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Reaction centers from three herbicide-resistant mutants of *Rhodobacter sphaeroides* 2.4.1: sequence analysis and preliminary characterization

MARK L. PADDOCK, SCOTT H. RONGEY, EDWARD C. ABRESCH, GEORGE FEHER & MELVIN Y. OKAMURA
Department of Physics – B-019, University of California, San Diego at La Jolla, CA 92093, USA

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Abstract. Many herbicides that inhibit photosynthesis in plants also inhibit photosynthesis in bacteria. We have isolated three mutants of the photosynthetic bacterium *Rhodobacter sphaeroides* that were selected for increased resistance to the herbicide terbutryne. All three mutants also showed increased resistance to the known electron transfer inhibitor o-phenanthroline. The primary structures of the mutants were determined by recombinant DNA techniques. All mutations were located on the gene coding for the L-subunit resulting in these changes $Ile^{229} \rightarrow Met$, $Ser^{223} \rightarrow Pro$ and $Tyr^{222} \rightarrow Gly$. The mutations of Ser^{223} is analogous to the mutation of Ser^{264} in the D1 subunit of photosystem II in green plants, strengthening the functional analogy between D1 and the bacterial L-subunit. The changed amino acids of the mutant strains form part of the binding pocket for the secondary quinone, Q_B. This is consistent with the idea that the herbicides are competitive inhibitors for the Q_B binding site. The reaction centers of the mutants were characterized with respect to electron transfer rates, inhibition constants of terbutryne and o-phenanthroline, and binding constants of the quinone UQ_0 and the inhibitors. By correlating these results with the three-dimensional structure obtained from x-ray analysis by Allen et al. (1987a, 1987b), the likely positions of o-phenanthroline and terbutryne were deduced. These correspond to the positions deduced by Michel et al. (1986a) for *Rhodopseudomonas viridis*.

Abbreviations: ATP – adenosine 5′-triphosphate, Bchl – bacteriochlorophyll, Bphe – bacteriopheophytin, bp – basepair, cyt c^{2+} – reduced form of cytochrome c, DEAE – diethylaminoethyl, EDTA – ethylenediamine tetraacetic acid, Fe^{2+} – non-heme iron atom, LDAO – lauryl dimethylamine oxide, Pipes – piperazine-N,N′-bis-2-ethane-sulfonic acid, PSII – photosystem II, RC – reaction center, SDS – sodium dodecylsulfate, Tris – tris(hydroxymethyl)aminomethane, UQ_0 – 2,3-dimethoxy-5-methyl benzoquinone, UQ_{10} – ubiquinone 50

Introduction

The reaction center (RC) is a membrane bound pigment-protein complex that mediates the primary photochemistry in photosynthesis. RCs from the

photosynthetic bacterium *Rhodobacter* (*Rb.*) *sphaeroides* have been extensively studied by biochemical and biophysical techniques (for reviews see Feher and Okamura 1978, Michel-Beyerle 1985) including X-ray crystallography (Allen et al. 1987a, Allen et al. 1987b). RCs consist of three protein subunits L, M and H and the following cofactors: four bacteriochlorophylls (Bchl), two bacteriopheophytins (Bphe), one non-heme iron (Fe^{2+}), and two ubiquinones (UQ_{10}). A specialized bacteriochlorophyll dimer, $(Bchl)_2$, serves as the primary electron donor, a bacteriopheophytin as an intermediate electron acceptor and two ubiquinones, Q_A and Q_B, as the primary and secondary electron acceptors, respectively. Absorption of a photon initiates electron transfer from the donor to one of the Bphe molecules. Subsequent electron transfer occurs to Q_A followed by electron transfer to the secondary quinone acceptor, Q_B. Q_B is believed to act as a mobile electron carrier; after receiving two electrons and two protons (Wraight 1981) it dissociates from the RC and transports the protons across the bacterial membrane to create a pH gradient, which drives ATP synthesis (for reviews see Ort and Melandri 1982 and Ort 1986).

The electron transfer from Q_A^- to Q_B in bacterial RCs is inhibited by triazine herbicides (Wraight 1981) of which terbutryne, studied in this work, is an example. These herbicides, which also inhibit electron transfer in PSII in green plants, are believed to act by competing with Q_B for its binding site (Tischer and Strotman 1977, Pfister and Arntzen 1979, Velthuys 1981, Wraight 1981, Vermass et al. 1983, Brown et al. 1984, Diner et al. 1984, Vermaas et al. 1984, Kyle 1985). The binding site for Q_B in *Rb. sphaeroides* has been shown by X-ray crystallography to be in a region between the D and E helices of the L subunit (Allen et al. 1987b). The inhibitors o-phenanthroline and terbutryne were found by X-ray studies to bind near the homologous region in *Rhodopseudomonas* (*R.*) *viridis* RC (Michel et al. 1986a). These structural studies verified the previous proposals based on photoaffinity labeling with azido-atrazine (Brown et al. 1984, deVitry and diner 1984) that Q_B binds to the L subunit.

Herbicide resistant mutants of *Rb. sphaeroides* have been isolated by several groups (Brown et al. 1984, Okamura 1984, Stein et al. 1984, Gilbert et al. 1985, Schenck et al. 1986) and of *R. viridis* by Sinning and Michel (1987). RCs from these mutants show decreased sensitivity to inhibition of electron transfer to Q_B by triazine herbicides (like terbutryne) and altered electron transfer properties. In this work we report on the sequence analysis and preliminary characterization of RCs from three terbutryne resistant mutants of *Rb. sphaeroides* 2.4.1. The changes in the primary structure obtained from the nucleotide sequence are correlated with changes in binding properties for quinone and inhibitor taking the known three-dimension-

al structure into account. A preliminary account of this work has been presented (Paddock et al. 1987). A more detailed report on the kinetics and thermodynamic properties of Q_B in these mutants will be published elsewhere.

Materials and methods

Materials

T4 DNA ligase, T4 DNA polynucleotide kinase (*E. coli B*), restriction enzymes BamHI, PvuII, SalI, and XhoI and radioactive nucleotides [γ-^{32}P]ATP and [α-^{35}S]dCTP were obtained from Amersham; restriction enzymes HindIII, PstI, and NruI were from New England Biolabs; large fragment *Escherichia* (*E.*) *coli* DNA polymerase I (Klenow subfragment) was from Bethesda Research Laboratories; pUC8 (Messing and Vieira 1982), M13mp18 and M13mp19 (Yanisch-Perron et al. 1985) were from p-L Biochemicals; deoxynucleotides and dideoxynucleotides were from Pharmacia; and alkaline phosphatase from calf intestine was from Boehringer Mannheim Biochemicals. The plasmid pUC119, which contains the M13 origin for replication, and the helper phage M13K07 were kindly supplied by Jeff Vieira. Centricon 30 microconcentrators were obtained from Amicon; nitrocellulose discs were either Schleicher and Schuell BA85 or Millipore HATF filters; DEAE was from Toyosoda; horse heart cytochrome c (type 6) and UQ_0 were from Sigma; DNase was from Worthington; terbutryne was from Chem Service; and atrazine and o-phenanthroline (1,10-phenanthroline) were from Baker. All other chemicals were of reagent or HPLC grade.

Isolation of mutants

Mutants of the photosynthetic bacterium *Rb. sphaeroides* were isolated by their ability to grow photosynthetically under increased terbutryne (100 μmol) or atrazine (300 μmol) concentrations (Okamura 1984). *Rb. sphaeroides* 2.4.1 was grown anaerobically to early log-phase in Hutners medium. At this stage either 100 μmol terbutryne or 300 μmol atrazine was added (10 cm^3 tubes, tungsten lamp light intensity $\sim 1\,mW\,cm^{-2}$, T = 30 °C). This temporarily stopped growth for a few weeks. The tubes were then grown to saturation and cells from each tube were spread on plates to isolate individual colonies. Single colonies were isolated and regrown to saturation in liquid culture with herbicide. Production of mutants resistant to o-phenanthroline using this method failed; growth did not resume after its addition, presumably because o-phenanthroline chelates the metals that are required for photosynthetic growth.

DNA cloning

DNA was isolated from anaerobically grown cells using a procedure similar to that described by Williams et al. (1983) with the following modifications: an extraction with phenol:chloroform (1:1) preceded the CsCl gradient. The CsCl was removed after the band extraction using Centricon 30 microconcentrators rather than dialysis. This was followed by a phenol:chloroform (1:1) extraction, a chloroform extraction, and ethanol precipitations in TE (10 mmol Tris-HCl pH 8, 1 mmol EDTA) with 2.5 mol ammonium acetate (75% ethanol) followed by a precipitation in TE with 0.3 mol sodium acetate (67% ethanol). The DNA at this stage had an optical absorbance ratio of $A_{260}/A_{280} \sim 2$.

All enzymatic reactions were performed as suggested by the manufacturers. Genomic DNA from the IM229 and SP223 mutants was digested with the restriction enzymes BamHI and HindIII and from the YG222 mutant with PstI. The digestions were stopped by a phenol:chloroform (1:1) extraction followed by ethanol precipitation. The fragments of the IM229 DNA were ligated into the pUC8 plasmid which was digested with BamHI and HindIII. The pUC119 plasmid was digested with BamHI and HindIII for the ligation of the SP223 DNA fragments and with PstI for the YG222 DNA fragments. All restriction enzyme digestions were checked on 0.8% (w/v) agarose gels using ethidium bromide to visualize the DNA bands. The digested vector DNA was dephosphorylated using alkaline phosphatase before starting the ligation. The ligation mix was adjusted to give a concentration of about $30\,\mu g\,cm^{-3}$ of vector DNA with a molar ratio ranging from 1:1 to 3:1 of chromosomal fragments to vector DNA.

The ligation mixes were either transformed directly into $CaCl_2$ treated *E. coli* (Maniatis et al. 1982) or were cleaned with a phenol:chloroform (1:1) extraction followed by an ethanol precipitation and transformed into cells treated by the Hanahan procedure (Hanahan 1985) to improve the transformation efficiencies. The *E. coli* hosts used were JM103 (Messing 1983), JM105 or JM109 (Yanisch-Perron et al. 1985). The transformed cells were grown in either LB, 2YT (Miller 1972) or SOB broth (Hanahan 1985) for 45–60 minutes and then plated on nitrocellulose discs on top of either LB, 2YT or SOB plates with $50\,\mu g\,cm^{-3}$ ampicillin. Cells were grown until the colony size was ~ 1 mm; this took 14–20 hours. At least two copies of the library were made and screened as described by Hanahan and Meselson (1980). The oligonucleotide, complementary to a region of the gene coding for L (*pufL*), Ala^{141} through Phe^{146} (5′-GCCTGGGGCTATGCCTTC-3′), was labelled using T4 polynucleotide kinase and [γ-^{32}P]ATP, which had a typical specific activity of $2–3 \times 10^5$ cpm pmole^{-1}. The incubation time for hybridization was 12–36 hours at 42 °C. After hybridization the filters were

rinsed in a solution of 0.9 mol NaCl with 0.09 mol sodium citrate three times at 23 °C for 30 minutes each and once at 42 °C for three minutes. The filters were air dried and exposed to Kodak XAR-5 film with an intensifying screen at −80 °C.

Colonies which lined up with the positive signals on the film were isolated and rescreened. Plasmid DNA from these colonies was prepared using either the boiling procedure of Holmes and Quigley (1981) as modified by Crouse et al. (1983) or the alkaline-SDS procedure of Birnboim and Doly (1979) to confirm that the clone had the appropriate restriction enzyme recognition sites. Large scale plasmid preparation was performed as described by Maniatis et al. (1982).

Subfragments of the cloned BamHI-HindIII fragment from the IM229 mutant were cloned into the M13 phage to isolate single stranded template for sequencing. Eight subclones were needed to sequence the entire structural coding genes for the L and M subunits (*pufL* and *pufM* respectively). The following subclones were used: a PstI-PvuII fragment (~ 1 kbp) cloned into M13mp18, a PvuII-SalI fragment (~ 450 bp) cloned into M13mp18 and M13mp19, two SalI-XhoI fragments of opposite orientation (~ 400 bp) cloned into M13mp18, an XhoI-NruI fragment (~ 425 bp) cloned into M13mp18 and M13mp19 and an NruI fragment (~ 1.5 kbp) cloned into M13mp18. These clones were identified using the complementation test (Messing 1983) with subclones that had been made for the sequencing of *pufL* and *pufM* from *Rb. sphaeroides* 2.4.1 (Williams et al. 1983; Williams et al. 1984). The use of pUC119 for the subsequent cloning of the *puf* operon from SP223 and YG222 eliminated the need for subcloning into M13 phage.

DNA sequencing

The IM229 mutant genes were sequenced by the dideoxy method of Sanger (1977) utilizing the M13 recombinant phage with its universal primer. The SP223 and YG222 mutants were sequenced by the dideoxy method of Sanger (1977) using template DNA prepared by superinfection of the clone with M13K07 and the following oligonucleotide primers:

1. 5′-CCGACTGCAAGCGGAGAG-3′ (complementary to a region 18 to 7 bases upstream of *pufL*),
2. 5′-TACCCGCCGGCCCTTGAA-3′ (from Tyr^{67} to Glu^{72} of L),
3. 5′-GCCTGGGGCTATGCCTTC-3′ (from Ala^{141} to Phe^{146} of L),
4. 5′-TTCACGAACGCGCTGGCTCTGGC-3′ (from Phe^{181} to Ala^{188} of L),
5. 5′-TGGGTCGACTGGTGGCAA-3′ (from Trp^{259} to Gln^{264} of L),
6. 5′-CTCTTCTCGGGCCTGATG-3′ (from Leu^{60} to Met^{65} of M),
7. 5′-GCGCTGGGCATGGGCAAG-3′ (from Ala^{139} to Lys^{144} of M),

8. 5′-TCGCGATGCACGGTGCGACCA-3′ (from Phe^{216} to Thr^{222} of M),
9. 5′-GCCGGTAATGATCATGCA-3′ (for sequencing the other strand near all of the mutation sites; from Gly^{252} to Cys^{247} of L).

The DNA was labelled in the sequencing reactions with ^{35}S using [α-^{35}S]dCTP in the presence of the large fragment of *E. coli* DNA polymerase I (Klenow subfragment). The DNA sequence analysis followed the procedure described by Williams et al. (1983).

Reaction center purification

RCs were isolated from each of the mutant strains using the following procedures: Bacteria were lysed using a French press and chromatophores were collected by centrifugation. RCs were solubilized from these chromatophores using LDAO by the method of Cogdell et al. (1975) and were further purified by an $(NH_4)_2SO_4$ precipitation followed by either DEAE chromatography (Feher and Okamura 1978) for SP223 and YG222 RCs or cytochrome c sepharose affinity chromatography (Brudvig et al. 1983) for IM229 RCs. The RCs had an optical absorbance ratio $A_{280}/A_{802} \simeq 1.3$ for SP223 and YG222 and $\simeq 1.7$ for IM229. In the final step the RCs were concentrated and dialyzed against TLE (10 mmol Tris-HCl pH 8, 0.025% LDAO, 1 mmol EDTA). The purified RCs from these mutants contained only one quinone (Q_A) as determined from either the amount of cytochrome c (cyt c^{2+}) oxidized by the RCs under saturating continuous illumination (10 μmol cyt c^{2+}, 10 mmol Pipes pH 6.8, 0.025% LDAO, 1–2 μmol RCs, $T = 20\,°C$, $I = 1\,W\,cm^{-2}$ of white light) or from the percentage of fast and slow recovery of the stabilized donor, (the recovery time from Q_B is $\simeq 1$ s; the recovery from Q_A is $\simeq 100$ ms) (see for example Okamura et al. 1982). One quinone RCs of *Rb. sphaeroides* 2.4.1 and R26 were prepared as described by Okamura et al. (1975) and assayed as described above.

UQ_0 binding

To determine the occupancy of the Q_B site, we prepared one quinone RCs and measured the rate of cyt c^{2+} photooxidation under continuous illumination (see for example Okamura et al. 1982; $I = 1\,W\,cm^{-2}$, white light) in the presence of varying concentrations of exogenous UQ_0. (Conditions: 10 μmol cyt c^{2+}, 10 mmol Pipes pH 6.8, 0.025% LDAO, 1 μmol RCs, $T = 20\,°C$). Light absorbed by the RC causes an electron to flow from the donor (D) through an intermediate acceptor (Bphe) and primary quinone acceptor (Q_A) to the secondary quinone acceptor (Q_B). In the presence of cyt c^{2+} the oxidized donor is reduced allowing more electrons to flow from the donor to the quinons (see Fig. 1). Thus, the oxidation of cyt c^{2+}, monitored at 550 nm with a modified Cary 14 (McElroy et al. 1974, Kleinfeld et al. 1984),

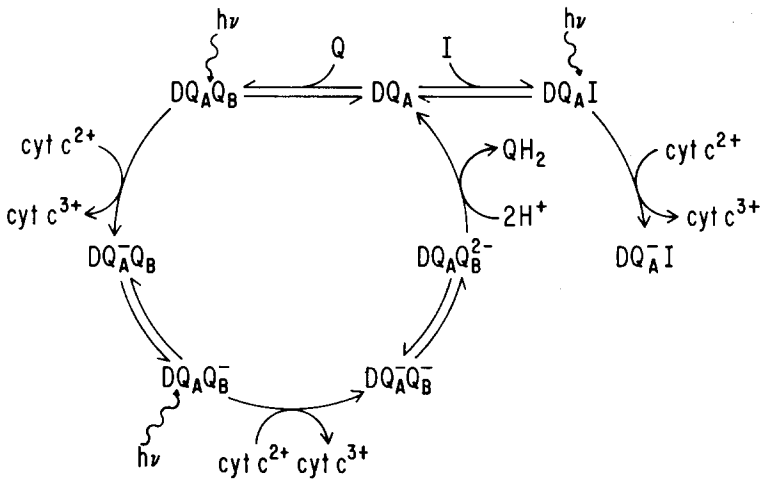


Fig. 1. Electron transfer scheme of the RC showing competitive inhibition between the secondary quinone (Q) and herbicide (I). When Q is bound, the RC can undergo a light induced catalytic cycle oxidizing cytochrome (cyt c^{2+} → cyt c^{3+}) and reducing exogenous quinone. Each Q_B accepts two electrons and two protons; when fully reduced to QH_2 it exchanges with oxidized Q from the quinone pool. For each electron cycled through the RC, one cyt c^{2+} is oxidized to cyt c^{3+}. When herbicide is bound to the RC, at the Q_B site, electron transfer from Q_A^- is inhibited and cyt c^{2+} photooxidation stops after one electron is transferred. Thus, the photooxidation rate provides a convenient assay for the fraction of RCs that have a bound Q_B. Cytochrome c photooxidation (cyt c^{2+} → cyt c^{3+}) was monitored optically at 550 nm as a function of time (see Fig. 3).

provides a measure of the electron turnover rate. RCs were cross illuminated with a projection lamp through 1 inch of water and a Corning 2-64 filter. The cytochrome turnover rate (in cyt $RC^{-1}s^{-1}$) was calculated at various quinone concentrations (Fig. 3). The data were fitted with a Michaelis-Menten equation (see for example Rawn 1983):

$$V = \frac{[UQ_0]}{K_Q + [UQ_0]} \times V_m, \tag{1}$$

where V is the cytochrome photooxidation rate at the UQ_0 concentration ($[UQ_0]$), V_m is the maximum cytochrome photooxidation rate and K_Q is the concentration of UQ_0 needed to reach one half of V_m; it was used as a measure of quinone binding. Since we are using a *kinetic* assay to determine an *equilibrium* constant, K_Q represents an approximation of the actual binding constant for UQ_0. However if equilibrium is reached on the time scale of the electron transfer, K_Q represents the real binding constant.

Inhibitor binding

The inhibition of terbutryne or o-phenanthroline was studied using the same cyt c^{2+} photooxidation assay as described in the previous section (Fig. 1). The turnover rate was measured for different inhibitor concentrations in the presence of 100 μmol UQ_0 (the quinone concentration that approximately equals the value of K_Q for RCs of the wild type strains 2.4.1 or R26). The activity was defined as the observed photooxidation rate with inhibitor present (V) divided by the photooxidation rate without inhibitor (V_0). The % Activity versus herbicide concentration was fitted with the relation:

$$\% \text{ Activity} = \frac{V}{V_0} \times 100 = \left(1 - \frac{1}{1 + K_{inh}/[I]}\right) \times 100, \quad (2)$$

where [I] is the concentration of inhibitor and K_{inh} is the inhibition constant found from this fit. A binding constant for the inhibitor (K_I) can be determined if direct competition between the inhibitor and Q_B is assumed (Wraight 1981, Brown et al. 1984, Diner et al. 1984) and equilibrium is reached on the time scale of the electron transfer to Q_B. The expression for K_I, which includes a correction for the number of active RCs at any time (which depends on the concentration of UQ_0, $[UQ_0]$, and the binding constant for the quinone, K_Q) is given by (see for example Rawn 1983):

$$K_I = \frac{K_{inh}}{1 + [UQ_0]/K_Q}. \quad (3)$$

Results

Cloning and sequencing

Three herbicide resistant mutants of *Rb. sphaeroides* 2.4.1, that were phenotypically distinct from each other as judged by electron transfer and herbicide binding characteristics (Okamura 1984), were chosen for DNA sequence analysis. Since preliminary experiments have implicated either the L or M subunit as the site of the mutation(s) (Okamura et al. 1985), the structural genes coding for the L and M subunit proteins (*pufL* and *pufM*) were cloned from the three mutants.

Restriction enzyme digests of genomic DNA were cloned into either pUC8 (for IM229) or pUC119 (for SP223 and YG222). The use of pUC119 eliminated the need to subclone smaller fragments into the M13 phage for DNA sequencing. The libraries were screened with a synthetic oligonucleotide probe complementary to a region of *pufL*, which codes for Ala^{141}

through Phe[146] (5′-GCCTGGGGCTATGCCTTC-3′). Colonies that hybridized with the probe were obtained with a frequency of 3/7000, 7/13 000 and 4/5000 from the IM229, SP223 and YG222 libraries respectively. A 6.3 kb BamHI-HindIII genomic DNA fragment (from IM229 and SP223) or a 4.5 kb PstI fragment (from YG222) was isolated; these fragments include *pufL* and *pufM*.

The complete sequence of the noncoding strands of *pufL* and *pufM* from each mutant was determined using either the M13 phage system (for IM229) or internal oligonucleotide primers and pUC119 (for SP223 and YG222). The sequence of the mutant coding strand around the altered region as well as that of the parent 2.4.1. genes Williams et al. 1984 region were used to verify the mutation(s). The nucleotide changes and the deduced amino acid changes are shown in Fig. 2. All three mutants showed changes in the primary structure of the L subunit between the D and E helices (Allen et al. 1987b); no changes were found in the M subunit.

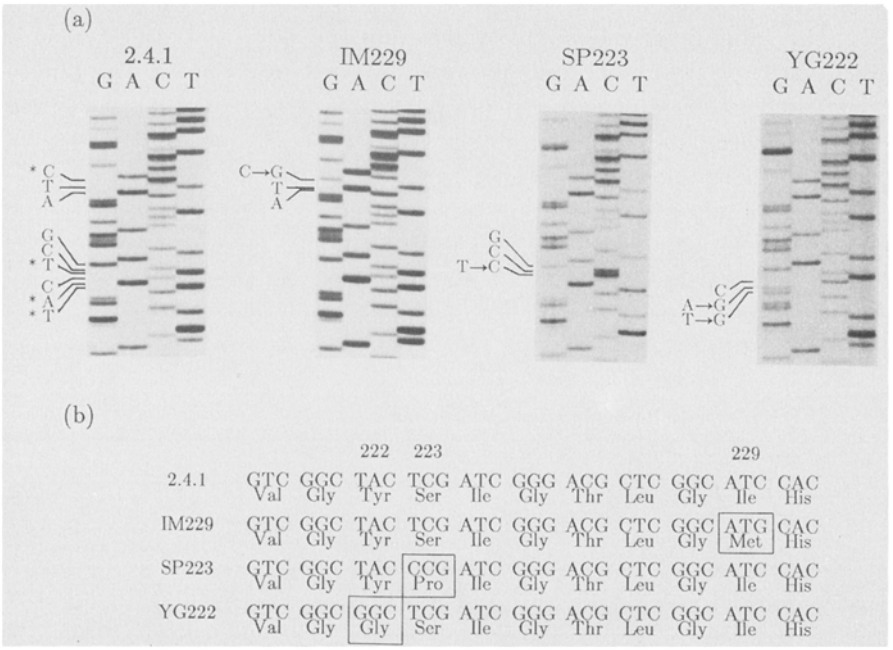


Fig. 2. Regions of the sequencing gels containing the mutations (a) and partial sequences of the mutated region (b) of *pufL* from *Rb. sphaeroides*. The changed nucleotides are indicated next to the gel (a): a C → G transversion was seen in *pufL* from the IM229 mutant, a T → C transition was seen for the SP223 mutant and two changes (a T → G transversion and a A → G transition) were seen in *pufL* from the YG222 mutant. (The gel is read upwards). The resulting amino acid replacements are boxed in (b). All three mutants show changes in the region between Tyr[222] and Ile[229] i.e: Ile[229] → Met (IM229), Ser[223] → Pro (SP223), and Tyr[222] → Gly (YG222).

A single nucleotide change was seen in *pufL* (Fig. 2) from the IM229 mutant (ATC → ATG), resulting in the replacement at position 229 of the L subunit of an isoleucine (Ile) (Williams et al. 1984) with the longer methionine residue (Met). This mutation was independently reported by Gilbert et al. (1985) and Schenck et al. (1986) for herbicide resistant mutants of *Rb. sphaeroides*. The cloned DNA from the SP223 mutant had two nucleotide changes, one at position 38 of the M subunit (CTG → CTC) resulted in no amino acid substitution and the other at position 223 of the L subunit (Williams et al. 1984) (TCG → CCG; Fig. 2) resulted in the replacement of serine (Ser) with proline (Pro). Two nucleotide changes were found in *pufL* from YG222 (TAC → GGC). Both were located in the same codon and resulted in the replacement at position 222 of the L subunit of tyrosine (Tyr) (Williams et al. 1984) with glycine (Gly).

UQ_0 binding studies

RCs with one quinone were prepared as described in Materials and Methods. The binding of the water soluble quinone UQ_0 to the Q_B site was investigated by the cytochrome photooxidation assay (Fig. 1). The rate of cytochrome oxidation, measured as a function of UQ_0 concentration varied greatly between the different mutant RCs (Fig. 3) and was used as a phenotypic characterization (Table 1).

The maximum photooxidation turnover rate, V_m (defined as the rate at saturating UQ_0 concentration), was different for all three mutant RCs (see Fig. 4; Table 1), showing that each mutation has a different effect on the net electron transfer rate from cyt c^{2+} to UQ_0. RCs from the SP223 mutant had the slowest turnover rate at high UQ_0 concentration although the rate was

Table 1. Turnover rates and inhibition and binding constants.

Strain[a]	Mutation	Turnover rate V_m^b (cyt/RC/sec)	Inhibition constants K_{inh}^c		Binding constants K_Q^d (mM UQ_0)	K_i^e	
			Ter (μM)	O-phen (μM)		Ter (μM)	O-phen (μM)
Wild type	-	260	0.1	24	0.09	0.05	11
IM229	Ile^{229} → Met	350	12	270	1.9	11	260
SP223	Ser^{223} → Pro	> 14	> 300	150	> 2.0	> 290	~ 140
YG222	Tyr^{222} → Gly	60	> 300	620[f]	0.7	> 260	540

[a] Wild type refes to either 2.4.1 or R26, both of which give the same values. SP223 and YG222 were previously called 5a and 11a (Okamura et al. 1984) and S223P and Y222G (Paddock et al. 1987).

[b] Maximum cytochrome turnover rate (± 25%).

[c] Inhibition constants (± 30%). Assay uses < 0.1 μmol RC, 0.025% LDAO, 20 mmol cyt c^{2+}, 10 mmol Pipes pH 6.8, and 0.1–1.0 mmol EDTA under continuous illumination (I = 1 W cm^{-2}).

[d] Concentration of UQ_0 needed to reach half maximum of cytochrome turnover rate (± 30%).

[e] Estimated inhibitor binding constant using equation 2 (± 30%).

[f] The previously reported value of 300 μmol (Paddock et al. 1987) may have been due to a contamination with Wild type RCs.

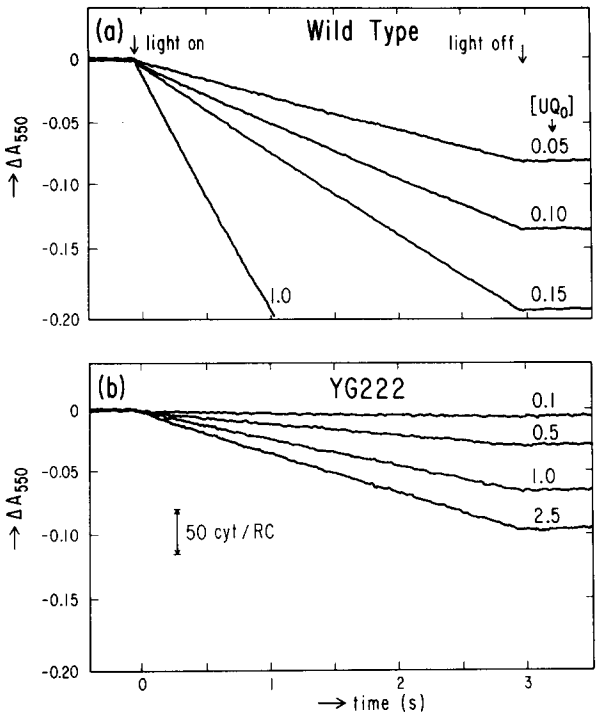


Fig. 3. Cytochrome photooxidation as a function of time for different quinone concentrations, [UQ_0]. The change in optical absorbtion from the oxidation of cyt c^{2+} → cyt c^{3+} is shown for RCs from the wild type strains (a), R26 and 2.4.1 (they give the same result), for different concentrations of UQ_0 in [mmol] (20 μmol cyt c, 10 mmol Pipes pH 6.8, 0.025% LDAO, 35 nmol RCs, T = 20 °C, I = 1 W cm^{-2}) and for RCs from the mutant YG222 (b) with UQ_0 concentrations in [mmol] as shown (conditions as above). The number of cyt c^{2+} oxidized per reaction center was determined from the relation (see for example Okamura et al. 1982):

$$\text{cyt c oxidized/RC} = 14.4\ (\Delta A_{550}/A_{802}).$$

The rate of cyt c^{2+} photooxidation is calculated from the number of cyt c^{2+} oxidized per unit time.

never saturated up to the limit of solubility of UQ_0 (V_m > 14 cyt $RC^{-1}s^{-1}$). RCs from the YG222 mutant had a V_m about four times slower (60 cyt $RC^{-1}s^{-1}$) than the rate obtained for RCs isolated from the wild type strains 2.4.1 or R26 (V_m = 260 cyt $RC^{-1}s^{-1}$). The binding curve for RCs isolated from the IM229 mutant was fit with a V_m value slightly higher (350 cyt $RC^{-1}s^{-1}$) than V_m obtained from 2.4.1 or R26 (Table 1).

An approximate binding constant (K_Q) for UQ_0 to the RCs was determined from the fit of the cytochrome photooxidation rate (Fig. 4) with equation 1; K_Q is the concentration of UQ_0 needed to reach a cytochrome turnover rate of one half V_m. All three mutants exhibited reduced binding (see Table 1). The least affected mutation (YG222) still had an eight times

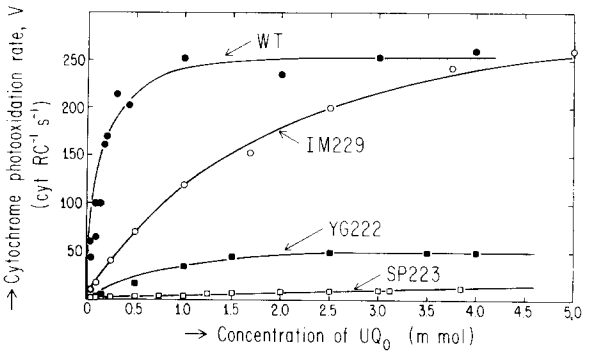


Fig. 4. Cytochrome photooxidation rate as a function of UQ_0 concentration for the wild type, WT (R26 and 2.4.1, which give the same result), and the mutant RCs (IM229, SP223, YG222). Data were obtained as described in Fig. 3. The binding constant, K_Q, was determined from the fit of the data to equation (1) and is the concentration of UQ_0 needed to reach 50% of the maximum rate, V_m (see Table 1). All of the mutant RCs show a reduced affinity for UQ_0 (higher K_Q than for the wild type strains). Note that the rate for neither the SP223 mutant nor the IM229 mutant is saturated at 4 mmol UQ_0.

lower binding constant (K_Q = 0.7 mmol) than RCs from the wild type strains 2.4.1 or R26 (K_Q = 0.09 mmol).

Herbicide binding studies

The reduction of the electron transfer rate, as measured by the cyt c^{2+} photooxidation assay, upon addition of inhibitors is shown for the wild type and the three mutants in Fig. 5. The solid lines represent theoretical fits to equation (2) with K_{inh} being equal to the herbicide concentration at 50% Activity. Values of the inhibition constants, K_{inh}, and the binding constants K_I (equation 3), are summarized in Table 1.

RCs from the SP223 and YG222 mutants were most resistant to terbutryne. In both cases the turnover rate could not be reduced to 50% of V_0 within the limit of terbutryne solubility (K_{inh} > 300 μmol; K_I > 260 μmol). RCs from the IM229 mutant were not as resistant to terbutryne as the other two mutants but its K_I value (= 11 μmol) was still more than 100 × larger than that of the wild type (K_I = 0.05 μmol). These results confirm that the mutated RCs confer terbutryne resistance to the bacterium. For o-phenanthroline the relative ordering of the mutants according to their resistance is different from that for terbutryne. RCs from the YG222 mutant showed the greatest resistance with a K_I = 540 μmol. RCs from both the SP223 and IM229 mutants are not as resistant to o-phenanthroline as they are to terbutryne (compare the relative values for K_I in Table 1), but they still show a significantly decreased binding of o-phenanthroline relative to UQ_0.

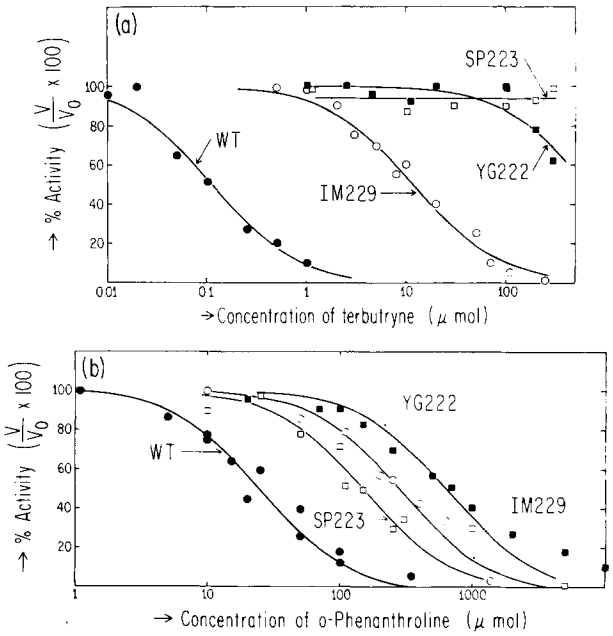


Fig. 5. Cytochrome turnover rate (% Activity = $(V/V_0) \times 100$) as a function of inhibitor concentration, terbutryne (a) and o-phenanthroline (b), for RCs from the three mutants (IM229, SP223, and YG222) and the wild type (WT) strains (R26 and 2.4.1). (Same conditions as described in Fig. 3). The relative resistance levels to herbicide inhibition are evident from these plots. All of the mutant RCs show an appreciable increase in resistance to inhibition by terbutryne and some resistance to inhibition by o-phenanthroline. Solid lines represent fits of the data to equation (2). Values of the inhibition constants, K_{inh}, determined from these plots and inhibitor binding constants, K_I (equation 3), are summarized in Table 1. Note the different scales in (a) and (b).

Summary and discussion

We have determined the changes in amino acid sequence of three herbicide resistant mutants of *Rb. sphaeroides* 2.4.1. The mutants were selected for increased resistance to the herbicide terbutryne. All mutants showed also increased resistance to o-phenanthroline. The mutated residues, Tyr^{222}, Ser^{223}, Ile^{229}, are located on the L subunit in the segment of amino acids between and including part of the D and E helices, which form the quinone binding pocket for Q_B as determined from the X-ray structure analysis of *Rb. sphaeroides* (Allen et al. 1987a, 1987b). An analogous pocket seen in the X-ray crystal structure of *R. viridis* (Michel et al. 1986a) (which lacks Q_B) serves as the binding site for the herbicide terbutryne and the inhibitor o-phenanthroline.

The reaction centers of the mutants were characterized with respect to electron-transfer rate, resistance to inhibition by terbutryne and o-phenanthroline and binding of quinone and inhibitors. A cytochrome photooxidation assay (Fig. 1) was used to determine the electron transfer rate (Fig. 3). Assuming that the mode of action of terbutryne and o-phenanthroline is to compete for the quinone binding site (Tischer and Strotman 1977, Pfister and Arntzen 1979, Velthuys 1981, Wraight 1981, Vermaas et al. 1983, Brown et al. 1984, Diner et al. 1984, Vermaas et al. 1984, Kyle 1985), we obtained from the reduction in electron transfer rate approximate values for the binding constants, K_Q, of quinone (equation 1) and inhibition constants, K_{inh}, for terbutryne and o-phenanthroline (equation 2). The approximate binding constants, K_I, of the inhibitors were obtained using equation (3) and the K_Q values. The characteristics of the mutants are summarized in Table 1.

All three mutants showed reduced binding of terbutryne and, except for IM229, a decrease in the maximum electron-transfer rate. The competitive inhibition assays were performed with the water soluble quinone UQ_0 that lack the isoprenoid chain. More detailed studies with the native quinone UQ_{10} will be published elsewhere.

The structure of the Q_B binding site, based on the X-ray diffraction analysis of the RCs from *Rb. sphaeroides* R26, is shown schematically in Fig. 6 (Allen et al., manuscript in preparation) together with the suggested positions of Q_B (a), terbutryne (b) and o-phenanthroline (c). At one end of the pocket (proximal to the Fe^{2+}) one of the keto groups of Q_B is H-bonded to His^{190}, the residue that form a ligand to Fe^{2+}. At the other end of the pocket (distal to the Fe^{2+}) the second keto group of Q_B interacts with Ser^{223} that has been mutated to Pro in the SP223 mutant. Another mutated residue, Ile^{229}, is in close contact with the quinone ring at the proximal side of the pocket. Other residues near Q_B include Leu^{193}, which makes contact with the quinone ring, Phe^{216}, which contacts the isoprenoid side chain, Glu^{212}, which is near the methoxy groups at the bottom of the binding pocket, and the carbonyl group of Ile^{224}, which contacts the quinone ring (not shown in Fig. 6 for simplicity of the figure). The remainder of the pocket is formed by the backbone atoms that connect these residues. The third mutated residue, Tyr^{222}, is located outside of the Q_B binding pocket and does not make direct contact with Q_B. However it is close to residues Ser^{223} and Ile^{224} which are involved in the binding of terbutryne (Michel et al. 1986a). The hydroxyl group of Tyr^{222} makes a H-bond with the backbone of Asn^{44} in the M subunit.

In *R. viridis* o-phennthroline binds close to the Fe^{2+} (localised mainly at the proximal side of the pocket), interacting exclusively with the L subunit

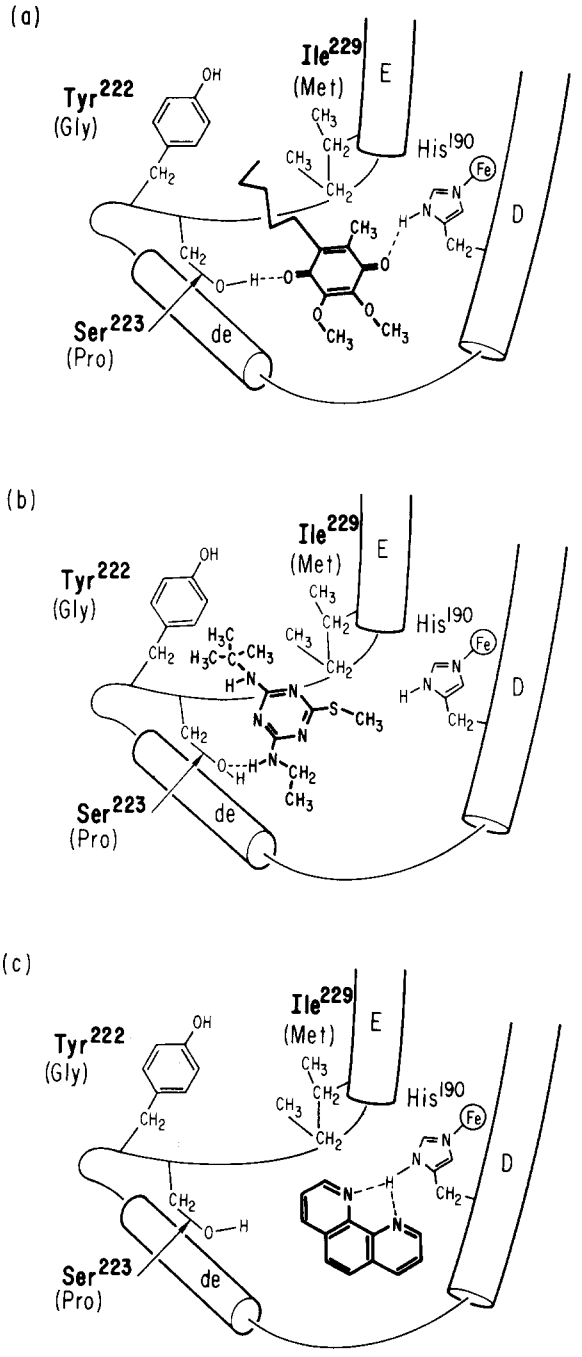
(Fig. 6c). Its two nitrogen atoms share an H-bond with the imidazole nitrogens of His^{190}. It is also in close contact with Ile^{229} and Leu^{193} (Michel et al. 1986a). Terbutryne binds further from the Fe^{2+} interacting mainly with residues at the distal side of the pocket making H-bonds with Ser^{223} and the peptide nitrogen of Ile^{224} (Fig. 6b). It makes close contacts with Val^{220}, Ile^{229}, Phe^{216} and Glu^{212} of the L subunit (Michel et al. 1986a).

The change of the quinone and herbicide binding in the RCs of the mutants can be qualitatively understood in terms of the structure of native RCs. The largest decrease in binding of UQ_0 was observed in the SP223 mutant ($Ser^{223} \rightarrow$ Pro). This is probably due to the loss of interaction between the quinone and the serine hydroxyl group (see Fig. 6a) as well as possible changes in conformation of the residues near the distal end of the pocket. The changes in UQ_0 binding was smaller in the IM229 ($Ile^{229} \rightarrow$ Met) and smallest in the YG222 mutant ($Tyr^{222} \rightarrow$ Gly) reflecting a decreased alteration of the quinone binding pocket for these mutations. The IM229 is likely to move Q_B farther away from His^{190}, decreasing the binding energy for quinone (smaller K_Q, see Table 1). The increase in distance between Q_B and His^{190}, a ligand to the Fe^{2+}, is consistent with the observation that RCs from this mutant have an altered EPR spectrum due to $Q_B^- Fe^{2+}$ and a decreased stabilization of the $Q_A Q_B^-$ state with respect to the $Q_A^- Q_B$ (Paddock et al., unpublished). The YG222 mutation may effect the quinone binding by altering the interactions with the nearby residue Ser^{223}.

The inhibition constants, K_{inh}, and inhibitor binding constants, K_I, for terbutryne binding of the SP223 and YG222 mutant RCs are large compared to those of the IM229 mutant RCs. This follows from the position of the terbutryne binding site near the distal end of the quinone binding pocket as has also been found in the structure of *R. viridis* (Michel et al. 1986a).

The K_{inh} and K_I values for o-phenanthroline binding are similar for all mutant RCs and are increased less compared to the wild type than those for terbutryne binding; this is not surprising since these mutants were selected for increased resistance to terbutryne. The changes in binding are probably due to alterations in the contacts between o-phenanthroline and the residues lining the binding pocket. It should be noted that the pattern of inhibition when UQ_0 is used as an acceptor is different compared to that observed when UQ_{10} is used (Paddock et al., manuscript in preparation). This indicates the importance of the isoprenoid tail in the quinone binding as has been shown earlier by McComb and Wraight (1983) and Warncke et al. (1987).

At present it is not possible to quantitatively account for the changes in binding affinities of the mutant RCs. This is because the overall three-dimensional effect of the mutations cannot be predicted. Even small changes in


(a)
Tyr222
(Gly)
Ile229
(Met)
E
His190
Fe
D
Ser223
(Pro)
de
(b)
Tyr222
(Gly)
Ile229
(Met)
E
His190
Fe
D
Ser223
(Pro)
de
(c)
Tyr222
(Gly)
Ile229
(Met)
E
His190
Fe
D
Ser223
(Pro)
de

structure can have dramatic effects on binding affinities as seen in the SP229 mutant ($Ile^{229} \rightarrow Met$) where an Ile residue is replaced by a slightly longer Met residue. In addition, in some instances even a qualitative understanding of the effects of the mutations is difficult at present. For instance it is difficult to explain why either the YG222 mutation ($Tyr^{222} \rightarrow Gly$) or the SP223 mutation ($Ser^{223} \rightarrow Pro$) has an effect on o-phenanthroline binding since o-phenanthroline is believed not to make direct contact with either Tyr^{222} or Ser^{223} (Michel et al. 1986a). Presumably these effects are due to small changes in the three-dimensional structure that propagate across distances of several angstroms. Detailed analysis of the three-dimensional structure of the mutant RCs may be required to explain the changes in binding affinities.

All three mutations described in this work involve residues that are conserved between *Rb. sphaeroides* (Williams et al. 1983, 1984, 1986), *Rb. capsulatus* (Youvan et al. 1984) and *R. viridis* (Michel et al. 1986b). This suggests the importance of these residues for optimal functioning of the RC and is consistent with the observed reduced binding constant for quinones when these residues are mutated. It is possible that more conserative mutations may produce herbicide resistance without decreased photosynthetic efficiency.

A topic of recent interest is the relationship between the RC from photosystem II (PSII) from higher organisms and bacteria (see for example Barber 1987, Evans 1987, Trebst 1987). Recent evidence indicates the proteins subunits D1 and D2 of PSII are analogous to the L and M subunits in bacterial RC. This analogy is partially based on the amino acid sequence homology between the L, M and D_1, D_2 subunits (Williams et al. 1984, Youvan et al. 1984, Michel et al. 1986b, Williams et al. 1986, Trebst 1986). Strong support for this proposal has come from the isolation of an active

Fig. 6. Schematic representation of the quinone (a) and inhibitor (b, c) binding sites. These sites are based on the X-ray diffraction analysis of the RCs from *Rb. sphaeroides* (Allen et al. 1987a) and *R. viridis* (Michel et al. 1986a). The sites are made up of a pocket that is lined with residues from part of the D and E helices of the L subunit and the loop joining the helices (in particular, residues His^{190}, Ile^{229}, Leu^{193}, Glu^{212}, Ser^{223}, Phe^{216}, and the backbone of Ile^{224}). Only residues which are in hydrogen bonding distance to the molecules, Ser^{223} and His^{190}, and the sites of the mutations, Ile^{229}, Ser^{223}, Tyr^{222} (in bold print with the mutation listed beneath in parentheses), are shown for simplicity. The suggested position of UQ_0, terbutryne and o-phenanthroline are shown in a, b and c, respectively. The putative hydrogen bonds are indicated by dashed lines. Those for UQ_0 are based on preliminary structural information (Allen et al., manuscript in preparation). Terbutryne binds more on the distal side (away from the Fe^{2+}) side of the quinone pocket, whereas o-phenanthroline binds more on the proximal side (closer to the Fe^{2+}). The view of the pocket in this sketch is normal to the two fold axis from the donor to the quinones rotated such that the Q_B is in the plane of the page.

PSII RC which contains only D1 and D2 polypeptides and 2 subunits of cyt b559 (Namba and Satoh 1987, Okamura et al. 1987). Mutants resistant to triazine herbicides (like terbutryne or atrazine) have been isolated from both bacteria and PSII containing organisms. The bacterial mutants contain an altered L subunit (Gilbert et al. 1985, Bylina and Youvan 1987; Paddock et al. 1987, Schenck et al. 1986, Sinning and Michel 1987), whereas the PSII mutants contain an altered D1 subunit (Hirschberg and McIntosh 1983, Erickson et al. 1984, Goloubinoff et al. 1984, Hirschberg et al. 1984, Erickson and Rochaix 1985, Erickson et al. 1985, Golden and Haselkorn 1985, Johanningmeier et al. 1987; for a review see Trebst 1987). The mutation sites of D1 are located in a region that is homologous to the location of the mutation sites of the bacterial L subunit, indicating that the structure of the Q_B and herbicide binding sites are similar in the PSII and bacterial RCs.

A detailed comparison of the mutated residues and the concomittant changes in function also show great similarities between PSII and bacterial RCs. A mutation commonly found in PSII RCs involves a change of Ser^{264} in D1 (Hirschberg and McIntosh 1983, Erickson et al. 1984, Goloubinoff et al. 1984, Hirschberg et al. 1984, Golden and Haselkorn 1985). This residue is homologous to the Ser^{223} of the L subunit in bacterial RCs (Williams et al. 1986). The mutation of Ser^{264} (to either Ala or Gly) in D1 result in a reduced binding constant of herbicides as well as altered electron transfer properties (Hirschberg and McIntosh 1983, Erickson et al. 1984, Hirschberg et al. 1984, Robinson et al. 1987). This is similar to the effects that we observed in the SP223 ($Ser^{223} \rightarrow$ Pro) mutant (see Table 1). Another mutation site, Leu^{275} of the D1 protein (Erickson and Rochaix 1985) is one helical turn away from Leu^{271}, the D1 equivalent (Williams et al. 1986) to Ile^{229} (the IM229 mutation site). These mutation sites may be homologous. No mutation of Tyr^{262}, corresponding to Tyr^{222} of L (Williams et al. 1986), has been found in the D1 protein although other mutations in the D1 protein occur in the vicinity of this residue. Some of these mutations (e.g., Ala^{251}, Val^{219}) involve residues that are not in direct contact with the quinone binding pocket similar to the bacterial YG222 mutant. The homology between the mutated residues in the L subunit and the D1 subunit provide further evidence of the structural and functional similarities between PSII and bacterial RCs (Brown et al. 1984, Williams et al. 1984, Michel et al. 1986, Trebst 1986). Thus, insights gained about the structure and function of bacterial RCs should in general be applicable to higher photosynthetic organisms.

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