Pathway of Proton Transfer in Bacterial Reaction Centers: Further Investigations on the Role of Ser-L223 Studied by Site-Directed Mutagenesis†

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ABSTRACT: The role of Ser-L223 in proton transfer to reduced Q_B in the reaction center (RC) from *Rhodobacter sphaeroides* was studied by site-directed replacement of Ser with residues having different proton donor properties, *e.g.*, the aliphatic residues Ala and Gly, the hydroxyl residue Thr, the amide residue Asn, the sulhydryl residue Cys, the imidazole residue His, and the carboxylic acid residue Asp. Compared to native reaction centers, RCs with Ala or Asn at L223 had greatly reduced (~300-fold) proton-coupled electron transfer rates, $k_{AB}^{(2)}$, associated with the second electron reduction of Q_B ($Q_A^-Q_B^- + H^+ \rightarrow Q_AQ_BH^-$). In contrast, RCs containing Thr, Asp, or Gly at L223 retained fast proton-coupled electron transfer rates. RCs with His or Cys at L223 did not bind the secondary quinone Q_B. These results show that $k_{AB}^{(2)}$ is larger when a good proton transfer group, *e.g.*, a hydroxyl residue (Ser, Thr) or a carboxylic acid (Asp), occupies the L223 site, supporting the proposal that Ser-L223 is a component of a proton transfer chain [Paddock, M. L., McPherson, P. H., Feher, G., & Okamura, M. Y. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87,* 6803−6807]. The surprising result that $k_{AB}^{(2)}$ is not significantly reduced in RCs with Gly at L223 suggests that a water molecule functionally replaces the missing Ser hydroxyl group in the mutant RCs. The importance of Ser-L223 in internal proton transfer reactions within the RC is discussed.

The photosynthetic bacterial reaction center (RC)[1] from *Rhodobacter sphaeroides* is a membrane-bound pigment−protein complex that performs the primary photochemistry by coupling proton transfer to light-induced electron transfer across the bacterial membrane. The bacterial RC is composed of three polypeptide subunits (L, M, and H), four bacteriochlorophylls, two bacteriopheophytins, one non-heme Fe^{2+}, and two ubiquinone (UQ_{10}) molecules [reviewed in Breton and Vermeglio (1988) and Feher *et al.*, (1989)]. In the RC, light-induced electron transfer proceeds from a primary donor (a bacteriochlorophyll dimer) through a series of acceptor molecules (a bacteriopheophytin and a quinone molecule, Q_A) to a loosely bound secondary quinone, Q_B, which serves as a mobile electron and proton carrier (Crofts & Wraight, 1983; Maroti & Wraight, 1990; Gunner, 1991). The reduction of Q_B to dihydroquinone, Q_BH_2, involves two electron transfer reactions. The second electron transfer is coupled with protonation as shown:

$$Q_A^-Q_B^- + H^+ \xrightarrow{k_{AB}^{(2)}} Q_AQ_BH^- \quad (1a)$$

$$Q_AQ_BH^- + H^+ \rightarrow Q_AQ_BH_2 \quad (1b)$$

After reduction to Q_BH_2, the dihydroquinone dissociates from the RC (McPherson *et al.*, 1990). The vacant Q_B site then becomes occupied by quinone from a membrane pool, resetting the photocycle for additional turnover (Figure 1). In photosynthetic membranes, reoxidation of QH_2 releases protons on the periplasmic side of the membrane, resulting in the formation of a proton gradient that drives ATP synthesis [reviewed by Dutton (1986) and Ort (1986)].

The Q_B binding site is in the interior of the protein, out of direct contact with the solvent (Deisenhofer *et al.*, 1985; Michel *et al.*, 1986; Allen *et al.*, 1987a,b, 1988; Deisenhofer & Michel, 1989; Arnoux *et al.*, 1990; Chang *et al.*, 1991; El-Kabbani *et al.*, 1991; Chirino *et al.*, 1994; Ermler *et al.*, 1994; Lancaster *et al.*, 1995). It has been established that specific amino acid residues of the L subunit are important for proton transfers necessary for the formation of the dihydroquinone. Based upon investigations of the kinetics of electron transfer and proton uptake from solution in native and site-directed mutant RCs, a separate proton transfer pathway has been proposed for each reaction (eq 1a,b). One pathway involves Ser-L223 and Asp-L213, both located in the vicinity of Q_B. The change of Ser-L223 with Ala resulted in significantly slower electron transfer associated with reaction 1a, $k_{AB}^{(2)}$ (Paddock *et al.*, 1990a; Leibl *et al.*, 1993; Bibikov *et al.*, 1994) and proton uptake (Paddock *et al.*, 1990a). Similar effects were observed when Asp-L213 was replaced with Asn (Paddock *et al.*, 1990a; Takahashi & Wraight, 1990, 1991, 1992; Rongey *et al.*, 1991). From these results, both Asp-L213 and Ser-L223 have been proposed to be components of a proton transfer chain linking the buried quinone Q_B to the exterior solution. This proton transfer is coupled to electron transfer (reaction 1a) as evidenced by the concomitant slowing of proton and electron transfer. More recent evidence from quinone substitution work on native RCs shows that, for reaction 1a, protonation precedes

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[1] Abbreviations: D, primary donor; Q_A, primary quinone acceptor; Q_B, secondary quinone acceptor; Q, quinone molecule; QH_2, dihydroquinone; UQ_{10}, ubiquinone-50; RC, reaction center; cyt c, horse heart cytochrome *c*; bp, base pair; HME, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.5, 0.04% maltoside, and 0.1 mM ethylenediaminetetraacetic acid (EDTA); Caps, 3-(cyclohexylamino)-1-propanesulfonic acid; Ches, 2-(cyclohexylamino)ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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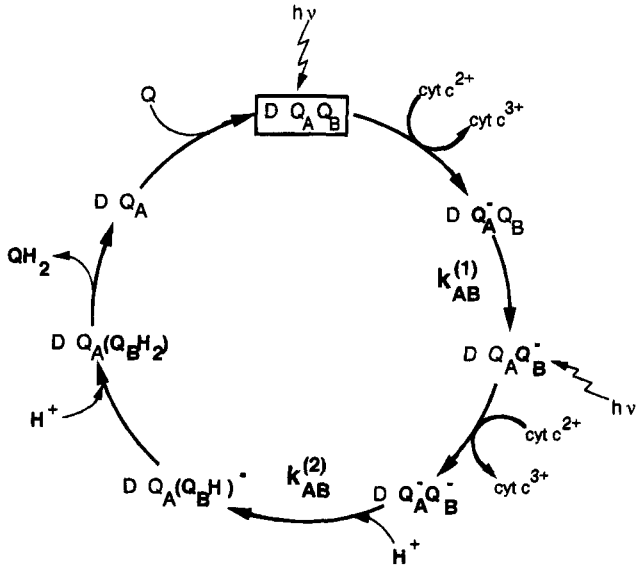

FIGURE 1: Photochemical cycle of reaction centers showing electron transfer, proton uptake, quinone exchange, and cytochrome oxidation. The first and second electron transfer rate constants are indicated as $k_{AB}^{(1)}$ and $k_{AB}^{(2)}$, respectively. The transfer of the second electron to Q_B involves the uptake of the first proton, $H^+(1)$ (Paddock *et al.*, 1990a). The transfer of a second proton, $H^+(2)$, to reduced Q_B is not a prerequisite for the second electron transfer (Paddock *et al.*, 1989). The cycling rate for native RCs is $\sim 10^3\ s^{-1}$.

electron transfer, with electron transfer being rate-limiting (Graige *et al.*, 1995).

The other proton pathway associated with reaction 1b involves Glu-L212, which is also in the vicinity of Q_B (Paddock *et al.*, 1989; Takahashi & Wraight, 1991). The change of Glu-L212 to Gln resulted in relatively little change in the measured electron transfer rates of the first and second electron (Paddock *et al.*, 1989; Takahashi & Wraight, 1991) but in a significantly slower proton uptake following the two-electron reduction of Q_B (reaction 1b) (McPherson *et al.*, 1990). Normal proton rates were observed in RCs with Asp replacing Glu-L212 (Paddock *et al.*, 1989, 1990b). From these results Glu-L212 was proposed to be a component of a separate proton transfer chain leading from solution to the quinone molecule.

In the present work we further investigated the role of Ser-L223 in proton transfers associated with the double reduction of Q_B by varying the proton donor capabilities of the side chain at L223 as well as the length of its side chain. Modified RCs were constructed using site-directed mutagenesis in which Ser-L223 was replaced by a variety of residues with differing proton donor properties—ranging from Thr, Asp, and His (good proton transfer residues) to Ala, Asn, Cys, and Gly (poor proton transfer residues). In all mutants, the pH dependences of $k_{AB}^{(1)}$ and $k_{AB}^{(2)}$ (reaction 1a) were measured. Charge recombination rates were measured as a function of pH for native and mutant RCs to determine the effect of mutation on Q_B^- stability. The results are discussed in terms of the role of Ser-L223 as a component of a proton transfer chain connecting Q_B to the solvent.

MATERIALS AND METHODS

Materials. Dodecyl β-D-maltoside was obtained from Calbiochem or Anatrace and *N,N*-dimethyldodecylamine-*N*-oxide (LDAO) from Fluka Chemie. Ubiquinone-50 obtained from Sigma was solubilized at 50 °C in either 10% deoxycholic acid (at ~1 mM quinone) or 1% LDAO (at ~0.2 mM quinone) and stored at −70 °C. The inhibitors terbutryn, 1,10-phenanthroline, and stigmatellin were from Chem Services, Fisher Scientific, and Fluka, respectively. Restriction enzymes and other DNA-modifying enzymes used in the site-directed mutagenesis procedures were obtained as described in Paddock *et al.* (1989). The site-directed mutagenesis kit was obtained from Amersham. The BBL GasPak 100 and 150 jar systems were obtained from Fisher Scientific. All other chemicals were of reagent or HPLC grade.

Site-Directed Mutagenesis. The construction of the site-directed mutants was performed as described by Paddock *et al.* (1990a) with a few modifications as noted. The construction of the Ser-L223 → Ala and Ser-L223 → Thr mutants [SA(L223) and ST(L223), respectively] was previously described with preliminary characterization of the mutant RCs (Paddock *et al.*, 1990a). Five new mutations were constructed in this work and are reported below. The mutagenesis was performed using the Amersham oligonucleotide-directed mutagenesis kit based upon the method developed by Nakamaye and Eckstein (1986). Oligonucleotides were synthesized to replace the native TCG codon for Ser-L223 using the following set of oligos: 5′-GTCG-GCTAC **XXX** ATCGGGACGCT-3′ with the **XXX** being (1) **GAC** Asp codon for the Ser-L223 → Asp mutation [SD(L223)], (2) **AAC** Asn codon for the Ser-L223 → Asn mutation [SN(L223)], (3) **GGC** Gly codon for the Ser-L223 → Gly mutation [SG(L223)], (4) **TGC** Cys codon for the Ser-L223 → Cys mutation [SC(L223)], and (5) **CAC** His codon for the Ser-L223 → His mutation [SH(L223)].

The mutations were incorporated into an M13 vehicle containing the *Pvu*II−*Sal*I fragment (Paddock *et al.*, 1990) [for the SD(L223) and SN(L223) mutations] or the *Asp*718−*Hin*dIII fragment (Lin *et al.*, 1944) [for the SG(L223), SC(L223), and SH(L223) mutations] which contains the DNA coding for the latter two-thirds of the L subunit; a *Hin*dIII site was added to the end of *pufL*. Mutants were identified by single-lane sequencing of phage DNA from several dozen plaques. A uniform phage culture for each replacement was obtained by reinfection into *Escherichia coli* followed by isolation of an individual plaque. The sequences of the entire insert region were determined for these uniform phage cultures. No changes other than the desired mutations were noted in the *Asp*718−*Sal*I or the *Asp*718−*Hin*dIII region. This fragment was then exchanged with the kanamycin fragment of pRKENKm (Paddock *et al.*, 1989) or pRKSCHKm (Lin *et al.*, 1994) which has the added *Hin*dIII site at the end of *pufL*. These were transformed into *E. coli* S17-1 (Simon *et al.*, 1983) and mated into the *Rb. sphaeroides* deletion strain ΔLM1, resulting in the complemented deletion strains. These strains were grown semi-aerobically to induce RC production without applying selection for photosynthetic growth as described by Paddock *et al.*, (1989).

Photosynthetic Growth. The growth of the complemented deletion strains carrying the native or site-directed modified RC genes were tested for their ability to grow under anaerobic photosynthetic conditions as described by Rongey *et al.* (1993). Briefly, the deletion strain complemented with

native or mutant genes was restricted to anaerobic photosynthetic conditions using the BBL GasPak 100 or 150 jar systems. Control plates grown aerobically were used to determine the original viable cell density. Between $\sim 10^5$ and 10^7 viable bacteria were spread evenly onto each plate before the RCs were subjected to either aerobic or anaerobic photosynthetic conditions.

Reaction Center Preparation. RCs were isolated in LDAO as described (Paddock *et al.*, 1988); they contained approximately 1 quinone/RC as determined by standard methods (Kleinfeld *et al.*, 1984) and had an observed ratio of $A_{280}^{1cm}/A_{802}^{1cm} \leq 1.3$. The RC concentration was determined (McPherson *et al.*, 1994) by the amount of cyt c oxidized (measured at 550 nm) after one saturating laser flash using the extinction coefficients $\epsilon_{cytc^{2+}}^{550} - \epsilon_{cyt^{3+}}^{550} = 21.1$ mM^{-1} cm^{-1} (Van Gelder & Slater, 1962). Reconstitution of Q_B into the RCs was accomplished as follows: a 5−10-fold excess of UQ_{10} was added to the RC solution which was then dialyzed for 2 days against 10 mM Hepes, pH 7.5, 0.04% dodecyl β-D-maltoside, and 0.1 mM EDTA.

Electron Transfer Rate Measurements. The kinetics of electron transfer were determined from absorption changes recorded on a modified Cary 14 spectrophotometer (Varian) as described by Kleinfeld *et al.* (1984). Voltage output from the amplifier was recorded on a digital oscilloscope (LeCroy 9310M) and transfered to a personal computer for storage and analysis. Kinetic data traces were fit to exponential functions on a personal computer using nonlinear curve-fitting software (Peakfit or Sigmaplot, Jandel). Actinic illumination was provided by a pulsed dye laser (Phase R DL2100c; 590 nm, ~0.2 J/pulse, 0.5-μs pulse width). All measurements were performed with 1−3 μM RCs in 10 mM buffer(s), 0.04% dodecyl β-D-maltoside, and 0.1 mM EDTA at 23 °C. The rate constant for the transfer of the first electron to Q_B, $k_{AB}^{(1)}$, was measured by monitoring the bacteriopheophytin band shift at 750 nm, which is sensitive to the reduction state of the quinones Q_A and Q_B (Vermeglio & Clayton, 1977; Kleinfeld *et al.*, 1984). To obtain better signal-to-noise ratios, traces (between 9 and 36) were averaged on the LeCroy oscilloscope prior to transfer to the personal computer. The measured rate constant for the double reduction of Q_B, $k_{AB}^{(2)}$ (eq 1a), was determined by measuring the decay after a second laser flash of the semiquinone absorption at 450 nm (Kleinfeld *et al.*, 1985) in the presence of 20−50 μM cytochrome *c* (cyt c) or 20−200 μM ferrocene, which reduces the donor after each flash.

The charge recombination rate $D^+Q_A^- \rightarrow DQ_A$ (k_{AD}) was determined from the rate of recovery of the oxidized donor monitored at 865 nm following a saturating laser flash in RCs containing only Q_A. The charge recombination rate $D^+Q_AQ_B^- \rightarrow DQ_AQ_B$ (k_{BD}) was determined from the kinetics of the slow phase of the donor recovery (~10−90% of the amplitude) in RC samples in the presence of excess UQ_{10}; any remaining fast phase was attributed to RCs lacking active Q_B and undergoing $D^+Q_A^- \rightarrow DQ_A$ with a rate constant k_{AD}. The pH of the solution was adjusted by adding acid (1 N HCl) or base (1 N NaOH) to a mixture of buffers containing Caps, Ches, Mes, Pipes, and Tris at 2.5 mM each.

Determination of Q_B^- Stability. The stability of the Q_B^- state depends on its energy with respect to the Q_A^- state. The equilibrium between the states $Q_AQ_B^-$ and $Q_A^-Q_B$ can be obtained from the measured charge recombination rate constants k_{AD} and k_{BD}:

$$D^+Q_AQ_B^- \underset{k_{BA}^{(1)}}{\overset{k_{AB}^{(1)}}{\rightleftharpoons}} D^+Q_A^-Q_B \xrightarrow{k_{AD}} DQ_AQ_B \qquad (2)$$

If the electron transfer rates between the quinones is fast compared to the recombination rates [*i.e.*, $k_{AB}^{(1)}$, $k_{BA}^{(1)} \gg k_{AD}$), an equilibrium will be established between the $D^+Q_A^-Q_B$ and $D^+Q_AQ_B^-$ states, from which the energy difference (ΔG^0) between these two states can be determined. The mechanism of charge recombination $D^+Q_AQ_B^- \rightarrow DQ_AQ_B$ has contributions both from direct recombination back to the ground state and from indirect recombination via thermal population of the $D^+Q_A^-Q_B$ state. The indirect mechanism was shown to be the dominant mechanism for k_{BD} in native RCs (Kleinfeld *et al.*, 1984). Consequently, the charge recombination rate constant k_{BD} is related to k_{AD} and the fraction of RCs in the $D^+Q_A^-Q_B$ state, α (Kleinfeld *et al.*, 1984), *i.e.*

$$k_{BD} = \alpha\, k_{AD} \qquad (3)$$

and the equilibrium constant K between the $D^+Q_AQ_B^-$ and $D^+Q_A^-Q_B$ states is given by

$$K = \frac{[D^+Q_AQ_B^-]}{[D^+Q_A^-Q_B]} = \frac{1-\alpha}{\alpha} = \exp(-\Delta G^0/kT) \qquad (4)$$

where k is Boltzmann's constant and T is the absolute temperature. Combining eqs 3 and 4, we find

$$\Delta G^0 = -kT \ln\left(\frac{1-\alpha}{\alpha}\right) = -kT \ln\left(\frac{k_{AD} - k_{BD}}{k_{BD}}\right) \qquad (5)$$

Assuming that the mechanism of charge recombination remains the same in the mutant RCs, the free energy difference ΔG^0 in the mutant RCs can be obtained from the measured values of k_{BD} and k_{AD}.

RESULTS AND ANALYSIS

Photosynthetic Growth of the Ser-L223 Mutants. The complemented deletion strains carrying the replacements of Ser-L223 with Ala, Thr, Asp, Asn, Gly, His, and Cys were tested for their ability to grow under anaerobic photosynthetic conditions. The number of colonies present on a plate grown under photosynthetic conditions was compared to the number on an identical plate grown under nonselective aerobic conditions. Total number of bacteria used was between $\sim 10^5$ and $\sim 10^7$ spread onto each plate. The complemented deletion strain carrying the native *puf* operon, which includes the genes coding for the native L and M subunits, showed a comparable number of colonies on duplicate plates grown under aerobic and anaerobic (photosynthetic) conditions. The complemented deletion strains carrying the ST(L223), SD(L223), or SG(L223) mutations also showed a similar number of viable colonies when grown under either aerobic or anaerobic conditions (Table 1). These results are in accord with those observed for mutant strains of *Rhodobacter capsulatus* (Bylina & Wong, 1992). The complemented

Table 1: Characteristics of Ser-L223 Mutants (pH 7.5)[a]

RC type	L223 residue	$k_{AB}^{(1)}$ (s^{-1})	$k_{AB}^{(2)}$ (s^{-1})	k_{AD} (s^{-1})	k_{BD} (s^{-1})	ΔG[b] (meV)	photosynthetic growth
native	Ser	7000	1300	9.5	0.8	−60	yes
SA(L223)	Ala[c]	15000	4	9.9	0.8	−60	no
SN(L223)	Asn	>300	~10[d]	11	6	0	no
ST(L223)	Thr	1000	9500[d]	11	5	0	yes
SD(L223)	Asp	≥500	5200[d]	11	8	+25	yes
SG(L223)	Gly	5000	650	9.3	2.5	−25	yes
SH(L223)	His	[e]	[e]	9.2	[e]	[e]	no
SC(L223)	Cys	[e]	[e]	9.5	[e]	[e]	no

[a] Errors in the rates are estimated to be ~5% for k_{AD}, ~10% for k_{BD}, and ~25% for the forward electron transfer rates. The estimated errors are larger for the ST(L223), SN(L223), and SD(L223) RCs due to lower Q_B activity (~20% for k_{BD} and ~50% for the forward electron transfer rates). [b] ΔG is the free energy difference between the $DQ_AQ_B^-$ and $DQ_A^-Q_B$ states determined from the charge recombination rate constants k_{AD} and k_{BD}. [c] The rates for the SA(L223) RCs have been previously reported (Paddock *et al.* (1990a). [d] Q_B activity in these RCs was increased by reconstitution with quinones suspended in deoxycholic acid. Native RCs in which Q_B was reconstituted by the same procedure yield $k_{AB}^{(2)}$ rates which are ~3-fold faster than reported in the table. [e] No data due to low Q_B occupancy in these mutant RCs.

deletion strains carrying the SA(L223), SN(L223), SH(L223), and SC(L223) mutations showed only a small fraction ($<10^{-5}$) of colonies on the anaerobic plate compared to the aerobic plate. The viable photosynthetic colonies observed on the anaerobic plate are attributed to revertants containing suppressor mutations that restore photosynthetic function to the RCs. Similarly, the SA(L223) replacement in *Rb. capsulatus* was found to be photosynthetically nonviable (Bylina *et al.*, 1989; Bylina & Wong, 1992).

Forward Electron Transfer Rates. Previous studies on SA(L223) RCs showed that the proton-coupled electron transfer rate constant $k_{AB}^{(2)}$ (eq 1a) is drastically reduced compared to native RCs in either *Rb. sphaeroides* (Paddock *et al.*, 1990a) or *Rhodopseudomonas viridis* (Leibl *et al.*, 1993). To test the effects of the other mutations, the first and second electron transfer rates were measured by monitoring transient optical absorption changes in native and mutant RCs.

First Electron Transfer $k_{AB}^{(1)}$: $Q_A^-Q_B \rightarrow Q_AQ_B^-$. The electron transfer rate constant $k_{AB}^{(1)}$ was measured in native and mutant RCs (Table 1) by monitoring the absorbance at 750 nm, which is sensitive to the reduction state of the quinones (Vermeglio & Clayton, 1977; Kleinfeld *et al.*, 1984). The mutant RCs displayed $k_{AB}^{(1)}$ values that are slightly different from native RCs at pH 7.5 (Table 1). The $k_{AB}^{(1)}$ value for the SA(L223) RCs is a factor of 2 larger than in the native RC, as previously reported for *Rb. sphaeroides* (Paddock *et al.*, 1990a) and *Rps. viridis* (Leibl *et al.*, 1993). The $k_{AB}^{(1)}$ value for ST(L223) RCs is 7-fold smaller (Table 1). The error in this value is relatively large (±50%) due to the low level of Q_B activity of the samples. $k_{AB}^{(1)}$ values for SN(L223) and SD(L223) RCs could not be accurately measured, but they remained relatively large. The electron transfer rates could not be measured for the SH(L223) and SC(L223) RCs because of low Q_B activity, presumably due to poor Q_B binding in these two mutant RCs.

At pH > 9, $k_{AB}^{(1)}$ for the SA(L223) and SG(L223) mutant RCs is essentially the same (within 20%) as for native RCs (Figure 2). This observation suggests that the rate-limiting step for $k_{AB}^{(1)}$ may be different at higher pH (>9) compared to lower pH (<8). Furthermore, this suggests that the observed rate constant $k_{AB}^{(1)}$ represents a multistep process. This has been previously proposed on the basis of complicated, wavelength-dependent, kinetic behavior of $k_{AB}^{(1)}$ (Paddock *et al.*, 1994); the decay rate attributed to $k_{AB}^{(1)}$ was found to depend on the monitoring wavelength varying by

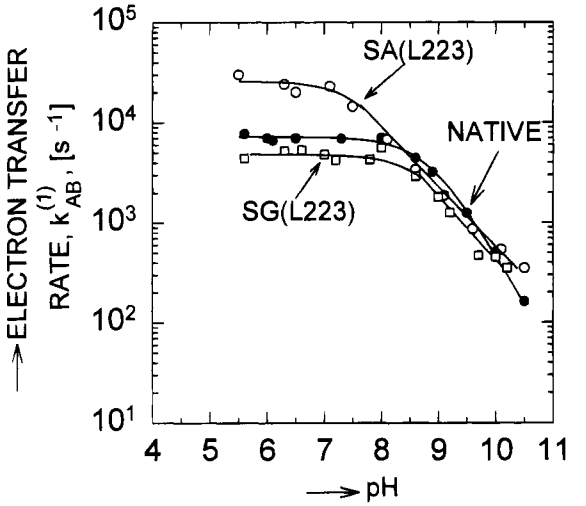

FIGURE 2: Forward electron transfer rate constant $k_{AB}^{(1)}$ (see Figure 1) as a function of pH for native, SA(L223) [Ser-L223 → Ala], and SG(L223) [Ser-L223 → Gly] RCs. The electron transfer rates were determined from the observed kinetics at 750 nm, a bacteriopheophytin absorption which is shifted upon quinone reduction (Vermeglio & Clayton, 1977; Kleinfeld *et al.*, 1984). The forward rate constant was estimated from the observed rate constant under a given set of assumptions [see, *e.g.*, Kleinfeld *et al.* (1984)] and was, within 30% (at pH 7.5), the same as the observed rate shown above. Conditions: ~3 μM RCs, 2.5 mM Caps, 2.5 mM Ches, 2.5 mM Mes, 2.5 mM Pipes, 2.5 mM Tris, 0.04% dodecyl β-D-maltoside, and 0.1 mM EDTA, 23 °C. The pH was adjusted by adding acid (1 N HCl) or base (1 N NaOH) to the sample mixture. Note the the pH profiles for the mutant RCs resemble that for native RCs except that the rate at lower pH is slightly faster (~3-fold) in the SA(L223) RCs.

a factor of 3 (at 4 °C) over the range 410 nm ≤ λ ≤ 500 nm. Although the interpretation of the kinetics remains unclear, the observed rate constant $k_{AB}^{(1)}$ increases with increasing electron driving force ΔG at pH 7.5 (see Table 1); *e.g.*, it is smaller in ST(L223) RCs and larger in SA-(L223) RCs. This functional dependence of the rate on driving force is consistent with the Marcus theory for electron transfer when ΔG is less than the reorganization energy λ (Marcus & Sutin, 1985).

Second Electron Transfer $k_{AB}^{(2)}$: $Q_A^-Q_B^- + H^+ \rightarrow Q_AQ_BH^-$. The rate constant $k_{AB}^{(2)}$ was measured by monitoring the decay of the semiquinone absorbance at 450 nm after two laser flashes in the presence of exogenous donors (Kleinfeld *et al.*, 1985). The rate constant $k_{AB}^{(2)}$ was reduced ~200−300 fold in the SA(L223) and SN(L223) RCs

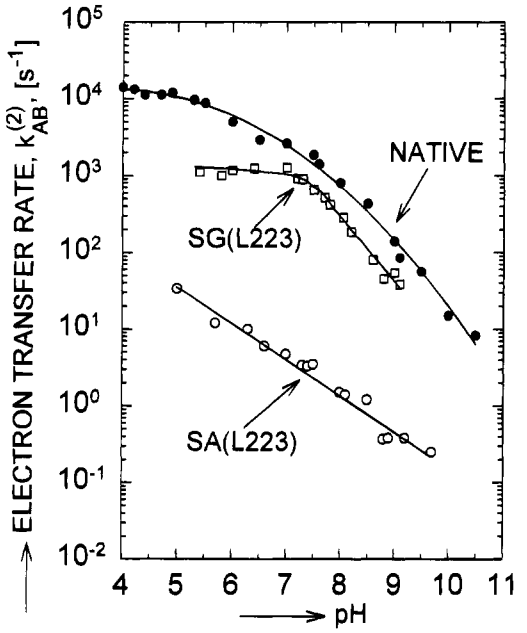

FIGURE 3: pH dependence of the proton-coupled electron transfer rate constant $k_{AB}^{(2)}$ determined by monitoring the decay of the semiquinone absorbance at 450 nm as a function of time for native, SA(L223), and SG(L223) RCs. Conditions: ~1−3 μM RCs; 20−500 μM ferrocene or 20 μM cytochrome *c*; 2.5 mM each Hepes, Caps, Ches, Mes, Pipes, and Tris; 0.04% dodecyl β-D-maltoside; and 0.1 mM EDTA, 23 °C. The pH was adjusted by adding HCl or NaOH. Note the drastic decrease (~10^2−10^3) in $k_{AB}^{(2)}$ observed for the SA(L223) RCs over the entire pH range compared to native RCs. In contrast, the SG(L223) RCs display rates that are similar to those in native RCs.

compared to native RCs (pH 7.5) (Table 1). In contrast, ST(L223), SD(L223), and SG(L223) display near native values for $k_{AB}^{(2)}$; the faster rates measured for the ST(L223) and SD(L223) are at least partially due to the method for Q_B reconsitution (see footnote to Table 1). The rate could not be measured in the SH(L223) and SC(L223) RCs, since Q_B activity could not be restored to the RCs.

At higher pH these observations are qualitatively the same, although some quantitative differences were observed (see Figure 3). Native RCs show a rate constant that is approximately proportional to $[H^+]^{0.4}$ at pH $\ll$ 8 and to $[H^+]^{0.9}$ at pH $\gg$ 8. In the SG(L223) RCs the observed rate constant $k_{AB}^{(2)}$ was found to be approximately pH-independent below pH ~ 8 and proportional to $[H^+]^{0.9}$ above pH ~ 9. In the SA(L223) RCs, the observed rate constant $k_{AB}^{(2)}$ was found to be reduced ~200−300 fold at all pH and was proportional to $[H^+]^{0.5}$ for 5 $\leq$ pH $\leq$ 9.5.

Charge Recombination Rates. The charge recombination rates contain important information about the environment around the reduced quinone molecules. The charge recombination rates $D^+Q_A^- \rightarrow DQ_A$ (k_{AD}) and $D^+Q_AQ_B^- \rightarrow DQ_AQ_B$ (k_{BD}) were measured by monitoring the change in absorption of the primary electron donor at 865 nm (Table 1).

Charge Recombination k_{AD}: $D^+Q_A^- \rightarrow DQ_A$. The recombination rate constant, k_{AD}, was measured in RCs containing only one quinone (Q_A) and was found to be essentially the same in native and in all of the mutant RCs (k_{AD} = 10 ± 1 s^{-1}).

Charge Recombination k_{BD}: $D^+Q_AQ_B^- \rightarrow DQ_AQ_B$. The recombination rate constant, k_{BD}, was measured in the presence of excess UQ_{10} [5−10 UQ_{10}/RC]. It was determined from the kinetics of the slow phase at 865 nm. The slow phase was observed in only five of the mutant RCs; it was not observed in the SH(L2232) and SC(L223) RCs due to a small fraction of RCs with active Q_B. The slow phase was absent in the presence of 1 mM 1,10-phenanthroline. In contrast, the slow phase was still present upon addition of 100 μM terbutryn for the SA(L223), ST(L223), SN(L223), and SD(L223) RCs; similar levels of terbutryn inhibited Q_B activity in native RCs and had some inhibitory effect in SG(L223) RCs. The photosystem II inhibitor stigmatellin was tested in native and SA(L223) RCs. It was a very potent inhibitor of Q_B activity in native RCs—a stoichiometric amount completely inhibited Q_B activity—whereas SA(L223) RCs were found to be resistant to stigmatellin.

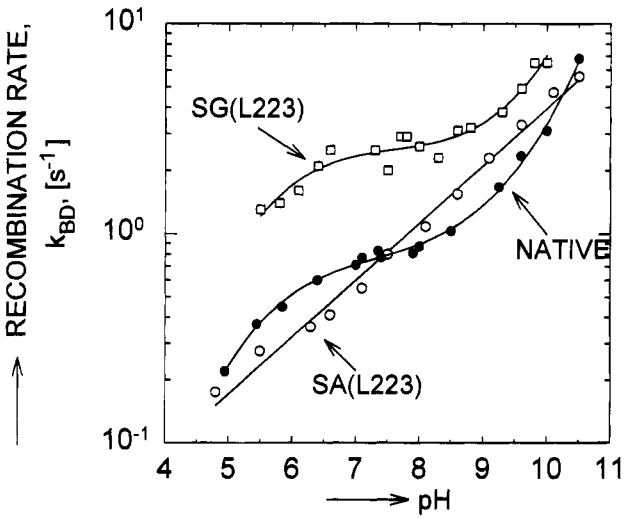

FIGURE 4: pH dependence of the charge recombination $D^+Q_AQ_B^- \rightarrow DQ_AQ_B$, k_{BD}, monitored *via* the absorbance of the donor at 865 nm as a function of time for native, SA(L223), and SG(L223) RCs. Conditions: ~1−3 μM RCs; 2.5 mM each Hepes, Caps, Ches, Mes, Pipes, and Tris; 0.04% dodecyl β-D-maltoside; and 0.1 mM EDTA, 23 °C. The pH was adjusted by adding HCl or NaOH. The SA(L223) RCs display similar values (within ~30%) for k_{BD} as native RCs at all pH. In contrast, the SG(L223) RCs display increased values (~3-fold) compared to native RCs over the entire pH range. This shows that Q_B^- is less stable in the SG(L223) mutant RCs. Destabilization is attributed to the loss of a hydrogen bond from Ser hydroxyl group to the keto oxygen of Q_B (Figure 5). The convergence of the rate constants at high pH is expected since k_{BD} can never exceed k_{AD} (~10 s^{-1}) (Kleinfeld *et al.*, 1984) unless the mechanism of charge recombination is changed. The increase at high pH (≥8) was shown to be related to the titration of Glu-L212 in native RCs (Paddock *et al.*, 1989). Note that the pH at which k_{BD} starts to increase is similar in the SG(L223) and native RCs and may be only slightly shifted in the SA(L223) RCs (see text for discussion). This suggests no major changes in the interactions between Q_B^- and nearby titrating sites.

The k_{BD} value measured for SA(L223) RCs (pH 7.5) was essentially the same as for native RCs (Figure 4). The SG(L223) RCs displayed slightly faster kinetics, suggesting that Q_B^- is less stable in the mutant RCs. Similarly, ST(L223), SD(L223), and SN(L223) RCs displayed larger k_{BD} values ranging from 5 to 8 s^{-1} (Table 1). The accuracy of these numbers is lower than those in the other mutant RCs where there is a larger difference between k_{BD} and k_{AD}. These rate constants are attributed to $D^+Q_AQ_B^- \rightarrow DQ_AQ_B$ and could be better resolved at lower pH (*e.g.*, pH 5), where k_{BD} becomes much smaller than k_{AD} (data not shown). Using eq 5 and the measured charge recombination rate constants k_{AD} and k_{BD}, the energy of the $D^+Q_AQ_B^-$ state can be

estimated. The $D^+Q_AQ_B^-$ state is destabilized compared to native RCs by ~35 meV in SG(L223) RCs, ~85 meV in SD(L223) RCs, and ~60 meV in SN(L223) and ST(L223) RCs (see Table 1). These results indicate a decrease in the equilibrium constant K, $[Q_AQ_B^-]/[Q_A^-Q_B]$ (see eq 4), by a factor of ~4 in the SG(L223), ~40 in the SD(L223), and ~10 in the SN(L223) and ST(L223) RCs compared to native RCs.

The pH dependence of k_{BD} for the native and the SA(L223) and SG(L223) mutant RCs is shown in Figure 4. Native RCs show two pH-dependent regions (titrations): one at pH > 9 and the other at pH < 7. The pH profile found for k_{BD} in SA(L223) RCs appears to increase monotonically with pH from 5 to 10.5. This can be explained by a convergence of the pH titrations, *i.e.*, a 0.75 pK_a unit increase of the lower pH titration and a 0.75 pK_a decrease of the higher pH titration. The pH profile for k_{BD} measured in the SG(L223) RCs shows titrations below pH 7 and above pH 9, nearly identical to those seen in native RCs. The similarity of the pH titrations suggests that the mutations had little effect on the interaction between the quinone and nearby titrating sites. For the SN(L223) and SD(L223) RCs, k_{BD} was measured only over a limited pH range of $7 \leq pH \leq 8$ and was found to be relatively independent of pH.

DISCUSSION

In this study we extended our investigation of the role of Ser-L223 in proton transfer to reduced Q_B. The approach used was to vary the proton donor properties (*e.g.*, size, pK_a) of the L223 residue by site-specific replacement and determine the effect(s) of these replacements on the electron and proton transfer reactions. The results are discussed in the context of the importance of Ser-L223 in proton transfer reactions associated with eq 1a.

Importance of Ser-L223 in Proton Transfer Reactions. The proton-coupled electron transfer rate constant $k_{AB}^{(2)}$ ($DQ_A^-Q_B^- + H^+ \rightarrow DQ_AQ_BH^-$) was reduced ~300-fold compared to native RCs when Ser-L223 was replaced with either Ala or Asn. As a consequence, the complemented deletion strain carrying either of these replacements did not grow photosynthetically. In contrast, $k_{AB}^{(2)}$ remained relatively unaffected when Ser-L223 was replaced with Thr or Asp. Thus, the complemented deletion strain carrying either of these replacements did grow photosynthetically. These kinetic (and growth) results show a correlation between fast proton-coupled electron transfer (and photosynthetic growth) and the presence of a side chain that can participate in proton transfer reactions *via* a hydrogen-bonded chain (see later discussion). There is no apparent correlation between the observed proton-coupled electron transfer rate constant $k_{AB}^{(2)}$ and either the length of the side chain or its pK_a. Thus, for the native, ST(L223), and SD(L223) RCs, proton transfer is not the rate-limiting step in $k_{AB}^{(2)}$. This suggestion is supported by the recent finding in native RCs that reaction 1a proceeds in two steps with proton transfer being faster and preceding electron transfer (Graige *et al.*, 1995). Hence $k_{AB}^{(2)}$ in native RCs is a measure of the *electron* rather than proton transfer. We propose that electron transfer is the rate-limiting step in $k_{AB}^{(2)}$ in the ST(L223) and SD(L223) RCs like in native RCs. This explains the lack of correlation between the proton donor properties of the L223 residue and $k_{AB}^{(2)}$ in these two mutants.

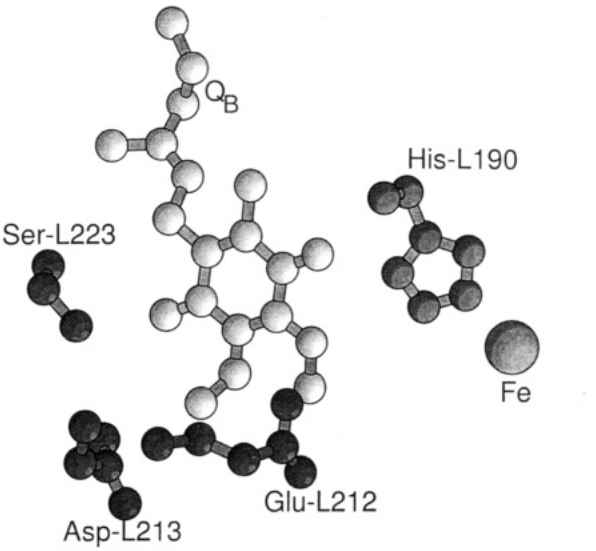

FIGURE 5: Partial RC structure near the secondary quinone, Q_B, binding site as determined by Allen *et al.* (1988) (Brookhaven Protein Data Bank entry 4RCR). Q_B forms two hydrogen bonds, one to Ser-L223 and one to His-L190. Nearby are two carboxylic acid groups, Asp-L213 and Glu-L212, that have been implicated in proton transfer to reduced Q_B (reactions 1a and 1b, respectively) (Paddock *et al.*, 1989, 1990a; Takahashi & Wraight, 1990, 1991, 1992; Rongey *et al.*, 1991). This structure suggests a possible proton transfer chain from Asp-L213 to Ser-L223 to reduced Q_B (see text for discussion). Asp-L213 may acquire its proton from several possible candidates, including Asp-L210, Glu-H173, and/or bound water molecules (not shown).

Slow proton-coupled electron transfer upon replacement of Ser-L223 with Ala and evidence suggesting that the structure was not significantly altered by the replacement was previously used as evidence that Ser-L223 constitutes a component of a proton transfer chain in *Rb. sphaeroides* (Paddock *et al.*, 1990a; Bibikov *et al.*, 1994) and in *Rps. viridis* (Leibl *et al.*, 1993). Structural changes in the *Rb. sphaeroides* SA(L223) RCs are unlikely since the charge recombination rate constants k_{AD} and k_{BD}, the forward electron transfer rate constant $k_{AB}^{(1)}$, and the electron paramagnetic resonance (EPR) spectrum of Q_B^- were all essentially the same in the mutant as in native RCs (Paddock *et al.*, 1990a). Similarly, on the basis of a comparison of the native and mutant crystal structures (Sinning *et al.*, 1990), structural changes in the *Rps. viridis* SA(L223) mutant RCs were excluded. The kinetic measurements reported in this paper thereby strengthen the previous proposal that Ser-L223 is involved in proton transfer to reduced Q_B, most likely as a component of a proton transfer chain.

Structure of the Q_B Binding Site. A full understanding of the function of Ser-L223 in proton transfer requires structural information. Of particular importance is the distance from Ser-L223 to reduced Q_B and other potential components of proton transfer chain(s) (*e.g.*, Asp-L213). The best structural information that is currently available comes from the analysis of the crystal structure of the RC from *Rb. sphaeroides* (Allen *et al.*, 1987a,b, 1988; Arnoux *et al.*, 1990; Chang *et al.*, 1990; El-Kabbani *et al.*, 1991; Chirino *et al.*, 1994; Ermler *et al.*, 1994) and *Rps. viridis* (Deisenhofer *et al.*, 1985; Michel *et al.*, 1986; Lancaster *et al.*, 1995). The structure of the RC near Q_B based on the coordinates from Allen *et al.* (1988)[2] (Brookhaven Protein Data Bank entry 4RCR) is shown in Figure 5. Although the details differ between the various published structures, there are several

common features that are relevant for the discussion. All structures have the Ser-L223 hydroxyl oxygen in the vicinity of a keto oxygen of Q_B ($\leq\sim$3.5 Å)[3] and place a carboxylic acid oxygen of Asp-L213 near the hydroxyl oxygen of Ser-L223 ($\leq\sim$3.5 Å). Asp-L213 is near several amino acid residues that have solvent accessibility, including Asp-L210 and Glu-H173. Water molecules may also be nearby as suggested by theoretical calculations (Beroza *et al.*, 1992) based on the structure of *Rb. sphaeroides* (Allen *et al.*, 1988). This suggestion was supported by the observation of ordered water molecules forming a contiguous water chain from near the methoxy oxygens of Q_B to the external solvent (Ermler *et al.*, 1994; Lancaster *et al.*, 1995). It should be noted that all structures were determined for the ground state of the RC with Q_B oxidized. For a more detailed analysis of the structure as related to internal proton transfer events, the relative positions of these species with Q_B reduced (eq 1) is necessary. This structure, which so far has not been determined, may differ in important details from the ground-state static structure.

Another approach to acquire details about the interaction of Q_B^- with the surrounding protein is through electron nuclear double resonance (ENDOR) spectroscopy, where protons magnetically coupled to an unpaired electron can be monitored (Feher *et al.*, 1985; Lubitz *et al.*, 1985). Protons located near Q_B, such as those hydrogen-bonded to the quinone, can be monitored upon formation of Q_B^-. Preliminary experiments in native and mutant RCs were initiated to monitor and assign observed ENDOR peaks to nearby protons, thereby obtaining structural information (Paddock *et al.*, 1995b). In addition, functional questions can also be addressed by ENDOR. For example, the role of nearby protons in forming Q_BH_2 was investigated using this technique (Paddock *et al.*, 1995b).

Mechanism of Proton Transfer by Ser-L223 to Reduced Q_B. Experimental observations of native and site-directed mutant RCs show that Ser-L223 is important for efficient proton-coupled electron transfer in eq 1a. However, the details of the mechanism are not clear. Given the proximity of the hydroxyl oxygen of Ser-L223 to a keto oxygen of Q_B, it is appealing to propose that Ser-L223 donates its proton directly to reduced Q_B. It is energetically very unfavorable to deprotonate the Ser hydroxyl group, since it has a pK_a $\sim$ 14 (in solution). Okamura and Feher (1992) used experimental evidence to assign charges to nearby carboxylic acid residues and then calculated electrostatic interactions between these charged sites and the ionized and protonated forms of the Ser hydroxyl group. The energy cost to form the OH_2^+ state in Ser-L223 was found to be less than the energy cost to form the O^- state. This suggests that Ser-L223 may become protonated, forming the strong acid OH_2^+ group prior to proton transfer to Q_B^- (Okamura & Feher, 1992). Alternately, mechanisms involving concerted proton hopping from one component to the next in a hydrogen-bonded network have been proposed for long-distance proton transfer (Nagle & Tristram-Nagle, 1983; Schulten & Schulten, 1986; Warshel, 1986). Such a mechanism greatly reduces the energy cost for moving a charged proton into a relatively low dielectric medium (*e.g.*, a protein). For this mechanism to be applicable, Ser-L223 must acquire a proton from a hydrogen-bonded donor as it donates its proton to Q_B^-. A likely candidate given its proximity to Ser-L223 is Asp-L213 (within 3.5 Å). Asp-L213 has been shown to be important for reaction 1a (Paddock *et al.*, 1990a; Takahashi & Wraight, 1990, 1991, 1992; Rongey *et al.*, 1991) and is connected to the solvent *via* several amino acid residues (*e.g.*, Asp-L210, Glu-H173) and possibly water molecules. Since Asp-L213 is believed to be predominantly ionized in the Q_B^- state (Takahashi & Wraight, 1990, 1992; Paddock *et al.*, 1994), such a hydrogen-bonded chain would be present only transiently. Thus, Ser-L223 could participate in concerted proton transfer through a transient hydrogen-bonded chain acting as the terminal proton donor to reduced Q_B.

Possible Functional Role of a Water Molecule in SG(L223) RCs. Given the results discussed above, it seems at first glance surprising that replacement of Ser-L223 with Gly, a poor proton transfer group, would display fast proton-coupled electron transfer (Table 1, Figure 3). A possible explanation of this behavior involves the functional substitution of another species for the missing Ser side chain. However, before proceeding with this hypothesis, we need to exclude possible structural changes as an explanation of the above findings.

Evidence for the lack of structural changes in this mutant is provided by the similarities of the pH profiles of $k_{AB}^{(1)}$ (Figure 2) and k_{BD} (Figure 4) for native and SG(L223) RCs. Since electron transfer rates are very sensitive to distance and orientation, these findings argue against any major structural changes. Further support was obtained from an analysis of the crystal structure of the SG(L223) RCs in which no major backbone changes compared to native RCs were observed (Axelrod *et al.*, 1995).

The Ser hydroxyl group in its protonated, neutral, or ionized state is structurally (*e.g.*, size, shape) and functionally (*e.g.*, pK_a, electronic hybridization of oxygen) very similar to a water molecule in the same state. Consequently, it seems reasonable to propose that a water molecule substitutes for the missing Ser hydroxyl group (Paddock *et al.*, 1995a). Unfortunately, the Q_B site in the SG(L223) RCs had a very low occupancy (<5%); consequently Q_B was not seen in the electron density maps nor was the resolution sufficient to resolve bound water molecules. More detailed functional and structural studies are required to test this hypothesis.

Comparison between Ser-L223 and Ser-264 of the D1 Polypeptide of Photosystem II. Structural and functional similarities between the bacterial L subunit and the D1 polypeptide of photosystem II (PS II) have been proposed on the basis of sequence homology and quinone and herbicide binding [see, *e.g.*, Trebst (1986), Michel and Deisenhofer (1988), Oettmeier (1992), and Kless *et al.* (1994)]. Several PS II mutants, resistant to triazine herbicides, have been isolated which contain replacements at the Ser-264 site of the D1 polypeptide of PS II. These include replacement with Gly in *Amaranthus hybridus* and *Solanum nigrum* (Hirschberg & McIntosh, 1983, Hirschberg *et al.*, 1984; Goloubinoff *et al.*, 1984); replacement with Ala in *Chlamydomonas reinhardtii*, *Synechococcus* PCC7942, and

[2] The more recent structure by Chirino *et al.* (1994) has a lower occupancy of Q_B, which makes its position less certain.

[3] There is considerable variation in distances between amino acid side chains and Q_B in the various published structures, due mainly to discrepancy in the placement of Q_B. Although most of the published structures place the hydroxyl oxygen of Ser-L223 within 3.5 Å of a keto oxygen of Q_B, in one structure this distance is reported to be greater than $\sim$5 Å (Ermler *et al.*, 1994). The authors of this reported structure propose that Q_B may have become reduced to quinol prior to X-ray analysis.

Anacystis nidulans (Erickson *et al.*, 1984, 1985; Golden & Haselkorn, 1985; Ohad *et al.*, 1992; Gleiter *et al.*, 1990; Etienne *et al.*, 1990); replacement with Thr in *Nicotiana tabacum* (Sato *et al.*, 1988); and replacement with Asn in *Nicotiana plumbaginfolea* (Pay *et al.*, 1988). Analogous changes were constructed in this work at the Ser-L223 site of *Rb. sphaeroides* and all demonstrated some level of resistance to triazine herbicides. Although a detailed study of herbicide binding was not performed in this paper, the qualitative similarity of herbicide resistance found in the herbicide-resistant PS II mutants and the analogous bacterial site-directed replacements support the suggestion that Ser-264 of the D1 polypeptide of PS II is structurally and functionally similar to Ser-L223 of the bacterial RC.

There are also some similarities in electron transfer characteristics between the Ala and Gly replacements at Ser-L223 and Ser 264 of the D1 polypeptide of PS II. Change of Ser → Ala in *C. reinhardtii* was found to increase the transfer rate of the first electron ~3-fold and reduce the transfer rate of the second electron >10-fold (Crofts *et al.*, 1993). The stability of Q_B^- was relatively unaffected by the mutation (Etienne *et al.*, 1990). These effects are very similar to those observed for the bacterial SA(L223) RCs for the forward electron transfer rates (Figures 2 and 3) and the stability of the Q_B^- state as determined from the charge recombination rates (Figure 4). A PS II mutant from *A. hybridus* containing a Ser → Gly replacement displayed normal electron transfer kinetics (Taoka & Crofts 1990) as found for the bacterial RC analog in this work. Thermoluminescence studies in *Synechococcus* PCC7942 containing a Ser → Gly replacement showed that Q_B^- stability was decreased (Ohad *et al.*, 1992). Similarly, the SG(L223) bacterial RCs showed little change of electron transfer rates (Figures 2 and 3) and k_{BD} studies (Figure 4) suggest that this replacement also destabilizes the Q_B^- state. These observations further strengthen the proposed similarity in function between Ser-264 of the D1 polypeptide of PS II and Ser-L223 of the bacterial RC.

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