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Influence of M subunit Thr222 and Trp252 on quinone binding and electron transfer in *Rhodobacter sphaeroides* reaction centres

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M subunit Trp252 is the only amino acid residue which is located between the bacteriopheophytin H_A and the quinone Q_A in the photosynthetic reaction centre of *Rhodobacter sphaeroides*. Oligodeoxynucleotide-directed mutagenesis was employed to elucidate the influence of this aromatic amino acid on the electron transfer between these two chromophores. For this, M subunit Trp252 was changed to tyrosine or phenylalanine, and Thr222, which presumably forms a hydrogen bridge to the indole ring of M subunit Trp252, to valine. In all three mutated reaction centres, the electron-accepting ubiquinone Q_A is less firmly bound to its binding site than in the wild-type protein. The electron transfer from the reduced bacteriopheophytin H_A^- to Q_A proceeds in the wild-type and in the mutant ThrM222Val within 220 ps. However, in the mutants TrpM252Tyr and TrpM252Phe the time constants are 600 ps and 900 ps, respectively. This indicates that M subunit Trp252 participates in the binding of Q_A and reduction of this quinone.

The photosynthetic reaction centres of purple non-sulfur bacteria are membrane-bound pigment-protein complexes which mediate a light-induced charge separation across the photosynthetic membrane via a sequence of highly efficient directional electron-transfer steps (Kirmaier and Holten, 1987; Feher et al., 1989; Holzapfel et al., 1989; Zinth et al., 1990; Holzapfel et al., 1990).

The reaction centre of *Rhodobacter sphaeroides* consists of three protein subunits called heavy (H), medium (M) and light (L) subunits. In addition, the reaction centre of *Rhodopseudomonas viridis* contains a cytochrome subunit. The recent X-ray structures have revealed that several amino acid residues of the L and M subunits of the protein interact specifically with the pigments (Deisenhofer et al., 1984; Deisenhofer and Michel, 1989; Allen et al., 1986 and 1987; Chang et al., 1986; Tiede et al., 1988; Rees et al., 1989). In addition to the function of amino acid residues for complexation of the chromophores, some of these residues have been implicated as participating in electron-transfer on the basis of structural data (Michel et al., 1986; Feher et al., 1989).

For some time, only the structure of the reaction centre from *Rps. viridis* was available at high resolution (0.23 nm). Recently, a high resolution structure for *Rb. sphaeroides* was also reported and a high degree of structural similarity between the two reaction centres was revealed (Buchanan et al., 1993; Ermler et al., 1992).

A reaction-centre deletion mutant of *Rb. sphaeroides* and its complementation in *trans* via a 5.3-kb *puf* operon shuttle fragment suitable for site-directed mutagenesis was established a few years ago (Farchaus and Oesterhelt, 1989). This opened the possibility to analyse the structural and functional role of specific amino acid side chains by genetic manipulation. Examples are the residue tyrosine M210 (Gray et al., 1990) and tyrosine L162 (Wachtveitl et al., 1993).

Here we report a detailed investigation on the function of the amino acid residues threonine M222 and tryptophan M252 in the reaction centre of *Rb. sphaeroides*. Fig. 1 shows the position of these two residues near the primary quinone-(Q_A)-binding site in the centre of *Rps. viridis*.

The indole ring of tryptophan M252 (tryptophan M250 in *Rps. viridis*) is in van der Waal's contact with both the bacteriopheophytin H_A and the quinone Q_A and, from this unique position, is suspected to participate as a (superexchange) mediator in electron-transfer (Plato et al., 1989). At the same time, due to its parallel orientation to Q_A, tryptophan M252 may contribute *via* a charge-transfer interaction to the binding of Q_A to the Q_A site. Threonine M222 (M220 in *Rps. viridis*) forms a hydrogen bridge to the nitrogen atom of the indole ring. This hydrogen bridge could help to hold the tryptophan parallel to Q_A. The same expectations are assumed to hold for the reaction centre in *Rb. sphaeroides*. It should be mentioned that one of the published structures of *Rb. sphaeroides* shows a hydrogen bond from threonine M222 to the quionone Q_A and not to the tryptophan M252. However, another previously published structure (Allen et al., 1988) and the recent high resolution (0.23 nm) structure (Buchanan et al., 1993; Ermler et al., 1992; H. Michel, unpublished results) indicate that the hydrogen bridge is di-

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Abbreviations. B_A (B_B), accessory bacteriochlorophyll on A (B) branch; H_A (H_B), bacteriopheophytin on A (B) branch; Kn, kanamycin; P, special pair; Q_A (Q_B), quinone on A (B) branch; Tc, tetracycline; UQ_{10}, ubiquinone-10.

Enzymes. T4 DNA ligase (EC 6.5.1.1.); DNA polymerase (Klenow fragment) (EC 2.7.7.7); T4 polynucleotide kinase (EC 2.7.1.78); lysozyme (EC 3.2.1.17); prokinase K (EC 3.4.21.14); RNase (EC 3.1.27.5).

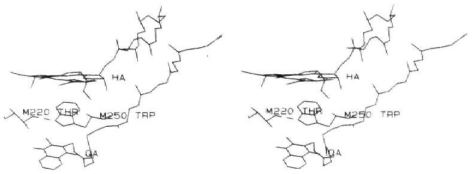

Fig. 1. Stereo plot of a section of the Q_A binding site of the reaction centre from *Rps. viridis*. Drawn are the cofactors bacteriopheophytin H_A and the ubiquinone Q_A and the amino acid residues threonine M220 and tryptophan M250. The possible hydrogen bond between the nitrogen atom of the indole ring and the hydroxyl group of threonine M220 is indicated by a dashed line. The stereo plot was drawn from the high-resolution crystal structure coordinates (0.23 nm) which were kindly provided by J. Deisenhofer and H. Michel (Deisenhofer and Michel, 1989).

rected from threonine M222 to tryptophan M252 and not to the quinone. This assumption allows a good understanding of the experimental observations presented below.

In order to analyse the influence of both residues on the structure and the electron-transfer rates in the reaction centre, threonine M222 was changed to valine, and tryptophan M252 to the aromatic amino acids tyrosine and phenylalanine.

EXPERIMENTAL PROCEDURES

Materials

Radiochemicals were purchased from Amersham. Restriction endonucleases and all other enzymes were obtained from Boehringer Mannheim. The Sequenase DNA sequencing kit was purchased from US Biochemical Corp. Nitrocellulose filters were obtained from Schleicher Schüll, Inc., DE52 anion-exchange cellulose was from Whatman Ltd. and TSK DEAE-650 (M) Fractogel was obtained from Merck. All other chemicals were of analytical or HPLC grade.

Bacterial strains and growth

The *Escherichia coli* strains DH5-α (F^-, recA1, 80d/lac Z M15, (lacZYA-argF9) U169, λ^-; Gibco-BRL) and S17-1 (recA, pro^-, res^-, mod^+, Tp^r, Sm^r, – pRP4-2-Tc::mu-Km::Tn7; Simon et al., 1983) were grown in Luria-Bertani medium (Maniatis et al., 1982) at 37°C. The *E. coli* K-12 strain WK6 mutL [Δ(lac-proAB), galE, strA, mutL::Tn10/ F'lacI^q, Z ΔM15, proA$^+$B$^+$; Zell and Fritz, 1987] was grown in M9 minimal medium (Maniatis et al., 1982) or in 2 × solution A (16 g bacto-tryptone, 10 g yeast extract, 5 g NaCl in H_2O; Lech and Brent, 1989) at 37°C. Plasmid-containing strains were grown in medium in the presence of appropriate antibiotics (pMa5-8, pRK404, pSup202, 100 µg/ml ampicillin; pMc5-8, 40 µg/ml chloramphenicol).

The wild-type *Rb. sphaeroides* strain ATCC 17023 (German Collection of Microorganisms), the green reaction-centre deletion strain *puf* ΔLMX 21/3 (Farchaus and Oesterhelt, 1989) and *Rb. sphaeroides* strains carrying mutated reaction-centre genes were grown chemoheterotrophically or photoheterotrophically as described previously (Farchaus and Oesterhelt, 1989). Cell growth was observed as reported (Chory and Kaplan, 1983). The absorbance was determined in a Klett photometer (model 800-3; Klett Manufacturing Inc.) equipped with a red filter. Light intensities were measured with an Opto-Meter (model 40x, United Detector Technology, Inc.).

Vectors

pMa/c5-8 (pBR322 derivative, colE1 replicon, F1 ori, Ap^r, Cm^r; Stanssens et al., 1989); pRK404 (RK2 derivative, oriV replicon, P^{lac}, Tc^r, oriT; Ditta et al., 1985) and pSup202 (pBR325 derivative, colE1 replicon, Mob^+, Ap^r, Tc^r, Cm^r; Simon et al., 1983).

Isolation of DNA and molecular biological methods

Whenever possible, standard molecular biological methods were used (Maniatis et al., 1982). Single-stranded DNA of the phasmid vectors pMa/c5.3 was isolated using M13K07 as helper phage (Stanssens et al., 1989). Genomic DNA was isolated from chemoheterotrophically grown *Rb. sphaeroides* cells using a modification of the procedure of Williams (Williams et al., 1983; Shiozawa et al., 1989). Labeling of DNA fragments was carried out using the random priming kits purchased from Boehringer Mannheim.

Oligodeoxynucleotides

Oligodeoxynucleotides were synthesized by the phosphoramidite method (Beaucage and Caruthers, 1981) using an Applied Biosystems 380A oligonucleotide synthesizer.

Site-directed mutagenesis

Single-stranded pMc5-8/ML phasmid DNA was annealed with *Nru*I-digested vector pMa5.3 to form the gapped duplex. The oligodeoxynucleotides A (5'-CACGGTGCGGT-CATCCTCGCG-3' (ThrM222Val), B (5'-GCCCTCTTCTA-TCGCTGGACC-3' (TrpM252Tyr) and C (5'-GCCCTCTTC-TTCCGCTGGACC-3' (TrpM252Phe) were phosphorylated, then mixed with the gapped duplex DNA for annealing. A subsequent DNA polymerase/ligase reaction closed the gap. The polymerase/ligase reaction mixture was used to transform *E. coli* WK6 mutL. Phasmid DNA was isolated from an overnight culture and was employed to retransform *E. coli* WK6 mutL. Recombinant phasmids were screened by colony hybridisation (Gergen et al., 1979; Grunstein and Wallis, 1979).

DNA sequence analysis

The sequence of the mutants was checked by dideoxynucleotide sequencing (Sanger et al., 1977; Tabor and Richardson, 1987). Compressions due to secondary-structure formation were resolved by the addition of deionized formamide to a final concentration of 40%.

Southern-blotting analysis

Southern blotting was performed essentially as described (Southern, 1975; Lang and Oesterhelt, 1989a).

Dot blotting

10 µg chromosomal DNA in solution B (15 mM Tris/HCl, 1 mM Na_2EDTA, pH 8.0) was denatured and loaded onto nitrocellulose filters as described previously (Lang and Oesterhelt, 1989a). Prehybridisation was carried out in hy-

bridisation buffer [1 × NaCl/Cit (Maniatis et al., 1982), 5 × Denhardt solution (Denhardt, 1966) and 100 µg/ml denatured and sonicated salmon DNA] for 4 h at 40°C. Hybridization was performed in fresh hybridisation buffer for 19 h at 45°C. Approximately 250000 cpm of ^{32}P-labeled oligodeoxynucleotides was added/cm^2 nitrocellulose. The filters were washed in 1 × NaCl/Cit, 5 × Denhardt solution and 0.1% SDS at 45°C, then at increasing stringency by increasing the temperature.

Isolation of chromatophores

The chromatophores used for photobleaching experiments were isolated from photoheterotrophic cultures. The cells were disrupted in a French pressure cell (2.9 kPa) and cellular debris was removed by centrifugation at 17000 × *g* for 25 min. Chromatophores were isolated from the supernatant by ultracentrifugation at 125000×*g* for 3 h and were finally suspended in 50 mM morpholinopropane sulphonic acid, pH 7.5, 100 mM KCl, 9 µM valinomycin to a total bacteriochlorophyll concentration of 12 µM using the absorption coefficient of 95 mM^{-1} cm^{-1} (Petty and Dutton, 1976).

Isolation of reaction centres

Reaction centres were isolated from photoheterotrophically grown cells as described previously (Holzapfel et al., 1989), except that the reaction-centre solutions were purified by passing them through a sequence of two DEAE 52 anion-exchange cellulose columns and one TSK DEAE-650 (M) fractogel column. Pure reaction centres ($A_{280/802}$ = 1.2) were dialysed against 20 mM Tris/HCl, pH 8.0, containing 0.08% lauryldimethylamine oxide and afterwards concentrated to 78 µM by ultrafiltration. The concentration of the reaction-centre solution was determined using the absorption coefficient E_{802} = 2.88 × 10^5 M^{-1} cm^{-1} (Straley et al., 1973).

Extraction of ubiquinone 10 (UQ_{10}) from reaction centres and quantitative analysis by HPLC

The ubiquinones were extracted from reaction-centre solutions by a slight modification of the method of Gast (Gast et al., 1985). An aliquot (3.29 ml, A_{802} = 1, measured in a cuvette of 1-cm path length) of the respective reaction centre solution was stirred at 36°C with 0.2 ml 0.1 M $FeCl_3$ for 5 min and for a further 30 min after addition of 30 ml acetone/methanol (50:50, by vol.). The ubiquinones were extracted at 4°C with three 60-ml portions of hexane. The combined hexane extracts were washed at 4°C, twice with 60 ml methanol/water (90:10, by vol.) and twice with 60 ml water. The hexane phase was dried over anhydrous sodium sulfate. The hexane was evaporated under reduced pressure and the ubiquinone residue was dissolved in a small amount of ethanol (300–500 µl).

Reverse-phase HPLC (25 cm × 5 mm Bishoff C_{18} ODS hypersil column, particle size 5 µm) of the extracts was performed at room temperature at a flow rate of 1 ml/min. The mobile phase was HPLC grade acetonitrile/HPLC grade diethyl ether (75:25, by vol.). The concentration of a standard solution (ethanolic solution of pure UQ_{10}) was determined according to Crane and Barr, 1971.

Reconstitution of UQ_{10} in reaction centres

UQ_{10} reconstituted reaction centres were prepared essentially as described (Okamura et al., 1975).

Spectroscopic methods

Room-temperature ground-state absorption spectra were recorded on a Shimadzu UV-160 spectrophotometer or on a Perkin Elmer Lambda 9 spectro-photometer.

Reaction-centre steady-state photobleaching was performed in the Q_y region of the special pair at 860 nm as described previously (Gray et al., 1990), except that a (860 ± 13) nm interference filter was placed in front of the measuring beam. The experimental system used for the investigation of time-resolved absorption changes has been described in detail previously (Holzapfel et al., 1989; Holzapfel et al., 1990).

RESULTS

Mutagenesis and construction of mutated strains of *Rb. sphaeroides*

For site-directed mutagenesis of the gene coding for the M subunit of the reaction centre of *Rb. sphaeroides*, a 5.3 kb *Bam*HI–*Hin*dIII *puf* operon shuttle fragment was used (Farchaus and Oesterhelt, 1989). This fragment was cloned into the multiple cloning site of the phasmids pMa/c5.3 (Stanssens et al., 1989; Farchaus et al., 1990), resulting in the phasmids pMa/c5.3. Mutagenesis was carried out by the gapped-duplex method (Kramer et al., 1984) following the procedure worked out by Fritz and coworkers (Stanssens et al., 1989). DNA sequence analysis confirmed that only the desired mutations and no others were introduced into the medium gene.

The mutated 5.3 kb *Bam*HI–*Hin*dIII fragments were then cloned into the broad host range vector pRK404 (Ditta et al., 1985). The *E. coli* strain S17-1 was used to transfer the pRK404/ML vectors into the *Rb. sphaeroides* reaction centre deletion strain *puf* DLMX 21/3 by conjugation using a diparental filter mating procedure (Farchaus and Oesterhelt, 1989; Davis et al., 1988). Kanamycin-(Kn)-resistant and tetracycline-(Tc)-resistant *Rb. sphaeroides* exconjugants were selected under chemoheterotrophic growth conditions. The *trans* complemented deletion strains of all three mutants grew phototrophically.

In order to obtain genetically defined strains, the mutated reaction-centre genes were integrated into the chromosome of *Rb. sphaeroides*. For this, the 5.3-kb *Bam*HI–*Hin*dIII fragment was cloned into the suicide plasmid pSup202 (Simon et al., 1983). The construct was transferred into the green deletion strain *puf* ΔLMX 21/3 and *Rb. sphaeroides* exconjugants having a phototrophic positive phenotype (PS^+) were selected by growing cells photoheterotrophically. PS^+ exconjugants were then replicaplated twice and grown photoheterotrophically, resulting in clones with a stable PS^+ phenotype. Clones with the phenotype PS^+, Kn^s, Tc^s were picked out. This phenotype was indicative of a double cross-over event in which the chromosomal transposon 5 (Tn5) Kn^r gene was replaced by the reaction-centre genes. In addition, the Tc sensitivity emphasizes that the pSup202 vector encoding the Tc resistance was lost. Chromosomal DNA of the mutant strains was analysed for correct insertion of the reaction-centre genes into the chromosome by Southern-blot analysis. Fig. 2a shows that the reaction-centre genes are located in wild-type *Rb. sphaeroides* as well as in the mutants on an approximately 13-kb *Bam*HI fragment, whereas they are missing in the chromosome of the deletion strain. As a control in Fig. 2b, hybridization of the same blot with a ^{32}P-

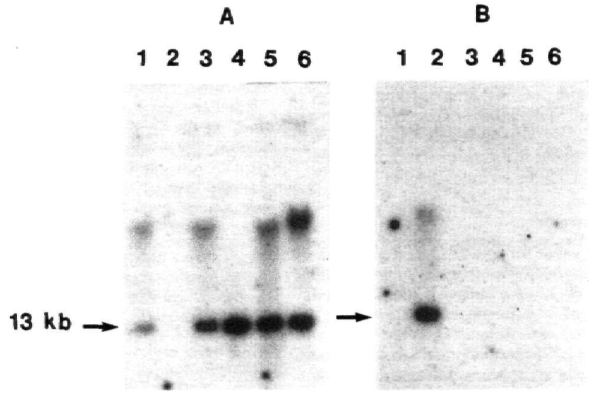

Fig. 2. Southern-blot analysis of chromosomal DNA from different *Rb. sphaeroides* strains: wild-type ATCC 17023 (lane 1), the green deletion strain *puf* ΔLMX 21/3 (Farchaus and Oesterhelt, 1989) (lane 2), a mutation strain not described here (lane 3), the mutations TrpM252Tyr (lane 4), TrpM252Phe (lane 5), and ThrM222Val (lane 6). Chromosomal DNA was digested with *Bam*HI and probed with random-primed ^{32}P-labeled 1.4-kb *Pvu*II–*Mae*III fragment containing a region of the reaction centre light and medium genes (Fig. 2a) or with a ^{32}P-labeled 0.9-kb *Pst*I fragment containing a region of the Tn5 Kn^r gene (Beck et al., 1982) (Fig. 2b). Hybridisation of the probes to DNA fragments larger than 13 kb is due to an incomplete digestion of the chromosomal DNA.

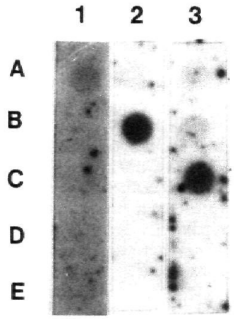

Fig. 3. Dot blot analysis of chromosomal DNA from different *Rb. sphaeroides* strains: the mutations ThrM222Val (line A), TrpM252Phe (line B), TrpM252Tyr (line C), a mutation strain not described here (line D), and from wild-type ATCC 17023 (line E). The chromosomal DNA was probed with ^{32}P-labeled oligodeoxynucleotides used for the construction of the mutants. Column 1, oligodeoxynucleotide(ThrM222Val); column 2, oligodeoxynucleotide B (TrpM252Phe); column 3, oligodeoxynucleotide C (TrpM252Tyr).

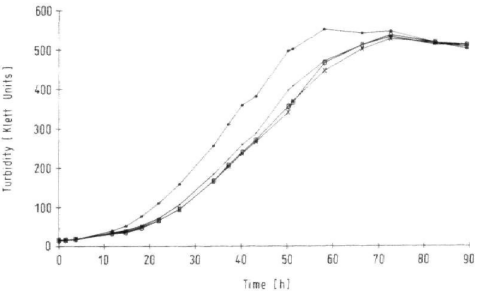

Fig. 4. Growth kinetics of *Rb. sphaeroides* wild-type ATCC 17023 (stars) and of the mutations ThrM222Val (points), TrpM252Tyr (crosses), and TrpM252Phe (open circles). Prior to the measurement of the growth curves the strains were grown once photoheterotrophically to early stationary phase. The cultures were illuminated with white light (9.5 W/m^2) at 30°C in Bellco anaerobic culture tubes. The generation times were 7.9 h for the wild-type, 9.6 h for the mutation ThrM222Val, 9.9 h for the mutation TrpM252Tyr and 9.6 h for the mutation TrpM252Phe.

labeled 0.9-kb *Pst*I fragment containing a region of the Tn5 Kn^r gene (Beck et al., 1982) emphasizes that the Kn^r gene is only present in the chromosome of the deletion strain. A dot-blot analysis of the chromosomal DNA of the three mutants verifies the successful amino acid substitution (Fig. 3). In addition, the lack of any cross-hybridization in the dot blot confirms that selection of stable ps$^-$ phenotypes by growth in the light does not cause enrichment of revertants. This is understandable, since the growth advantage of wild-type *Rb. sphaeroides* is not significant compared to the mutant strains (Fig. 4).

Photobleaching of reaction centres in chromatophores

Fig. 5 shows the light-induced bleaching of the low-energy Q_y transition of the special pair at 860 nm in the photosynthetic membrane of *Rb. sphaeroides* wild-type (Fig. 5a) and the *cis* complemented mutants (Fig. 5b–d). Nearly the same maximal bleaching is observed in all samples under saturating actinic light conditions. Since the concentration of the membranes was normalized to total Bchl (Petty and Dutton, 1976), this indicates that approximately an equal amount of functional reaction centre is present in the membranes of the wild-type and the mutants.

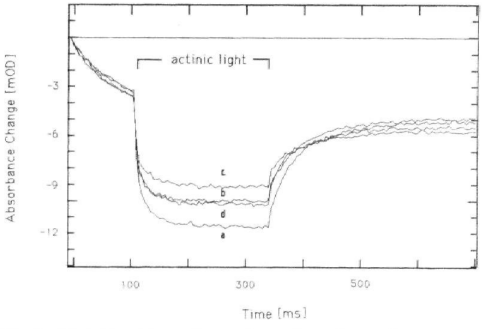

Fig. 5. Photobleaching of membrane-bound reaction centres from wild-type *Rb. sphaeroides* ATCC 17023 (a) and from the mutations ThrM222Val (b), TrpM252Tyr (c) and TrpM252Phe (d). The reaction centres were excited with saturating actinic light (150 W tungsten lamb, 2 mm Schott BG38 filter). The absorption changes were recorded in the Q_y region of the special pair at 860 nm. The decrease of the base line prior to the excitation is due to bleaching by the probing light.

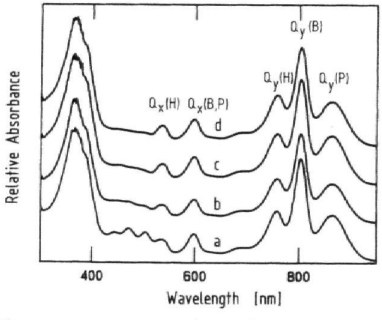

Fig. 6. Room temperature ground state absorption spectra of isolated reaction centres from wild-type *Rb. sphaeroides* ATCC 17023 (a) and from the mutations ThrM222Val (b), TrpM252Tyr (c) and TrpM252Phe (d). Indicated are the Q_y and Qx transitions of the special pair P, the two accessory bacteriochlorophylls B, and the two bacteriopheophytins H.

Biochemical characterization of the mutated reaction centres

Reaction centres from *Rb. sphaeroides* wild-type strains and from the strains complemented *in-cis* were isolated. (Reaction centres from the mutant strains complemented *in-trans* were also isolated and found to be biophysically identical.)

Fig. 6 depicts the respective absorption spectra. The absorption spectra of all four samples are identical in the spectral region where the bacteriochlorophylls and bacteriopheophytins absorb via their Q_y and Q_x transitions. In contrast, the wild-type absorption spectrum is distinguishable from the absorption spectra of the mutants around 500 nm, where a carotenoid molecule bound to the reaction centre absorbs. This difference is caused by an altered carotenoid composition in the green deletion strain *puf* ΔLMX 21/3 compared to the wild-type strain (Farchaus and Oesterhelt, 1989).

Since threonine M222 and tryptophan M252 belong to the Q_A-binding site, the mutations are likely to have an influence on Q_A binding. Therefore, the ubiquinone content in the various reaction centre samples was determined by standard methods of ubiquinone extraction and HPLC analysis. Pure UQ_{10} as external standard was recovered to 75%. Under the same conditions, approximately 0.7 ubiquinone molecules/reaction centre were found in the wild-type sample. This corresponded, therefore, to an amount of nearly one ubiquinone molecule/reaction centre. It is well established that only Q_A is firmly bound in the wild-type protein. Apparently, Q_B is completely missing in our preparations, in contrast to previous reports (Okamura et al., 1975). The analysis of the mutant proteins is based on the assumption that Q_B is also lost in these samples. The Q_A content of isolated reaction centers decreased in the following order: wild-type, ThrM222Val mutation, TrpM252Phe mutation, and TrpM252Tyr mutation (see Table 1). In addition, the observation that Q_A is partially missing in the mutants is further supported by the time resolved absorption data shown in Fig. 8.

Spectroscopic analysis of the mutated reaction centres

The time-resolved absorption measurements on the different reaction-centre samples were performed at three wavelengths of 920 nm (Fig. 7), 665 nm (Fig. 8) and 785 nm (data not shown). The reaction centres were excited at 860 nm in order to secure selective excitation of the Q_y transition of the special pair P. The time-resolved absorption changes are plotted in Figs 7 and 8 as a function of delay times between -1 ps and 1 ps on a linear scale and for delay times $t_D > 1$ ps on a logarithmic scale. The data in Fig. 7 are calculated according to a kinetic model incorporating three components, whereas the data in Fig. 8 are based on a kinetic model composed of four components (Holzapfel et al., 1989; Zinth et al., 1990; Holzapfel et al., 1990).

The primary step in the photosynthetic electron-transfer is the decay of the excited electronic state P* of the special pair P. This can be observed on the long-wavelength side of the special-pair absorption band at a probing wavelength of 920 nm. The occupation of the excited electronic state P* is seen in Fig. 7 as a very rapid absorption decrease due to stimulated emission. The stimulated emission decays in the wild-type sample (Fig. 7a) as well as in the ThrM222Val mutation (Fig. 7b), the TrpM252Tyr mutation (Fig. 7c) and TrpM252Phe mutation (Fig. 7d) with the same time dependence. A monoexponential fit yields a rate constant of 3.5 ± 0.4 ps. The experimental data indicate that the mutations do not change the lifetime of the excited electronic state P*. The small absorption decrease found in the wild-type sample at $t_D = 100$ ps is caused by the accumulation of the long-lived final photoproduct $P^+Q_A^-$, which, at 920 nm, absorbs less than the initial ground state PQ_A. In the mutants, however, the state $P^+Q_A^-$ is formed only to a minor extent since Q_A is partially missing.

The consequences of the mutations for Q_A binding and for the electron-transfer kinetics in the reaction centres are readily seen at the probing wavelength of 665 nm where the reduced bacteriochlorophylls and the reduced bacteriopheophytins absorb (Davis et al., 1979). Fig. 8a shows the results of measurements on the wild-type sample. The instantaneous part of the absorption increase originates from the population of the excited electronic state P* of the special pair P. It was shown recently that the subsequent slower absorption increase with a time constant of 0.9 ± 0.3 ps may be related

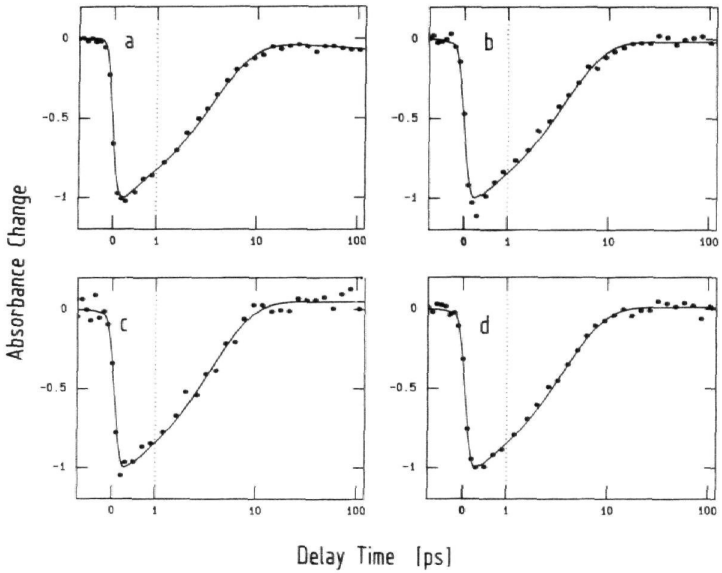

Fig. 7. Time-resolved absorption data (points) measured at a probing wavelength of 920 nm for reaction centres of wild-type *Rb. sphaeroides* ATCC 17023 (a) and of the mutations ThrM222Val (b), TrpM252Tyr(c) and TrpM252Phe (d). The reaction centres were excited at 860 nm. The maximal absorption changes at 920 nm were normalized to a value of 1 for the various samples. The respective experimental data varied over 0.014−0.022. The delay time scale is linear between −1 and +1 ps and logarithmic for $t_D > 1$ ps. The curves are calculated as described in the text.

to the transfer of an electron from the accessory bacteriochlorophyll B_A to the bacteriopheophytin H_A and that the weak absorption decrease with a time constant of 3.5 ± 0.4 ps is due to the electron-transfer from P to B_A (Holzapfel et al., 1989; Zinth et al., 1990; Holzapfel et al., 1990). At later delay times, a pronounced absorption decrease with a time constant of 220 ± 40 ps is found. This absorbance change reflects the electron-transfer from the reduced bacteriopheophytin H_A^-, which absorbs strongly at 665 nm, to the primary quinone acceptor Q_A. At delay times $t_d > 1$ ns, the state $P^+Q_A^-$ causes the small absorption decrease compared to the initial state PQ_A. Fig. 8b points out that the time-resolved absorption data taken on the wild-type protein are not affected by the addition of a 4.5 molar excess of UQ_{10}. Apparently, the higher detergent concentration (1.1% lauryldimethylamine oxide) in the latter sample, which is caused by the addition of lauryldimethylamine oxide solubilized UQ_{10} to the initial preparation (0.08% lauryldimethylamine oxide), has no influence on the electron-transfer kinetics.

At early delay times, the transient absorption curves of the ThrM222Val (Fig. 8c), TrpM252Tyr (Fig. 8e) and TrpM252Phe (Fig. 8g) mutations resemble that of the wild-type. The time constants of the corresponding electron-transfer steps are unchanged at 3.5 ps and 0.9 ps, respectively. Beyond this, the time-resolved absorption data taken at 785 nm demonstrate that the respective time constants connected to the electron-transfer step $B_A^- \rightarrow H_A^-$ amount to 0.9 ± 0.3 ps. At late delay times, however, part of the induced absorption persists in the mutated reaction centres. This illustrates that the decay of the reduced bacteriopheophytin H_A^- is not complete in the mutant proteins, emphasizing that the electron acceptor Q_A is partially missing. From the extent of induced absorption remaining at a delay time of $t_D = 5$ ns it is deduced that the Q_A binding site is occupied only to approximately 70%, 15% and 50% in the mutations ThrM222Val, TrpM252Tyr and TrpM252Phe, respectively. These values correspond well to the those found by chemical analysis (see Table 1). By addition of a 4.5-molar excess of UQ_{10} to the reaction-centre solutions, Q_A could be partially reconstituted into the Q_A binding site. After the reconstitution, Q_A was present to a level of 80% in the mutations ThrM222Val (Fig. 8d) and TrpM252Phe (Fig. 8h) and to 65% in the mutation TrpM252Tyr (Fig. 8f). This indicates that the mutation $Trp^{M252} \rightarrow Tyr$ has the lowest binding affinity for Q_A. It should be noted, however, that the incomplete reconstitution may be caused by the high lauryldimethylamine oxide concentration of 1.1% in the reconstituted samples.

The decay of H_A^- proceeds in the mutation ThrM222Val (Fig. 8c) as in the wild-type sample with a time constant of 220 ± 40 ps. The addition of UQ_{10} had no influence on the time constant (Fig. 8d). In the mutations TrpM252Tyr and TrpM252Phe, the electron is more slowly transferred from H_A^- to Q_A. The time constants could only be accurately determined for the UQ_{10} reconstituted samples (Fig. 8f and h). They are 600 ± 200 ps and 900 ± 200 ps for the mutations TrpM252Tyr and TrpM252Phe, respectively. The time constants apparently do not depend on the detergent concentration in the solution, since the same time constants were found (data not shown) for material complemented *in-trans* at a detergent concentration of 0.08% lauryldimethylamine oxide.

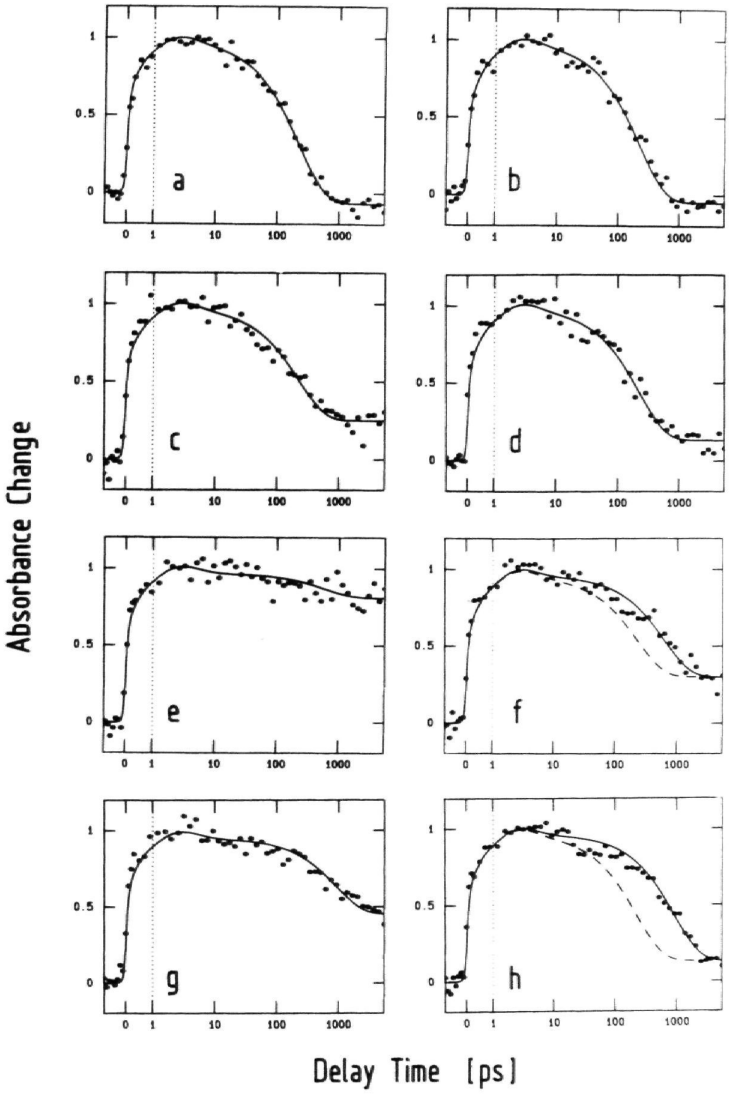

Fig. 8. Time-resolved absorption data (points) at the probing wavelength of 665 nm for reaction centres of wild-type *Rb. sphaeroides* ATCC 17023 (a, b) and of the mutations ThrM222Val (c, d), TrpM252Tyr (e, f) and TrpM252Phe (g, h). The reaction centres were excited at 860 nm. The absorption changes, which persist at late delay times, point out that Q_A is partially lost in the mutated reaction centres (left side). Q_A was reconstituted into the reaction centres by addition of a 4.5-molar excess of UQ_{10} (right side). The maximal absorption changes at 665 nm were normalized to a value of 1 for the various samples. The respective experimental data varied over 0.0045−0.006. The delay time scale is linear between −1 and +1 ps and logarithmic for $t_D > 1$ ps. The curves are calculated as described in the text. The dashed curves presuppose that the electron-transfer from H_A^- to Q_A proceeds within 220 ps.

Table 1. Summary of mutations. Characteristic properties of wild-type and mutated reaction centres. The total ubiquinone content of the reaction centres was determined by HPLC analysis. Since Q_B has been lost during the isolation procedure the total ubiquinone content corresponds to the Q_A content (wild-type taken to be 100%). The Q_A content in the reaction centres was determined from the transient absorption changes remaining at a delay time of $t_D = 5$ ns at the probing wavelength 665 nm (Fig. 8). The 0.9-ps kinetic was assigned to the electron-transfer from B_A^- to H_A.

Mutation	Nucleotide change	Total quinone content	Q_A content	Electron transfer rates		
				$P^*\rightarrow B_A$	$B_A^-\rightarrow H_A$	$H_A^-\rightarrow Q_A$
		%		ps		
None		100 ± 15	100 ± 8	3.5 ± 0.4	0.9 ± 0.3	220 ± 40
ThrM222Val	ACC→GTC	80 ± 12	70 ± 7	3.5 ± 0.4	0.9 ± 0.3	220 ± 40
TrpM252Tyr	TGG→TAT	13 ± 2	17 ± 9	3.5 ± 0.4	0.9 ± 0.3	600 ± 200
TrpM252Phe	TGG→TTC	58 ± 9	51 ± 7	3.5 ± 0.4	0.9 ± 0.3	900 ± 200

Consequently, the increased time constants are not due to a detergent artifact.

DISCUSSION

Replacement of threonine M222 by valine and tryptophan M252 by tyrosine or phenylalanine results in mutated reaction centres which preserve the ability of *Rb. sphaeroides* cells to grow photosynthetically. Mutation strains which carry the mutated genes in the chromosome grow under photoheterotrophic conditions with rates comparable to wild-type *Rb. sphaeroides* ATCC 17023. This, and since all the mutations are based on a 2-bp exchange, indicates the in-*cis*-complemented mutation strains are genetically stable. Indeed, a dot-blot analysis (Fig. 3) of chromosomal DNA of the various mutations proved that photosynthetic growth did not lead to reversions at positions M222 and M252. However, reversions at secondary sites cannot be definitely excluded. Nevertheless, these are unlikely to have occurred since identical electron-transfer rates were observed in two different reaction-centre preparations isolated from both in-*trans*-complemented and in-*cis*-complemented strains.

Complementation *in-cis* leads to genetically defined strains. In addition, the photosynthetic membrane of the *in-cis*-complemented strains is likely to have the same molecular composition as the wild-type membrane. Indeed, the mutated proteins could be isolated from the membrane in the same manner and with yields comparable to that of the wild-type protein. Beyond this, the photobleaching experiments (Fig. 5) further provide evidence that a comparable amount of functional reaction centres exist in the membrane of wild-type and mutation strains. From these observations it can be concluded that approximately the same amount of reaction centres reside in the respective membranes.

Furthermore, these experiments indicate that, in the photosynthetic membrane, the Q_A-binding site is entirely populated. In contrast, Q_A is partially missing in isolated reaction centres of the mutated proteins, whereas Q_A is not lost during the isolation of the wild-type protein. However, Q_A can be reconstituted into the mutated proteins by addition of excess UQ_{10} to the aqueous detergent phase. The distribution of the quinone Q_A between the aqueous detergent phase and the protein phase can be considered as a two-phase equilibrium. The equilibrium is progressively displaced from the protein phase to the detergent phase in the order wild-type, ThrM222Val, TrpM252Phe and TrpM252Tyr mutations. Only in the photosynthetic membrane where an excess of quinone is present (Takamiya and Dutton, 1979) is the Q_A-binding site in the mutations also entirely occupied. This result clearly emphasizes an important role for the threonine M222 and the tryptophan M252 in the reaction centre structure and function. Tryptophan M252 presumably mediates binding of Q_A to its binding site via a charge-transfer interaction (Szent-Györgyi et al., 1961; Foster and Fyfe, 1966; Ishida et al., 1980 and references cited therein) between the electron donor tryptophan and the electron acceptor Q_A. This is suggested by the crystal structure of *Rps. viridis* where the π-electron-rich C2−C3 bond of the indole-ring system is exactly located above the *p*-benzoquinone ring system of the menaquinone-9 (Deisenhofer and Michel, 1989). Threonine M222, in contrast, seems to hold the tryptophan in place via a hydrogen bond. This hydrogen bond is evident from the 0.23-nm crystal structure of *Rps. viridis* (Deisenhofer and Michel, 1989) and has recently also been reported as existing in two reaction-centre structures of *Rb. sphaeroides* (Allen et al., 1988; Michel, H., unpublished results). The absence of this hydrogen bond in the mutation ThrM222Val should import to the tryptophan M252 an increased freedom of movement. This may be responsible for the weaker binding of Q_A in this mutation. For the mutations TrpM252Tyr and TrpM252Phe, the strong electron-donor tryptophan is replaced by the weaker electron donors tyrosine and phenylalanine. Therefore, the reduction of the binding affinity of Q_A to its binding site in these mutations is to be expected. However, it is not directly obvious why the lowest binding affinity is observed for the mutation TrpM252Tyr. One possible explanation could be an unfavourable dipole-dipole interaction between the hydroxyl group of tyrosine M252 and a carbonyl oxygen of the ubiquinone.

The primary charge separation steps leading to the state $P^+H_A^-$ are unchanged in the mutated reaction centres. In addition, the ground state absorption spectra of the wild-type and the mutated reaction centres (Fig. 6) are identical. This observation supports the idea that the mutations are not accompanied by large overall structural changes and that position and energetics of the bacteriopheophytin H_A, as far as they are related to the primary electron-transfer, are not modified. The electron-transfer from the reduced bacteriopheophytin H_A^- to the quinone Q_A has been shown to proceed in the native reaction centre of *Rb. sphaeroides* at room temperature within 200 ps (Kaufmann et al., 1975; Rockley et al., 1975; Peters et al., 1978; Kirmaier et al., 1985). An increased reaction rate has been observed at lower temperatures (Kirmaier et al., 1985) which indicates that the electron-transfer does not lead to activation (Jortner, 1980).

The reaction rate of this process is given in the frame of conventional electron-transfer theory by Eqn 1 (Plato et al., 1989).

$$K_{ET} = \frac{2\pi}{\hbar} |V|^2 F. \quad (1)$$

The Franck-Condon-factor F contains the nuclear coordinates and the energetics of the molecules involved. The electronic coupling V represents the effective overlap of the electronic wave functions of the electron donor and the acceptor. V decreases exponentially with increasing donor-acceptor edge-to-edge distances (McLendon, 1988). In the reaction centre of *Rb. sphaeroides*, the edge-to-edge distance between the bacteriopheophytin H_A and the quinone Q_A amounts to 1.0 nm (Allen et al., 1987). In view of such a large separation, it is unlikely that the direct electronic overlap between H_A^- and Q_A can account for the observed fast electron-transfer rates (Plato et al., 1989; Budil et al., 1987). One possible role of the amino acid residue tryptophan M252 could be to speed up the electron-transfer. Due to its high redox potential, tryptophan M252 cannot act directly as a spectroscopically observable kinetic intermediate. The electrochemical reduction potential of tryptophan has not been determined. However, from the values of corresponding organic molecules, the reduction potential of tryptophan in polar solutions can be estimated to lie between 1.7 and −2.0 eV (Ender et al., 1954; Volke, 1963). Besides, tryptophan M252 could accelerate the electron-transfer via the so-called superexchange mechanism (Michel-Beyerle et al., 1988; Halpern and Orgel, 1960; McConnell, 1961; Larsson, 1981; Miller and Beitz, 1981; Beratan et al., 1985). In this case, the electronic coupling is mediated by a molecule M lying between the donor D^- and the acceptor A (Eqn 2)

$$V = V_{direct} + V_{super}, \quad (2)$$

$$\text{with } V_{super} = \frac{V_{DM} \cdot V_{MA}}{E_{DM}}.$$

Here, E_{DM} is the difference in free energy between the donor-state DMA and the mediating state D^+M^-A at the intersection of the potential-energy surface of the two states DMA and D^+MA^-. It has been calculated that tryptophan M252 may act as an intervening molecule in native reaction centers (Plato et al., 1989). Replacing tryptophan M252 by a tyrosine or a phenylalanine leads to reduced reaction rates. This could be caused by either a change of the respective Franck-Condon-factors F or by smaller electronic overlaps, V_{super}. A change of the Franck-Condon-factor F, e.g. via the energetics of the chromophores involved, cannot be excluded. However, one may find strong arguments for a change of the electronic overlap V_{super}. The electronic systems of tyrosine and phenylalanine are similar but less extended than those of tryptophan, reducing V_{DM} or V_{MA}. In addition, the redox potential changes in the order tryptophan to tyrosine to phenylalanine, increasing the free-energy difference E_{DM} in the same order. Both effects may combine and lead to the decreased electron-transfer rates in the mutation reaction centres of $1/k_{ET}$ = 600 ps (TrpM252Tyr) and 900 ps (TrpM252Phe). In this context, experimental observations by Youvan and coworkers (Coleman et al., 1990) are of particular interest. They found that the reaction rate was reduced by a factor of 14 when they substituted tryptophan M252 by leucine in the reaction centre of *Rb. capsulatus*. This finding is in agreement with the expectation that replacing tryptophan M252 by the non-aromatic leucine should reduce the electronic overlap V_{super} even further than in the case of tyrosine and phenylalanine.

The mutation ThrM222Val does not influence the electron-transfer rates in the reaction centre. This is remarkable in two respects. First, the mutation presumably increases the thermal mobility of tryptophan M252, which leads to the observed reduction of the quinone-binding strength. However, it appears that the conformations accessible in this way allow normal electron-transfer rates.

CONCLUSION

a) Tryptophan M252 mediates the high-affinity binding of Q_A to the reaction centre of *Rb. sphaeroides*. The precise position of the indole ring is presumably defined by a hydrogen bridge to threonine M222 and is essential for a stable binding of Q_A.

b) The rate constants of the electron-transfer step $H_A^- \rightarrow Q_A$ depend on the aromatic amino acid side chain bridging H_A and Q_A. The experimental results support the idea of a superexchange mechanism in the secondary electron-transfer mediated by tryptophan M252.

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Supplementary Material. **Influence of threonine M222 and tryptophan M252 on quinone binding and electron transfer in *Rhodobacter sphaeroides* reaction centres.** Fig. S1. Time-resolved absorption data (points) taken at a probing wavelength of 785 nm for reaction centres of wild-type *Rb. sphaeroides* ATCC 17023 (a) and of the mutants ThrM222Val (b). This information is available, upon request, from the Editorial Office.

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