}||/\sum_h|F(h)_{obs}|$.

X-ray Data Collection. Crystals of mutant reaction centers (Y → F and Y → T) were prepared according to Michel (1982). The crystals were isomorphous to the native crystals. The X-ray data collection for the mutant Y → F was carried out at the BL18B station in the Photon Factory (λ = 1.00 Å), Tsukuba, Japan. The intensity data were collected using a Weissenberg camera for macromolecular crystallography and an imaging plate as a detector (Sakabe, 1983). The imaging plate exposures were digitized with a BA100 Bioimage analyzer (Fuji Photo Film Co., Ltd., Japan), and the intensities were integrated and scaled with the program WEIS (Higashi, 1989). The X-ray data for the mutant Y → T were obtained in-house with a MAR-Research imaging plate detector using Ni-filtered Cu Kα radiation generated by a Rigaku RU-200 X-ray generator (40 kV, 100 mA). The images were processed with the program DENZO (Otwinowski, 1991). Table 1 contains a summary of data collection statistics.

Structure Determination. The crystal structures of mutant reaction centers were determined using the wild-type structure (Lancaster *et al.*, 1995), which is based on the structure refined by Deisenhofer and Michel (1989) (1PRC in the Brookhaven Protein Data Bank). The initial *R*-factors for Y → F and Y → T mutants were 21.7% (10.0−2.5 Å) and 28.5% (6.0−3.2 Å), respectively. Positional and temperature factor refinements were performed with the program X-PLOR (Brünger *et al.*, 1987) using geometric parameters for the protein subunits of Engh and Huber (1991) and for the cofactors of Treutlein *et al.* (1992) and of C. R. D. Lancaster (personal communication). After three cycles of refinement and manual manipulation of the models with the program O (Jones *et al.*, 1991), the refinement converged at crystallographic *R*-factors of 19.1% (10.0−2.5 Å) for the Y → F mutant and 18.7% (6.0−3.2 Å) for the Y → T mutant. The

final model of each mutant contains 11 282 protein atoms, four bacteriochlorophylls *b*, two bacteriopheophytins *b*, one menaquinone-9, one ubiquinone-9, one non-heme iron, one carotenoid, and three LDAO molecules. Five sulfate and 201 water molecules are also included in the model of the Y → F mutant as solvent. Table 1 contains a summary of refinement statistics.

Redox Titrations. Titrations were carried out with the same setup as described (Wachtveitl *et al.*, 1993). Samples of isolated reaction centers with an OD_{830} of 2−3 were placed in a cuvette with an optical path length of 0.2 mm, equipped with a gold mesh redox measuring electrode, a platinum wire as counter electrode, and a Ag/AgCl electrode as a reference. They were illuminated with white light from a halogen lamp passed through a 10% neutral density filter and an RG 610 filter before entering the samples. The light beam was dispersed after passing the sample and measured by diodes in the range from 680 to 990 nm at 10 nm intervals. Due to the intensity of the measuring beam, the photooxidized special pair accumulated in time, thus allowing the difference spectra to be recorded within the 1 s measuring period. Samples were kept at potentials between 270 and 600 mV with the help of a potentiostat. Individual spectra were taken every 20 or 30 mV.

Rereduction Kinetics of the Special Pair. Freshly isolated chromatophores were suspended in 20 mM Tris-HCl, pH 8, with 0.08% LDAO. Alternatively, the chromatophores stored at −20 °C were thawed and sonicated shortly before the measurements to prevent aggregation. Primary charge separation was induced by a 10 ns laser flash (broad band around 595 nm), and absorption changes were recorded at 1283 nm and analyzed as described (Ortega & Mathis, 1993), except that the photodiode had a diameter of 1 mm. The measurements were performed with chromatophores with an OD_{1020} of about 1.5 in a 10 × 10 × 30 mm cuvette, with addition of 100 μM sodium ascorbate and 20 μM terbutryn. The sample was kept at 21 ± 1 °C, the temperature used in all other measurements reported here.

The pH dependence of the kinetics was measured in a similar way except that the chromatophores were pelleted by ultracentrifugation and the pellet was resuspended in 20 mM MES, pH 6, or 20 mM Tris-HCl, pH 9. Ionic strength was varied by addition of sodium chloride to a final concentration of 35 or 85 mM.

RESULTS

Protein Isolation. Reaction centers could be isolated from all mutants by the procedure established for wild-type reaction centers. Reaction centers were purified on an ion-exchange column (DE 52) and gave final products which did not show any impurities in their spectra, in particular the absence of any 680 nm absorption indicating low concentration of free pigments (Figure 2). Analysis by SDS gel electrophoresis, however, showed that small amounts of contaminating proteins are present which, however, have no absorption peaks in the visible range and therefore do not interfere with the measurements described here (data not shown).

CD Spectra. The CD spectra show no differences between the wild type and any of the mutants in the arrangement of the pigments in the protein.

Structure of the Mutants L 162 Y → F and L 162 Y → T. The crystal structures of the Y → F and Y → T mutants are

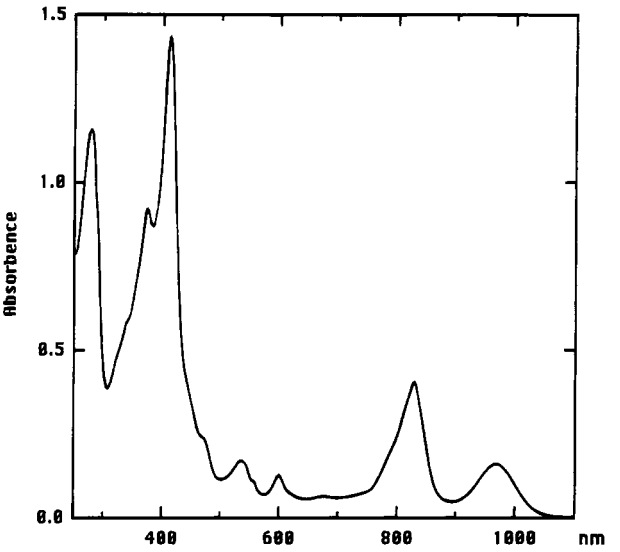

FIGURE 2: Absorption spectra of isolated RC in TL buffer. Spectra of wild type and all mutants are indistinguishable.

shown in Figures 3 and 4 in comparison with the wild-type structure (Deisenhofer & Michel, 1989) (1PRC in the Brookhaven Protein Data Bank). Apart from the mutation site, the overall structures of the Y → F and Y → T mutants are nearly unchanged compared to the wild-type structure. The rms deviations of Cα positions of Y → F and Y → T mutants from the wild-type structure are 0.249 Å (for 1178 residues which deviate less than 1.5 Å) and 0.339 Å (for 1177 residues which deviate less than 1.5 Å), respectively. These values are comparable to the coordinate errors of 0.27 and 0.33 Å for the Y → F and Y → T mutant structures which are estimated from Luzzati plots (Luzzati, 1952).

Focusing on the mutation site, a few differences between the two mutant structures are visible. In the case of the Y → F mutant, the replacement of the amino acid residues does not cause any further conformational changes. In contrast, the exchange of tyrosine to threonine in the second mutant is followed by a slight shift of *c*-559 up to 0.3 Å toward the special pair. The missing van der Waals contact between tyrosine L 162 and *c*-559 might be responsible for this movement. Due to the limited resolution of the Y → T mutant, a final discussion of the structural changes is not yet possible.

No significant differences in the position of water molecules is observed between wild type and the mutants. In neither wild type nor mutants are water molecules directly placed between the proximal heme and the special pair (in Figure 4a the projection misguides the eye).

Redox Titration. As shown in Table 2 the exchange of tyrosine in position L 162 for other amino acids changes the midpoint potential of the special pair. The largest change was found for threonine with +58 mV compared to the wild type. Significant changes caused by the mutations were also found for the redox potential of heme *c*-559 of the cytochrome. Mutants containing phenylalanine and tryptophan lead to a lowered midpoint potential of 325 and 340 mV compared to 380 mV in wild type. Thus, the midpoint potential of heme *c*-559 in these mutants comes close to that of heme *c*-556, which has a midpoint potential of 310 mV (Nitschke & Dracheva, 1994) (Figure 1B). The exchange of tyrosine for methionine, on the other hand, leads to an increase in the midpoint potential of *c*-559 by 30 mV. It

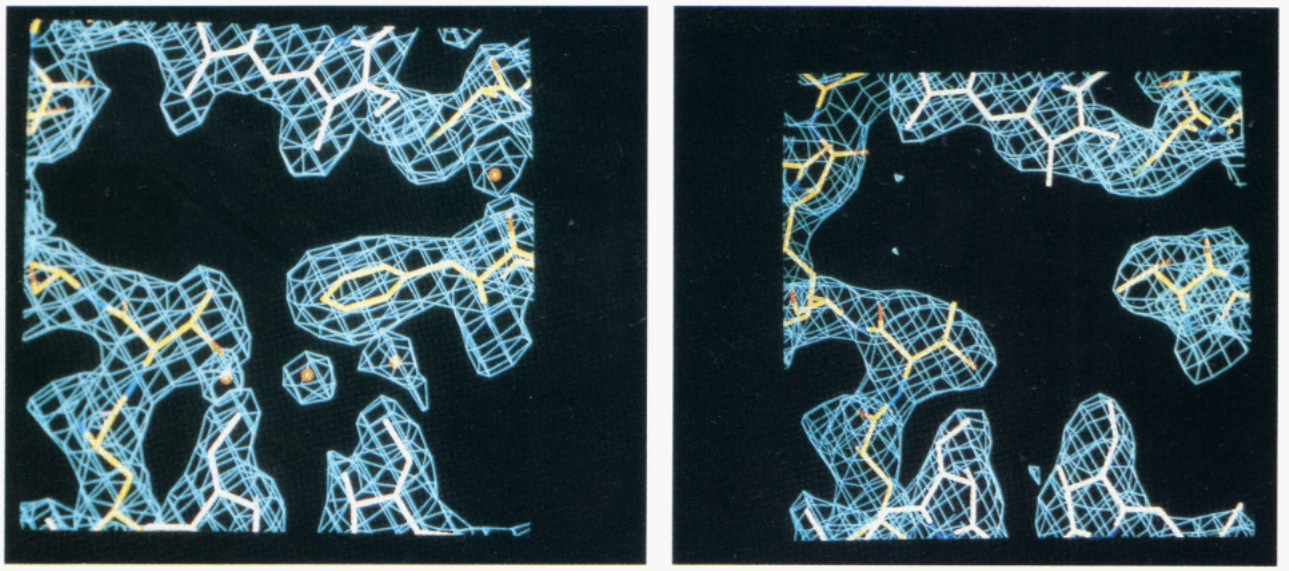

FIGURE 3: Two $|F_o| - |F_c|$ electron density maps showing density for the L 162 residues and surroundings in the Y → F (a, left) and Y → T (b, right) mutants. The maps were contoured at the 1.5σ level. In the models, carbon atoms in the *c*-559s (top) of the cytochrome subunit and the special pairs (bottom) are shown in white.

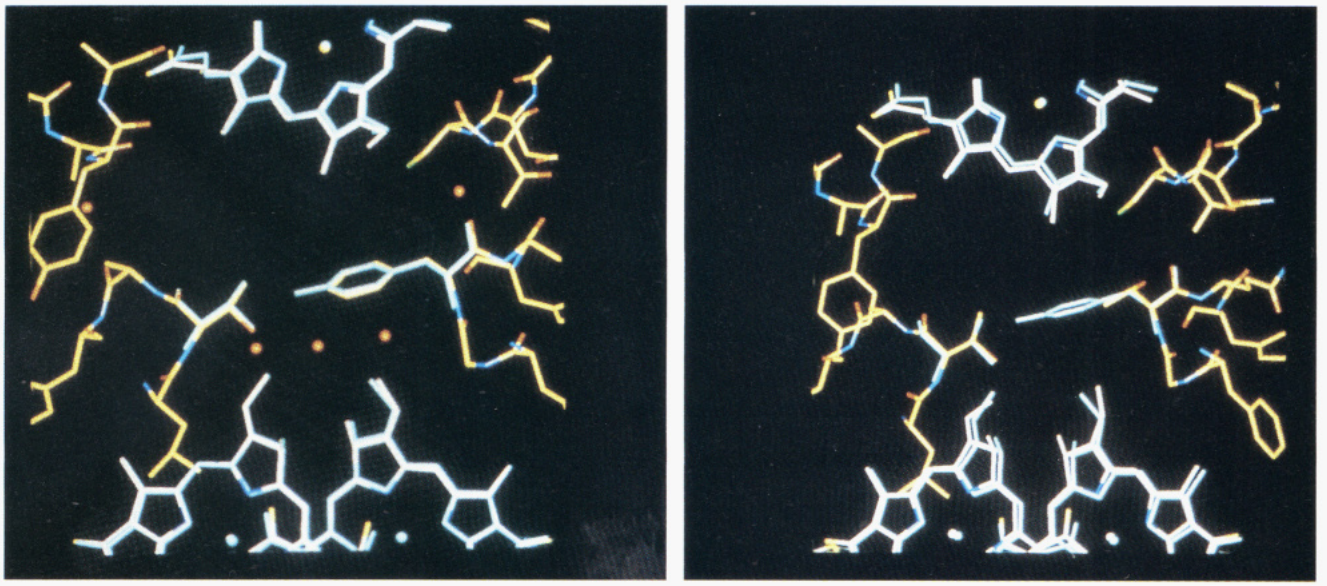

FIGURE 4: Images of the L162 residues and surroundings superimposed from the wild type to the Y → F mutant (a, left) and to the Y → T mutant (b, right). The residues (L 162 Y and M 185 T) and cofactors (*c*-559 and special pair) of the wild-type structure are presented in cyan.

should be pointed out, however, that the redox titration performed here does not allow one to distinguish between heme *c*-559 and heme *c*-556. The assumption is that heme *c*-559, which is closer to the mutated amino acid and has a higher redox potential than *c*-556, is affected more. In all but one of the mutants of the amplitude of the bleaching signal at 960 nm was comparable with that of the WT, but the maximal amplitude was detected at different potentials. Only the methionine mutant showed two-thirds of the bleaching amplitude compared to WT.

Kinetics of P^+ Reduction. In wild-type chromatophores the absorption change at 1283 nm induced by a 595 nm flash decays in a multiphasic manner (Figure 5). Four phases are clearly found in a multiexponential analysis (Table 3): a dominant very fast phase (VF) with $t_{1/2} = 185$ ns and three minor phases with $t_{1/2} \simeq 1.9$ μs (fast, F), $t_{1/2} \simeq 12.7$ μs (intermediate, I), and $t_{1/2}$ above 150 μs (slow, S; the half-time is not well-defined because the measuring system is AC coupled at ≃300 Hz). These data are very similar to those found earlier in reaction centers under analogous redox conditions where the two high-potential hemes are reduced

Table 2: Redox Potentials of *c*-559 and *P*[a]

sample	E_m(*c*-559)	E_m(P)	ΔE
L 162 Y (WT)	380	520	140
L 162 F	325	515	190
L 162 G	395	530	135
L 162 M	410	542	132
L 162 L	370	525	155
L 162 W	340	510	170
L 162 T	380	578	198
L 162 H	nd	570	nd

[a] Midpoint potentials of the special pair and of *c*-559 in WT L 162 Y and mutants at position L 162, measured on isolated RC in solutions. The redox state of P/P^+ and *c*-559 [Fe(II)/Fe(III)] was determined by the maximal bleaching of the 960 nm special pair peak after illumination with white light from a halogen lamp. The actinic light passed a 10% neutral density glass filter and a RG 610 filter before entering the samples. All values are in millivolts.

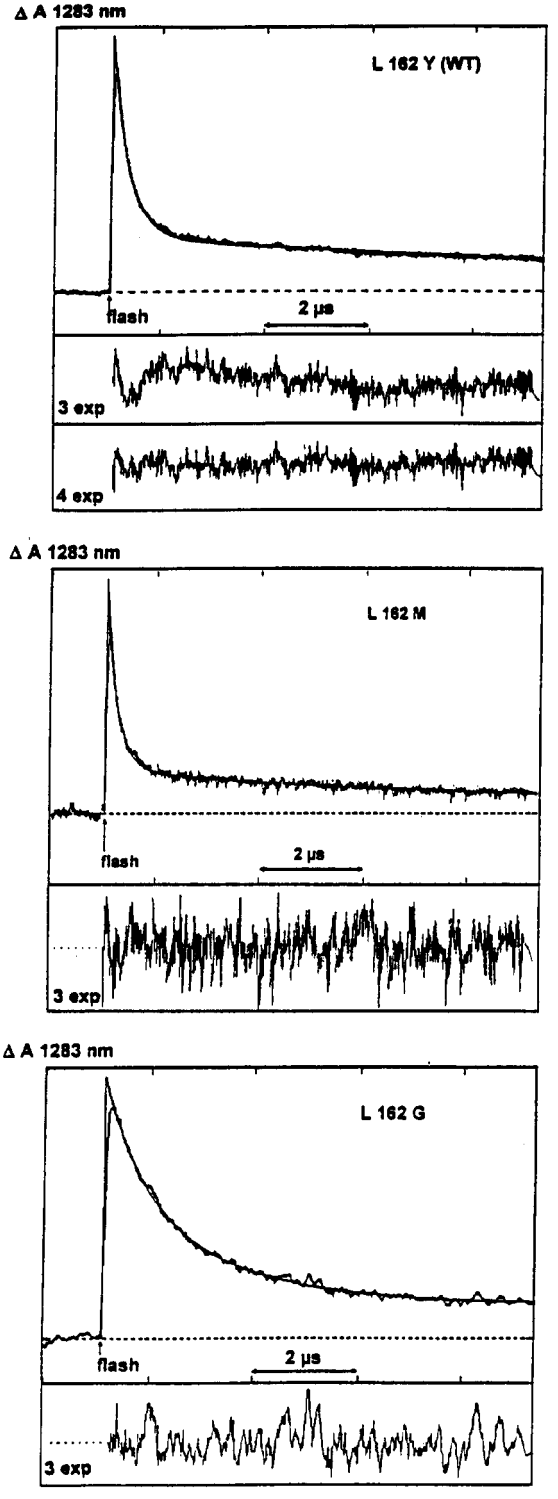

FIGURE 5: Kinetics of flash-induced ΔA at 1283 nm elicited by a saturating laser flash in suspensions of chromatophores (buffer at pH 8.0; addition of 100 μM ascorbate and 20 μM terbutryn). Averaged effect of 16 flashes (WT, top trace), 8 flashes (L 162 M, middle trace), or 2 flashes (L 162 G, bottom trace) with a time interval of 30 s between flashes. The concentration was such that the OD at 1020 nm was 1.5 (WT), 1.6 (L 162 M), and 1.5 (L 162 G). ΔA_{max}: WT, 1.8×10^{-2}; M, 0.6×10^{-2}; G, 1.7×10^{-2}. Below the panels of the kinetics the quality of the fit is shown as residuals (difference between the measured data and the fitted curve). The residuals are multiplied by a factor of 5. The absence of a systematical deviation indicates the quality of the fit. In WT kinetic comparison of a fit with four exponentials to one with three shows that a fourth time constant is necessary to describe the kinetics correctly. In contrast to that in mutants three time constants are sufficient to describe the measured data.

Table 3: Kinetics of Reduction of the Oxidized Special Pair[a]

	very fast (VF)		fast (F)		intermediate (I)		slow (S)
sample	t (ns)	part (%)	t (μs)	part (%)	t (μs)	part (%)	part (%)
L 162 Y (WT)	185	76	1.9	8	12.7	13	3
L 162 F	174	86	4.5	13			1
L 162 G	499	41	1.4	46			13
L 162 M	147	81	4.9	14			5
(4 exp)	(140)	(80.4)	(2.2)	(9.2)	(11)	(5.9)	(4.5)
L 162 L	341	78	2.3	15			7
L 162 W	233	87	noisy				13
L 162 T	1000	56	2.7	38			6
L 162 H	268	70	1.5	30			

[a] Results of the analysis of the kinetics of absorption recovery at 1283 nm, following a single flash, with *Rps. viridis* chromatophores (WT: L 162 Y and various mutants at position L 162). Conditions as in Figure 5. For the WT a fourth component was present; it is tentatively attributed to ^{3}P. For the M mutant the data in parentheses give the values that are derived when the curve is fitted with four time constants. Slow phases have a $t_{1/2}$ above 150 μs.

(Ortega & Mathis, 1993). The very fast phase (VF) corresponds to the kinetics of direct electron transfer from the *c*-559 to the oxidized special pair (P^+). The intermediate phase has been attributed to the decay of triplet state ^{3}P, formed in reaction centers where Q_A is reduced before the flash (Ortega & Mathis, 1993). This interpretation is reinforced by a control experiment where diaminodurene (an efficient electron donor to the cytochrome) was added and the sample submitted to repetitive flashes; under these conditions the I phase is largely dominant (data not shown).

The data obtained with the mutants were analyzed by a fit function with three instead of four time constants. This procedure is justified, since the quality of the fit did not improve by introducing a fourth component (residuals in Figure 5 middle and bottom traces with three exponentials). The data are given in Table 3 and the kinetic components assigned to the VF, F, and S phases. (Figures in Table 3 are the direct result of the data analysis in exponential components; their accuracy is estimated to $\pm 10\%$ for both half-times and relative signal sizes.) The omission of the I phase in the mutants has experimental reasons: first, the signal-to-noise ratio in some of the experiments with mutants is lower than in wild type, which then makes it difficult to distinguish between the F and the I phase (in the case of W, L, and H mutants). Second, the time constants of the two phases may have changed; for example, a slower F phase would lead to a time constant comparable to that of the I phase and result in only one detectable component (in the case of F and M mutants). With three time constants the VF and F phases of the F and M mutants have a kinetic behavior very similar to that of the wild type, the latter one showing a slightly shorter half-time for the very fast phase. The L, W, and H mutants also give fairly similar kinetics, but the fast phase is slightly slower than in the wild type. The data for the W mutant are less precise because the sample was highly scattering (chromatophores were difficult to resuspend); consequently, the signal-to-noise ratio was rather poor, and the F phase could not be resolved from the VF phase.

However, to show that the analysis with four components is in principle possible, it was tried for the M mutant (the data are also given in Table 3) and resulted in a fit that resembles the wild-type characteristics with respect to the F and I phases. The four time constants of all the phases are

very similar to those of the wild type. This procedure was not applied to all mutants, not only because the fit with four components did not reduce residuals significantly but also because the I phase in wild type so far is only tentatively assigned to a ^3P state.

Only two mutations give rise to largely different kinetics. For the G mutant the very fast phase (VF) is slower ($t_{1/2}$ = 499 ns) and makes up only 41% of the total, while the F phase is larger than in other cases (46%). For T the kinetics are even slower since the very fast phase (VF) (56%) has a half-time of 1.0 μs. The fast phase (F) is not much slower ($t_{1/2}$ = 2.7 μs), but these two phases were clearly needed to obtain a good fit of the experimental data. In spite of these differences, it is remarkable that the total range of variation in the kinetics is quite limited. As in all experiments on the reduction of P^+ by cytochrome, a small slow phase (S) was found with all strains (it was especially small in the H mutant). It was much larger in control experiments (not shown) done without ascorbate. No obvious explanations are yet available for the fast and slow phase (F and S) (Ortega & Mathis, 1993). The slow phase may be due partly to reaction centers where the heme *c*-559 is oxidized before the flash. Fast phases were absent after addition of ferricyanide which oxidizes the cytochrome.

A pH change to 6 or 9 or conditions with higher ionic strength lead to the same kinetics, as described in Table 3 (data not shown).

DISCUSSION

Replacement of tyrosine L 162 of the reaction center in *Rps. viridis* by amino acids F, M, W, H, L, G, or T leads to cells which are photosynthetically competent: reaction centers are correctly assembled and fully functional. The absorption spectrum and the CD spectrum of isolated reaction centers are not modified, indicating that the pigment arrangement is the same. This has been confirmed by X-ray crystallographic analysis for the mutants Y → F and Y → T (Figures 3 and 4). In all cases, the special pair P has an absorption maximum at 960 nm, which bleaches upon illumination. Significant alteration of the redox potential of heme *c*-559 is observed, to both higher and lower values compared with WT. The redox potential of P is also modified; e.g., the Y → T mutation leads to a significant increase by 58 mV. An additional surprising observation is found in that mutant. Despite a 0.3 Å shorter distance between *c*-559 and the special pair in this muant compared to wild type, a five times slower time constant of rereduction of P^+ is observed. No explanation can be given for this finding.

This series of mutants was designed in order to check for an eventual specific role of the tyrosine L 162 Y in facilitating electron transfer from the cytochrome to P^+. There is abundant (but controversial) literature on such a role of aromatic amino acids in various electron-transfer proteins. For yeast cytochrome *c* it has been claimed that an aromatic residue at position 82 greatly accelerates electron transfer to cytochrome *c* peroxidase (Liang *et al.*, 1988). The data were first explained in terms of superexchange for hole transfer (Liang *et al.*, 1988), but they were later analyzed in terms of structural heterogeneity (Everest *et al.*, 1991; Ogrodnik *et al.*, 1994). Other studies have also led to controversial interpretations (Mauro *et al.*, 1988; Davies *et al.*, 1990, 1992; Concar *et al.*, 1991; Broo & Larsson, 1991; Miles *et al.*, 1992). Recently, it was shown that an aromatic residue permits rapid electron transfer from ferredoxin to ferredoxin–NADP oxidoreductase (Hurley *et al.*, 1993). In the reaction center of photosynthetic bacteria, theoretical analysis proposed that a conserved tyrosine residue (L 162) plays a key role in electron transfer from cytochrome to P^+ (Knapp *et al.*, 1987; Cartling, 1991). For RCs which lack a bound cytochrome, one suggestion favors interaction of electrostatic potentials rather than specific residue interaction determining the binding of cytochrome c_2. The binding site could then lie above the periplasmatic surface of the M subunit (Tiede *et al.*, 1993). In previous work (Farchaus *et al.*, 1993; Wachtveitl *et al.*, 1993), kinetic analysis of site-directed mutants of the *Rb. sphaeroides* RC showed that L 162 Y indeed plays an important role in electron transfer from (soluble, monohemic) cytochrome c_2 to P^+. This reaction, however, implies a correct docking of the cytochrome to the reaction center, and a detailed analysis of the data led to the conclusion that the state with incorrectly docked cyt c_2 was favored in the mutants (Wachtveitl *et al.*, 1993). In *Rps. viridis*, the tetrahemic cytochrome is permanently bound to the reaction center and may therefore allow an investigation of the role of tyrosine at L 162.

We show in this work that P^+ is efficiently reduced by the cytochrome in all of the mutants. We worked with chromatophores in order to avoid possible disturbances of interaction of subunits by the detergent treatment needed to extract the reaction center. The experimental redox poising (100 μM ascorbate) was such that the cytochrome has the two high-potential hemes (*c*-559 and *c*-556) reduced before flash excitation (Figure 1B). Under these conditions it is known that P^+ is rapidly reduced by heme *c*-559, which is itself subsequently rereduced by heme *c*-556 with $t_{1/2}$ = 1.7 μs (Ortega & Mathis, 1993; Case *et al.*, 1970; Dracheva *et al.*, 1986; Shopes *et al.*, 1987). Monitoring the kinetics of P^+ disappearance after a flash thus directly records the kinetics of electron transfer from heme *c*-559 to P^+. The results reported here clearly demonstrate that this electron transfer remains very fast in all the mutants investigated and the variation of the rate of the very fast phase was within an order of magnitude in all cases. For example, the L 162 L mutant gives kinetics very close to that of the wild type; therefore, neither tyrosine nor aromaticity is required for fast electron transfer.

Apart from this clear-cut negative conclusion, the data deserve a more detailed discussion. The rate of electron transfer according to Marcus theory

$$k_{et} = 2\pi/\hbar|V_R|^2FC$$

depends on many factors (distance between donor and acceptor; nature of the bridging medium, via the attenuation factor β and perhaps via specific contributions such as the one we could possibly attribute to the residue L 162 Y) and Franck–Condon factors (driving energy $\Delta G°$, reorganization energy λ, temperature, nature of vibrations coupled to electron transfer). Searching for a specific effect of tyrosine, one should be sure that these parameters are not modified by the mutations. At constant temperature the only parameter on which detailed information is available is $\Delta G°$, the variation of which can be approximated as the difference in the E_m of the donor/acceptor couple (actual $\Delta G°$ includes

electrostatic contributions due to Q_A^- and to heme *c*-556, which are assumed to be constant).

In the "normal" $\Delta G°$ region, according to the Marcus theory, electron transfer should be faster when $-\Delta G°$ increases. The differences reported in Table 2 do not correlate with the observed variations of the kinetics (Table 3). The differences in $\Delta G°$, however, are rather small, and they could be counterbalanced by variations in the other factors, which are not accessible experimentally.

Another remarkable property of the kinetics in mutants is their biphasic character [disregarding the small contribution of the slow phase (S); the intermediate phase (I) is not found in the mutants]. In wild-type reaction centers, a small microsecond phase (F) has been reported previously ($t_{1/2}$ = 1.5 μs; extent of 10%) and tentatively assigned to the decay of a substate of the reaction center (Ortega & Mathis, 1993). Chromatophores behave like isolated reaction centers in this respect. In all the mutants the kinetics are changed compared with the wild type. The change is not as drastic as in *Rb. spheroides*, but each mutant has at least one change in its kinetic characteristics. For example, either the time constant or the amplitude of the F phase is changed significantly in every mutant RC. A suggestion for the physical basis of this F phase is that the binding of the cytochrome to the RC is usually well defined, but a substate exists in which there is some variability in the detailed arrangement of the two subunits. A mutation in the interfacial area may lead to more variability in the interaction, so that the fast phase (F) makes a larger contribution.

Why is tyrosine L 162 conserved in reaction centers with a bound tetraheme cytochrome if, as shown by this work, it is not necessary for fast electron transfer to P^+? The residue may play, in a more limited manner, the same role as in *Rb. sphaeroides*, i.e., support an ideal docking of the cytochrome subunit with the L and M subunits. It may fill up the free space between the subunits optimally such that the cytochrome dovetails exactly at the reaction center.

If there is another amino acid residue which has nearly the same size and volume as the tyrosine, the cytochrome subunit would fit in the same way and the reduction kinetic to P^+ would run normally. This is the case for F, M, L, and H. Tryptophan is bigger than tyrosine, but it does not seem to lead to incorrect binding of the cytochrome subunit. In the case of G and T the space between the subunits is perhaps not optimally filled, and the cytochrome subunit has more variability than in wild type. Thus the kinetics become slower, and the percentage of the F phase is more important.

A more detailed structural analysis of the mutants will allow us to address these questions more specifically.

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