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Accelerated Publications

Role of Tyrosine M210 in the Initial Charge Separation of Reaction Centers of *Rhodobacter sphaeroides*[†]

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ABSTRACT: Femtosecond spectroscopy was used in combination with site-directed mutagenesis to study the influence of tyrosine M210 (YM210) on the primary electron transfer in the reaction center of *Rhodobacter sphaeroides*. The exchange of YM210 to phenylalanine caused the time constant of primary electron transfer to increase from 3.5 ± 0.4 ps to 16 ± 6 ps while the exchange to leucine increased the time constant even more to 22 ± 8 ps. The results suggest that tyrosine M210 is important for the fast rate of the primary electron transfer.

The primary photochemical event during photosynthesis of bacteriochlorophyll- (Bchl-) containing organisms is a light-induced charge separation within a transmembrane protein complex called the reaction center (RC). The crystal structures of RC's from *Rhodospseudomonas (Rps.) viridis* and *Rhodobacter (Rb.) sphaeroides* have been solved to high resolution [reviewed in Deisenhofer and Michel (1989), Chang et al. (1986), Tiede et al. (1988), and Rees et al. (1989)]. The RC from *Rb. sphaeroides* contains three protein subunits referred to as L, M, and H, according to their respective mobilities in SDS-polyacrylamide gels. Associated with the L and M subunits are the cofactors, consisting of four Bchl *a*, two bacteriopheophytin (Bph) *a*, one atom of non-heme ferrous iron, two quinones (*Q_A* and *Q_B*), and in some species one carotenoid [reviewed in Parson (1987) and Feher et al.

(1989)]. The cofactors are arranged in two branches (Figure 1) with an approximate *C₂* axis of symmetry. The kinetic data support a model in which the primary electron transfer proceeds after light absorption by the primary donor [a special pair of Bchl referred to as P; reviewed in Kirmaier and Holten (1987)]. The absorption of light generates the excited electronic state P*, which has a lifetime of approximately 3 ps. An electron is transferred from P along only one branch (the so-called A-branch). It is generally accepted that after approximately 3 ps the electron arrives at the Bph on the A-side (*H_A*) and after 220 ps it reaches *Q_A*. The role of the accessory Bchl located between P and *H_A* (referred to as *B_A*) has not been definitely assigned. Recently, we have shown that at room temperature an additional kinetic ($\tau = 0.9$ ps) component is detectable (Holzapfel et al., 1989). The spectral properties and the kinetic constants lead to the conclusion that the corresponding intermediate is the radical pair P⁺B_A⁻ (Holzapfel et al., 1990).

Additional intriguing points concerning the process of

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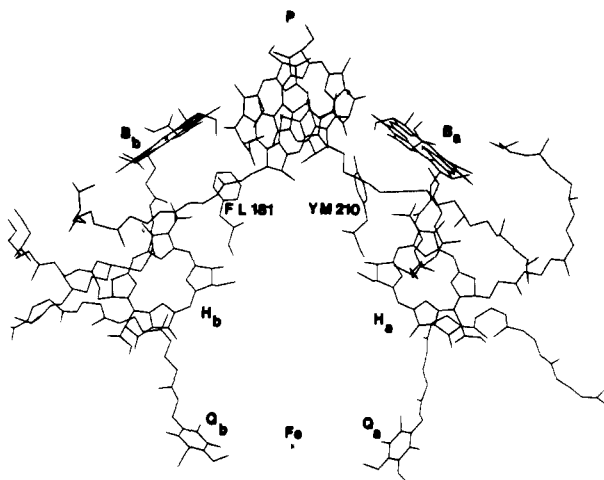


FIGURE 1: View of the chromophores from the reaction center of *Rb. sphaeroides*. Included are tyrosine M210 (YM210) and phenylalanine L181 (FL181). Abbreviations: P, special pair; B_A and B_B , monomer bacteriochlorophylls on the A- and B-branches, respectively; H_A and H_B , the bacteriopheophytins on the A- and B-branches, respectively; Q_A and Q_B , the ubiquinones on the A- and B- branches, respectively; Fe, non-heme ferrous iron. The X-ray coordinates were kindly provided by Drs. M. Schiffer and J. Norris of Argonne National Laboratories, Argonne, IL.

primary electron transfer in the RC include the basis for the unidirectionality of electron transfer and concomitantly the role of the pigments on the "wrong" (B) branch as well as the influence of the protein side groups surrounding the chromophores. It has been shown previously that site-directed mutagenesis is a powerful tool to study these questions (Bylina et al., 1988; Kirmaier et al., 1988, 1989; Bylina & Youvan, 1988; Breton et al., 1989). We investigated RC's in which one specific amino acid, tyrosine M210 (YM210), was exchanged. YM210 is in the direct vicinity of P, B_A , and H_A and therefore may influence the primary electron-transfer steps (see Figure 1). This residue is conserved in other photosynthetic bacteria, namely, *Rps. viridis*, *Rhodobacter capsulatus*, and *Rhodospirillum rubrum*. The corresponding residue on the opposite branch is a phenylalanine, L181. In the RC of the green photosynthetic bacterium *Chloroflexus (Chl.) aurantiacus*, a leucine is found at M210 (Shiozawa et al., 1989; Ovchinnikov et al., 1988a,b). In *Chl. aurantiacus* the rate of the primary electron-transfer step has been shown to be slower than in *Rb. sphaeroides* (7 ps compared with 3.5 ps in *Rb. sphaeroides*) (Becker et al., 1989). Recently, Parson et al. (1990) have used electrostatic calculations to suggest that the interactions of YM210 may lower the energy of the state $P^+B_A^-$, thereby facilitating electron transfer along the A-branch. The construction and properties of mutants in which YM210 has been exchanged to phenylalanine (Y \rightarrow FM210) and leucine (Y \rightarrow LM210) have been described in a previous report (Gray et al., 1990). Cells containing the Y \rightarrow LM210 mutation were shown to grow photoheterotrophically only under high light conditions while the FM210 mutants grew under both high and low light.

We report below the results obtained from femtosecond spectroscopy of the primary reaction dynamics of these mutants. We show that dramatic changes occur in the lifetime of the excited electronic state and in the first electron-transfer step. Both processes are slowed down by more than a factor of 4 compared to the wild-type RC.

MATERIALS AND METHODS

The construction of the mutant RC and the subsequent purification of the protein have been described in detail

elsewhere (Gray et al., 1990). In brief, two synthetic oligonucleotides (21-mers) were synthesized complementary to the region to be mutagenized except for the codon for YM210. The oligonucleotides were annealed to a gapped duplex DNA containing the genes coding for the L and M polypeptides of the RC and subsequently filled in *in vitro*. Mutants were selected initially by colony hybridization, and the changes were confirmed by sequencing with Sequenase (U.S. Biochemical Corp.). The mutagenized DNA fragments were subcloned into the mobilizable plasmid pRK404 and used to transform *Escherichia coli* S17-1. The respective *E. coli* strains were mated with a PUF Δ LMX deletion strain of *Rb. sphaeroides* as described (Farchaus & Oesterheld, 1989). The reaction center was only isolated from semiaerobically grown cells to prevent reversions and second-site mutations. The wild-type RC refers to the case in which the protein was isolated from the deletion strain complemented in trans with the wild-type genes. The isolated RC was concentrated on small DEAE-cellulose columns (DE-52, Whatmann) and solubilized in either 0.5% (w/v) octyl glucoside or 0.08% (w/v) *N,N*-dimethyldodecylamine *N*-oxide (LDAO). The mutant RC's used for these measurements were shown to contain only a small fraction of quinone at the Q_A site (approximately 10%). The spectral assay used for this determination has been described in Okamura et al. (1982).

All kinetic measurements were performed at 298 K. The RC solutions (0.2 mL) were kept in cuvettes of 1-mm path length. Transmission of the samples at 860 nm was adjusted to approximately 10%. The samples were constantly stirred to exchange the excited volume between two laser shots. The femtosecond laser system and the applied pump and probe technique, which were used to measure the light-induced transient absorption changes, have been described in detail (Holzapfel et al., 1990). In brief, an excitation pulse ($t_p \leq 100$ fs) was focused to a spot diameter of 0.5 mm. The pulse wavelength was 860 nm (bandwidth 15 nm) to excite P in its lowest energy transition. The excitation intensity was adjusted so that less than 12% of the RC in the irradiated volume was excited. The subsequent population of different intermediates (e.g., P^* , $P^+B_A^-$, $P^+H_A^-$, $P^+Q_A^-$) leads to characteristic time-dependent changes of the absorption spectrum.

These absorption changes were monitored by properly delayed probing pulses ($t_p \leq 150$ fs) of very low intensity and accurately measured by a sensitive difference detection system. The data points shown in Figure 2 are averaged over at least 500 individual absorption measurements. At a probing wavelength of 920 nm a parallel polarization of exciting and probing pulses was used; at $\lambda_{pr} = 665$ nm both parallel and perpendicular polarizations were used.

The transient absorbance data are plotted as a function of delay time t_D on a linear scale for $t_D < 1$ ps and on a logarithmic scale for $t_D > 1$ ps.

RESULTS

As described under Materials and Methods, two probing wavelengths were used to detect the formation of the various intermediates. The excited electronic level P^* of the primary donor is detected by the stimulated emission (gain) seen at 920 nm. The formation of $P^+B_A^-$ and $P^+H_A^-$, respectively, can be observed at 665 nm by using parallel or perpendicular polarization of the probing and excitation beams. $P^+Q_A^-$ is detected by monitoring the decay of $P^+H_A^-$ at 665 nm.

At a probing wavelength of 920 nm in the wild-type sample (Figure 2a) a nearly instantaneous absorption decrease was obtained. This signal is predominantly due to stimulated emission from the electronically excited state P^* . A small

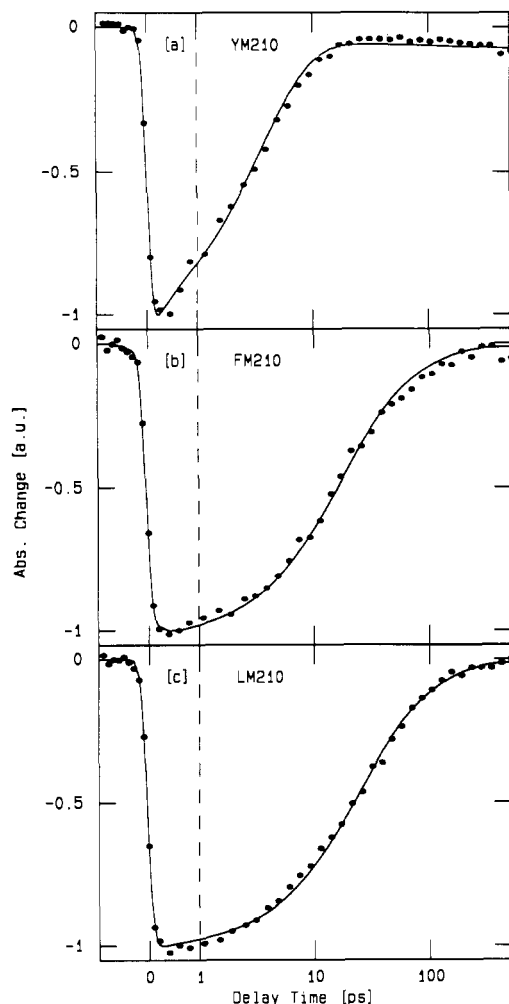


FIGURE 2: Transient absorption data (full circles) measured at a probing wavelength of 920 nm for wild-type RC (YM210) (a) and the mutant RC's FM210 (b) and LM210 (c). The peak absorbance change is $\Delta A = -0.019$. The solid curves are calculated by using a rate equation system with time constants as given in the text.

fraction of this signal results from the bleaching of the ground-state absorption. The decay of the stimulated emission in the wild-type molecule reflects the depopulation of P^* with a time constant of 3.5 ± 0.4 ps. The weak remaining bleaching at $t_D = 500$ ps is related to the absorbance change in the state $P^+Q_A^-$. Parts b and c of Figure 2 show the gain measurements for the mutants $Y \rightarrow FM210$ and $Y \rightarrow LM210$, respectively. It is not possible to fit the experimental data with a single time constant. The best fits are obtained with the time constants $t_{11} = 16 \pm 6$ ps and $t_{12} = 70 \pm 30$ ps (FM210) and $t_{11} = 22 \pm 8$ ps and $t_{12} = 90 \pm 40$ ps (LM210). For both mutant RC's the amplitude of the faster component is approximately three times larger than that of the slower one.

In the spectral region around 665 nm the unexcited RC's absorb only weakly but the reduced Bchl and Bph have pronounced absorption. At this wavelength the formation of B_A^- and H_A^- can be monitored. Since the angle between the Q_Y transition moment of P and that of B_A is approximately 30° and to that of H_A approximately 70° , the sample exhibits a strong transient dichroism. Measurements on wild-type RC's have shown that when perpendicular polarization of the excitation and probing pulses is used, the absorption change at 665 nm does not exhibit the 0.9-ps kinetic component attributed to the transition from $P^+B_A^-$ to $P^+H_A^-$ (Holzapfel et al., 1990). The signal, therefore, reflects the reduction of either tetrapyrrole B or H by P^* . Figure 3 shows the transient absorption measurements with perpendicular polarization at

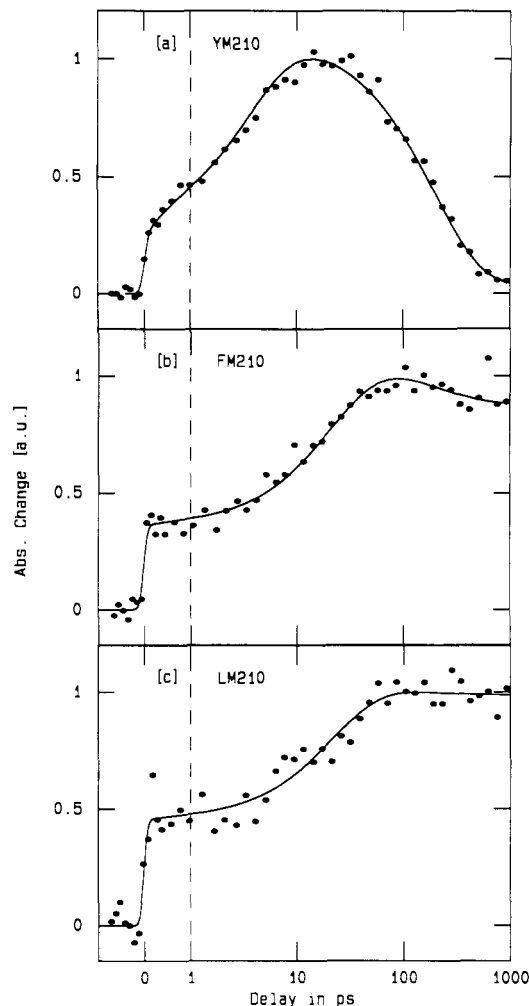


FIGURE 3: Transient absorption data for a probing wavelength of 660 nm using perpendicular polarized pulses: (a) results for wild-type RC (YM210); (b and c) results for mutants FM210 and LM210, respectively. The peak of the absorbance change is $\Delta A \approx 0.005$.

$\lambda_{pr} = 665$ nm for the wild-type and the two mutant RC's. In Figure 3a (wild type) the instantaneous absorption increase at $t_D = 0$, due to the formation of P^* , is followed by a further increase in the time domain of 0.1–10 ps. This increase is due to the reduction of the first tetrapyrrole with a time constant of 3.5 ps, i.e., paralleling the decay time of P^* . Between 10 and 1000 ps the absorption decreases due to electron transfer from H_A^- to Q_A with a time constant of 220 ps. RC's isolated from the $Y \rightarrow FM210$ mutant (Figure 3b) show an absorption increase occurring after the instantaneous rise taking place with a time constant of 16 ± 6 ps; i.e., the first tetrapyrrole is reduced much slower than in the wild-type molecule. Also at later times the signal shape deviates considerably from that of the wild type. Between 40 ps and 1 ns a weak relative absorption decrease occurs with a 220-ps time constant. The small amplitude of the 220-ps component indicates the $P^+Q_A^-$ state is reached by only a small fraction of all the reaction centers. This is in agreement with spectral analysis showing that a large percentage of quinone at Q_A was lost during preparation (see Materials and Methods). The statistical noise superimposed on the signal does not allow an unambiguous determination of the presence of the 70-ps kinetic component that was observed at 920 nm. Experiments on the $Y \rightarrow LM210$ RC are shown in Figure 3c. They are qualitatively similar to the data in Figure 3b. A quantitative analysis indicates that here the population of the reduced tetrapyrrole occurs with a 22 ± 8 ps time constant. There is no significant

contribution from a 220-ps component, indicating an even larger loss of quinone at Q_A , once again agreeing with spectral analysis of the occupation of the Q_A site. The results for $\lambda_{pr} = 665$ nm and parallel polarizations are not shown. With this polarization the wild-type RC exhibits the 0.9-ps absorption kinetic (Holzapfel et al., 1989, 1990), which has been assigned to the transfer of an electron from $P^+B_A^-$ to $P^+H_A^-$. In both mutants we do not observe a related absorption kinetic.

DISCUSSION

The experiments show that the lifetime of the excited electronic state P^* (observed at 920 nm) is more than four times longer in both RC mutants containing changes at tyrosine M210 as compared to the wild type. The time constants are 16 ps for the $Y \rightarrow FM210$ RC and 22 ps for $Y \rightarrow LM210$ RC. We also observe an additional slow kinetic component in the gain measurements, which has a time constant of 70 ps (90 ps). The first reduction process (observed at 665 nm) occurs with approximately the same time constants of 16 and 22 ps as the decay of P^* . We observe no additional fast kinetic with a 1-ps time constant. Nagarajan et al. (W. W. Parson, personal communication) have also measured similar mutants. They observed approximately the same time constants as those reported here, i.e., 10.5 ± 1.0 ps for FM210 and 16 ± 1 ps for RC in which an isoleucine is inserted at M210.

The following discussion of the influence of the mutations on the primary charge separation is based on the assumption that the effects of the mutations originate from the molecular properties of the exchanged amino acid and not from a secondary structural disorder. This assumption is supported by previous results from linear dichroism spectroscopy (Gray et al., 1990) and resonance Raman spectroscopy (T. Mattioli, K.A.G., D.O., M. Lutz, and B. Robert, unpublished results), which show that there are no gross alterations in the orientation and interactions of the various chromophores. We cannot rule out though long-range structural alterations that may not be detected by either of the aforementioned optical methods.

We address first the biphasic decay of the excited state P^* in the mutants. There are two main possibilities to explain the observed time constants of 16 ps (22 ps) and 70 ps (90 ps). If the sample is heterogeneous, the two time constants may originate from two populations of RC's. A genetic, that is, primary structural, heterogeneity can be excluded since the DNA region mutagenized was sequenced prior to RC isolation from the same batch of cells used for protein isolation (Gray et al., 1990), and these sequences showed no ambiguity. However, there is a sample heterogeneity caused by the loss of Q_A from a large portion of the RC's during isolation. Nevertheless, experiments on other mutants in which similar amounts of Q_A were lost showed that the loss of quinone did not affect the kinetics of the initial electron-transfer step (Stilz et al., 1990). In addition, a heterogeneity at the site of YM210 cannot be excluded. If YM210 interacts with the acetyl group of P_B (one of the individual Bchl of P that is on the B-branch), the lack of the hydroxyl group in the mutants may lead to two distinct configurations of the chromophore which could be related to the different kinetic constants of 16 ps (22 ps) and 70 ps (90 ps).

The second main possibility that could explain the two time constants concerns a nonheterogeneous sample. In a homogeneous sample the slow 70-ps (90-ps) time constant may originate from an additional intermediate state (a parking level), which may involve chromophores on the B-branch.

At present we cannot differentiate between the various possibilities to explain the slower component. However, since the dominant electron transfer proceeds with the faster time

constants (16 or 22 ps), we will focus on these processes.

The location of YM210 between P, B_A , and H_A may support the idea that it participates in the primary electron transfer as has been suggested by others (Allen et al., 1988). At first glance YM210 may act via three fundamental mechanisms.

First, it may fix the position of the chromophores in a geometric arrangement favorable for high overlap of the wave functions. One would then expect that the exchange of YM210 to F or L would strongly reduce the rate of electron transfer. However, the changes in structural rearrangement might be related with significant rearrangements of the spectral properties, which are not observed (Gray et al., 1990). Second, YM210 may participate directly in a superexchange-type electron transfer. The structural arrangement of the reaction center indicates that the electronic system of YM210 can only mediate the electron transfer between the special pair and the accessory bacteriochlorophyll B_A . The exchange of the polar and aromatic tyrosine by the nonpolar but aromatic phenylalanine should slow down the electron transfer. The mutation to the nonaromatic leucine, however, should lead to an additional strong decrease in the reaction rate, which is not observed experimentally.

Finally, the most probable influence of YM210 on the primary electron transfer would be the tuning of the energy levels of intermediate states. Due to the location of this tyrosine and its polarity, it may act by lowering the energy of the state $P^+B_A^-$. This view is supported by recent calculations by Parson et al. (1990), in which it was shown that the electrostatic interactions of the tyrosine may lower the energy level of the state $P^+B_A^-$ (these calculations were based on the structure of *Rps. viridis*). The consequences of this change should be discussed for the two current models of primary electron transfer. On the supposition that $P^+B_A^-$ in wild-type RC's is a distinct intermediate, an increase in the energy level of $P^+B_A^-$ would slow down the overall electron transfer. If the energy level of $P^+B_A^-$ is increased over that of P^* , thermal activation of the reaction is required or direct electron transfer from P^* to $P^+H_A^-$ by superexchange via the high-lying $P^+B_A^-$ becomes important. In this case one would expect substantial changes in the reaction rate constants.

On the supposition that the primary electron transfer in the wild-type RC's proceeds by $P^+B_A^-$ as a virtual (superexchange) state, the increase of its energy level again would decrease the reaction rate. For both reaction models the effect of the mutations would be the removal of the polarity of tyrosine and would not be very specific to either F or L. Therefore, similar reaction rates would be expected.

The observed strong decrease of the primary electron transfer upon exchange of tyrosine to both phenylalanine (16 ps) or leucine (22 ps) strongly supports the idea that the primary action of M210 is the tuning of the energy level of the radical pair $P^+B_A^-$. Tyrosine M210 apparently optimizes the primary electron transfer in the wild-type reaction centers. The absence of a tyrosine at the complementary position of the B-pigment branch and the insertion of a phenylalanine instead may be one major reason for the unidirectionality of the electron transport.

Finally we shall address the question of whether the observations on the mutated reaction centers give additional information on the reaction scheme of the primary electron transfer in the wild-type reaction centers. The observed time constants of 0.9, 3.5, and 220 ps can be arranged in two sequential reaction schemes: If the 3.5-ps process is assumed to precede the 0.9-ps reaction, the data analysis supports the stepwise electron transfer via $P^+B_A^-$ (Holzapfel et al., 1990).

In the second possible reaction scheme the 0.9-ps kinetic is followed by the 3.5-ps process. Since the decay of the excited state P^* , observed at 920 nm, was 3.5 ps, a plausible molecular interpretation of this 0.9-ps kinetic model involves two excited electronic states P_1^* and P_2^* coupled by a 0.9-ps relaxation process. Subsequently, the electron transfer would proceed in one superexchange step directly to the bacteriopheophytin H_A . The molecular arrangement of the RC's indicates that tyrosine M210 mainly interacts with the accessory bacteriochlorophyll B_A and not with the special pair. As a consequence the mutations FM210 and LM210 should not change a relaxation process in the excited electronic state of the special pair. Therefore, the 0.9-ps kinetic component (time constant and amplitude) should not be influenced by the mutations and should be observed in our measurements. The absence of this component in both mutant samples is an argument that the 0.9-ps process does not correspond to a relaxation in the excited electronic state P^* . However, the experimental results presented here do not contradict a sequential reaction scheme where the 0.9-ps process reflects the electron transfer from the accessory bacteriochlorophyll B_A to the bacteriopheophytin H_A .

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Registry No. L-Tyrosine, 60-18-4; L-phenylalanine, 63-91-2; L-leucine, 61-90-5.

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