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A crucial role for Asp^{L213} in the proton transfer pathway to the secondary quinone of reaction centers from *Rhodobacter sphaeroides*

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The aspartic acid at position 213 of the L-subunit (Asp^{L213}) of reaction centers from *Rb. sphaeroides* is one of two acidic residues in the binding site of the secondary quinone (Q_B). Alteration of Asp^{L213} to asparagine by site-directed mutagenesis drastically affected the light-induced proton and electron transfer functions leading to formation of quinol (Q_BH_2). The first electron transfer was slowed to a half-time in the millisecond time range, but the equilibrium ($Q_A^-Q_B \leftrightarrow Q_AQ_B^-$) was substantially increased in favor of Q_B reduction and the pH dependence of the equilibrium was altered. The stabilization of Q_B^- is suggested to result from the uncharged nature of the substitution, with the implication that Asp^{L213} is normally ionized and presents an electrostatic restriction to the first electron transfer. The second electron transfer ($Q_A^-Q_B^- + 2H^+ \leftrightarrow Q_AQ_BH_2$) was even more severely inhibited and was at least 10^4-times slower than the wild type at pH > 6.5, and after only two flashes the RCs were blocked in the $Q_A^-Q_B^-$ state. At lower pH some transfer activity was restored, although with a rate still 10^3-times slower than the wild type. The kinetics of the second electron transfer at low pH corresponded exactly to the kinetics of proton uptake. These data are interpreted as implying an essential role for Asp^{L213} in the proton transfer pathway leading to the formation of QH_2 after the second flash.

The reaction center complex (RC) of the purple photosynthetic bacterium *Rhodobacter sphaeroides* is composed of three polypeptide subunits, L, M and H. The L and M subunits, which bind all the cofactors involved in photochemical charge separation, are encoded by *puf*L and *puf*M genes within the *puf* operon [1]. Light activation results in photooxidation of the primary donor, P, and reduction of two quinones, Q_A and Q_B, acting in series to stabilize the initial charge separation. With multiple light flashes, and in the presence of an electron donor to re-reduce P^+, the quinones undergo reduction and oxidation as follows [2]:

odd flashes: $Q_AQ_B \xrightarrow{h\nu} Q_A^-Q_B \underset{(H^+)}{\overset{K_2}{\longleftrightarrow}} Q_AQ_B^-(H^+)$ (stable)

even flashes: $Q_AQ_B^-(H^+) \xrightarrow{h\nu} Q_A^-Q_B^-(H^+) \underset{(H^+)}{\overset{K_3}{\longleftrightarrow}} Q_AQ_BH_2$

$Q \quad QH_2$
$\leftrightarrow \quad Q_AQ_B$

The semiquinone formed after the first flash is anionic and the charge is partially neutralized by protonation of the RC [3] involving pK shifts of several protonatable amino acid residues [4–6]. After the second flash, two protons must be transferred to Q_B to form quinol, which leaves the RC and is replaced by a quinone. Significant functional differences between Q_A and Q_B are apparent from the scheme above. Since both quinones in *Rb. sphaeroides* RCs are ubiquinone-50 (Q-10), the differences in the functional properties of the two quinones can be attributed to differences in the local protein environments of the two quinone-binding sites. One of the more obvious differences, evident from the three-dimensional structure of the RC [7,8], is the presence of two ionizable residues, glutamate L212 (Glu^{L212}) and aspartate L213 (Asp^{L213}), in close proximity to Q_B. Mutagenesis of Glu^{L212} to glutamine (Gln) in RCs of *Rb. sphaeroides* by Paddock, et al. [9] resulted in lowering of the steady-state rate of cytochrome *c* oxidation by a factor of 25 when compred to the wild type (Wt) RC. This difference was attributed to a reduced rate of proton transfer to Q_B^{2-}. In the present work we have examined the role of Asp^{L213} in the *Rb. sphaeroides* RC function. Asp^{L213} was altered to a non-protonatable residue asparagine (Asn) by site-directed mutagenesis

Abbreviations: Mes, 2-[*N*-morpholino]ethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; Tris, 2-amino-2-hydroxymethyl-propane-1,3-diol; Ches, 2-[*N*-cyclohexylamino]ethanesulfonic acid; Caps, 3-[cyclohexylamino]-1-propanesulfonic acid.

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(designated as mutant L213DN), and we report here the effect of this mutation on the electron acceptor and proton transfer functions of the RC.

Details of the molecular biological techniques involved in expressing mutant RCs in *Rb. sphaeroides* have been described previously [10]. The L213DN mutation was obtained by in vitro mutagenesis employing the method of Kunkle [11]. The mixed oligonucleotide 5′-GATCACGAGXATACGTTCT-3′, containing a mixture of nucleotides A, T and C at **X**, was used to alter the codon for Asp^{L213} (GAT) to that of Asn (AAT). The mutation was screened by DNA sequencing. A kanamycin (Km) resistant *Rb. sphaeroides* RC deletion strain, GaKM(+) [10], which lacks most of the *puf* L and all of *puf* M, was complemented *in trans* with a tetracycline (Tc) -resistant broad host range plasmid pRK404 [12,13] carrying the *puf* operon with the L213DN mutation. In order to induce RC expression, the complemented deletion strain was grown under semiaerobic conditions in the dark in Sistrom's minimal medium [14] supplemented with 0.2% casamino acids, in the presence of antibiotics (25 μg/ml Km and 2 μg/ml Tc). RCs were isolated as previously described [4], using 220 mM NaCl, 0.1% LDAO, 10 mM Tris (pH 8.0) to elute the RCs from the DEAE-Sephacel column. Wild-type RCs were isolated from the strain Ga, parent to GaKM(+).

The kinetic spectrophotometer apparatus used was of local design as described in Ref. 3. The assay solution was 2.5 mM KCl, 20 μM Q-10 (Sigma, St. Louis, MO), 0.03% Triton X-100, 1 mM buffer (Mes, Pipes, Tris, Ches or Caps, depending on the pH) and about 1 μM RC. The kinetics of $P^+Q_AQ_B^-$ charge recombination, in the absence of an electron donor, were monitored at 430 nm. Cytochrome oxidation was monitored at 550 nm, with 20 μM cytochrome *c*. The semiquinone signal was monitored at 450 nm with 100 μM ferrocene as donor to P^+. Flash-induced proton binding was monitored spectroscopically at 586 nm using pH-indicator dyes, as described in Ref. 4, in assay solution containing 50 mM NaCl, 40 μM pH indicator dye, 20 μM Q-10, 0.03% Triton X-100, 100 μM ferrocene and 1 μM RC. Samples were gassed under nitrogen prior to the start of the assay. Similar measurements were made after addition of 10 mM buffer, and the net proton uptake was derived by subtracting the buffered traces from the unbuffered traces.

The decay of P^+ after a flash was measured in isolated RCs from L213DN, in the absence of exogenous electron donors. In the absence of added ubiquinone, the kinetics were largely fast, with $t_{1/2} \approx$ 60–80 ms, corresponding to charge recombination of the $P^+Q_A^-$ state. When ubiquinone was added there appeared a slow phase of recovery with $t_{1/2} \approx 10$ s at pH 7.0. In the Wt, the slow phase is known to arise from recombination of RCs in the $P^+Q_AQ_B^-$ state [3,15].

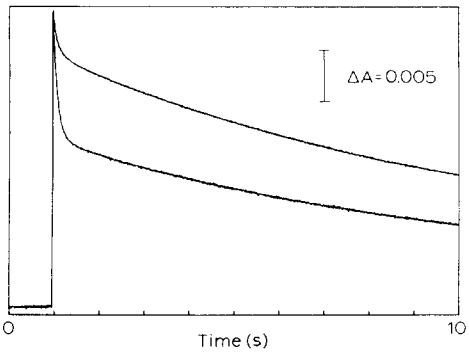

Fig. 1. P^+ recovery kinetics in RCs from L213DN, monitored at 430 nm. Conditions: 1 μM RC, 20 μM Q-10, 2.5 mM KCl, 0.03% Triton X-100, 1 mM Pipes (pH 7.0). Upper trace: kinetics following the first flash administered to a fresh sample. Lower trace: kinetics following the fifth flash, each given at 10 min intervals.

The slow phase titrated in with a half-saturated concentration of less than 1 μM for Q-10, and in thoroughly dark-adapted samples the maximum amplitude of the slow phase reached 85–90% (Fig. 1, top trace). These parameters are very similar to the Wt [16].

The pH dependence of the P^+ recovery rates ($P^+Q_AQ_B^- \rightarrow PQ_AQ_B$ charge recombination) for Wt and L213DN RCs are shown in Fig. 2. In the range accessible to study (pH 4–11), Wt RCs exhibited two pH-dependent regions– below pH 5 and above pH 9. In contrast, the charge recombination rate for L213DN was pH-independent below pH 7.0 and was continuously pH-dependent from pH 7.0 to 10.5. It may then become pH-independent above pH 10.5. The onset of pH dependence for Wt RCs at about pH 9 has been attributed to the ionization of Glu^{L212} with an apparent p*K* value of about 9.5, and subsequent electrostatic interaction with Q_B/Q_B^- [9]. Below pH 7.0 the L213DN charge recombination rate was essentially pH-independent, in contrast to the Wt RC, which displayed a second region of pH dependence below pH 5.0. Thus,

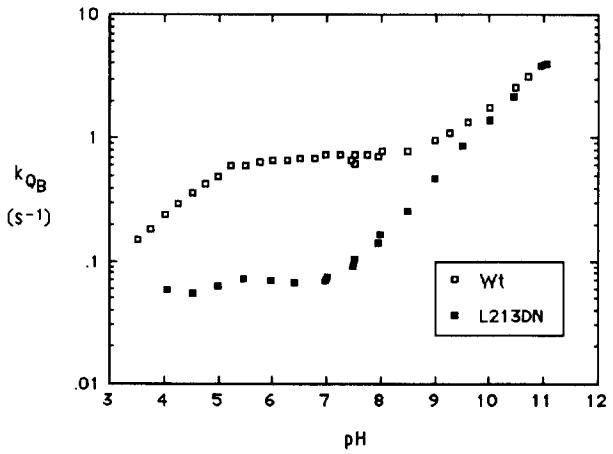

Fig. 2. pH dependence of the $P^+Q_AQ_B^-$ charge recombination kinetics in RCs from Wt and L213DN. Conditions: as for Fig. 1, with 1 mM each of Mes, Pipes, Tris, Caps and Ches.

Asp^{L213} could be responsible for the pH-dependent behavior in the Wt RC, with an apparent pK value of about 4.5.

It is generally accepted for the Wt that the decay of $P^+Q_AQ_B^-$ proceeds by charge recombination via $P^+Q_A^-Q_B$ [2]:

$$P^+Q_AQ_B \underset{k_{QA}}{\overset{h\nu}{\rightleftharpoons}} P^+Q_A^-Q_B \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} P^+Q_AQ_B^-$$

$$\uparrow \quad \_\_\_\_\_ \quad k_{dir}$$

and that the direct recombination rate is negligible ($k_{dir} < 0.1\ s^{-1}$ [15]). The observed rate of decay is then given by:

$$k_{QB} = k_{QA}[1 + K_2]^{-1} \quad (1)$$

where $K_2 = k_2/k_{-2}$ [17]. In the L213DN mutant, the observed decay is sufficiently slow at low pH that the direct charge recombination between P^+ and Q_B^- cannot be ruled out as contributing to the decay. Thus, the tendency towards pH independence may be misleading. However, taking the data at face value, it may be that in L213DN the region of pH-dependence of the charge recombination rate corresponds with the high pH region of the Wt, but with the pK shifted down from about 9.5 to 7.5 or lower. This would suggest that the pK in the Wt results, to a significant extent, from interaction with Asp^{L213}. The recombination rate for L213DN is substantially slower than the Wt over most of the measured pH range, and especially in the pH 5–7 region where the rates differ by more than an order of magnitude, indicating a significant additional stabilization of the electron on Q_B^-. Since k_{QA} is essentially unchanged in the mutant, application of Eqn. 1 yields an equilibrium constant of 100–125, 10-times greater than for the Wt in the region of pH 5–7. With the reasonable assumption that the mutation does not affect the redox midpoint potential (E_m) of Q_A/Q_A^-, the E_m for Q_B/Q_B^- must be at least 60 mV higher than in the Wt. This most likely results from the uncharged nature of Asn^{L213}.

When semiquinone behavior was examined with multiple saturating flashes in the presence of exogenous donor (ferrocene), the typical oscillatory behavior seen for the Wt RC [3,5,15] was not observed with L213DN RCs at pH values above 6.5 (Fig. 3A, bottom trace). After the first flash, a stable semiquinone absorbance signal was observed, but on all subsequent flashes the additional absorbance change decayed slowly to the first flash level. The spectrum of this decaying signal showed it to arise from a semiquinone species (Fig. 3B). The semiquinone signal produced by the second flash showed a very slow decay above pH 6.5 ($t_{1/2} > 1$ s at pH 7.0), indicating a drastically inhibited rate for the second electron transfer to Q_B when compared to the Wt RC ($t_{1/2} \approx 200$ μs [3,18]). At pH values below 6.5, the rate of decay of the semiquinone signal after the second flash increased ($t_{1/2} \approx 200$ ms at pH 6.0) and distinct oscillations of the stable semiquinone signal became apparent (Fig. 3A, top trace), indicating that the increased rate was due largely to an accelerated transfer of the second electron to Q_B. At pH ≥ 7.0, the slow decay of the semiquinone signal after the second and subsequent flashes is due, at least in part, to direct oxidation of Q_A^- by exogenous oxidants, including dis-

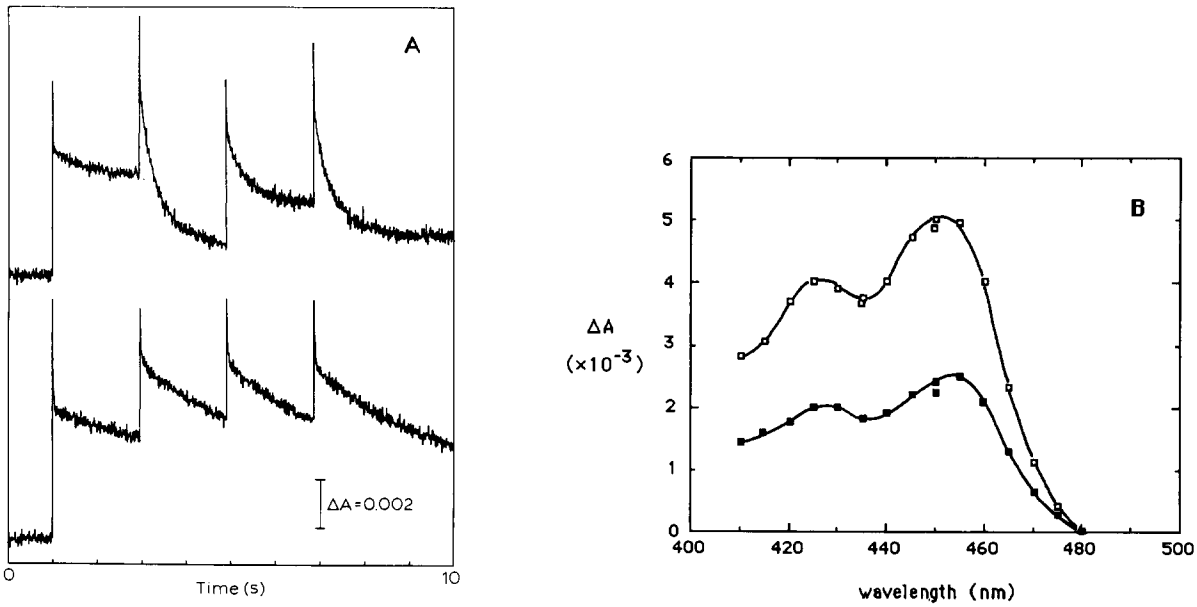

Fig. 3. Semiquinone absorbance changes in RCs from L213DN, monitored at 450 nm. Conditions: as for Fig. 1, except plus 100 μM ferrocene. (A) Top: pH 6.0, plus 1 mM Mes; Bottom: pH 8.0, plus 1 mM Tris. (B) Spectra of the absorbance changes 20 ms after the first (□) and second (■) flashes, at pH 8.0.

solved oxygen. Addition of excess ferrocyanide to keep the ferrocene reduced actually accelerated the decay due to very low levels of ferricyanide acting as an effective oxidant [5,19].

The remarkably potent inhibition of the second electron transfer in L213DN was also apparent in an impaired ability of RCs to oxidize cytochrome *c* in a series of flashes. At pH > 6.5, one cytochrome was oxidized after each of the first two flashes, but very little after subsequent flashes (not shown).

Flash-induced proton-binding by L213DN RCs was fully consistent with the observed semiquinone and cytochrome oxidation behavior. At pH > 6.5, a small rapid uptake of H^+ occurred after the first flash, but very little after subsequent flashes (Fig. 4, bottom trace). At pH < 6.5, where oscillations in the semiquinone signal are observed, the stoichiometry of H^+ binding is very similar to that of the Wt [4,6]. At pH 6.0, approx. 0.6 H^+/RC were taken up on the first flash and 1.4 H^+/RC on the second, with low-amplitude oscillations apparent in a series of flashes (Fig. 4, top trace). The major component of the kinetics of H^+ binding on the second flash was very similar in rate to the disappearance of the semiquinone signal after the second flash ($Q_A^-Q_B^- \rightarrow Q_AQ_B^{2-}$ electron transfer; $t_{1/2} \approx 200$ ms at pH 6.0).

The extreme retardation of the second electron transfer and the substantial stabilization of the Q_B/Q_B^- redox couple in the mutant gave rise to unexpected behavior of the RCs in the absence of added donors. When the charge recombination kinetics were recorded after several flashes given at 10 min intervals, the amplitude of slow phase decreased progressively from 90% on the first flash to 50–60% after four or five flashes (Fig. 1, lower trace). Addition of ferricyanide increased the slow phase amplitude somewhat, but the exclusion of O_2 (by nitrogen sparging) had no significant effect. The gradual increase in amplitude of the fast phase arises from accumulation of RCs with Q_B^- present before the flash. This state accumulates from previous activations due to competition between the slow intrinsic recombination rates of the mutant RCs ($t_{1/2} > 10$ s at pH 7.0) and slow electron donation to P^+ from unspecified, weak reductants in the assay medium. Adventitious donation with a half-time of 100–200 s would result in the trapping of 5–10% of the RCs in the $PQ_AQ_B^-$ state after each flash. Subsequent activation of these RCs produces $P^+Q_A^-Q_B^-$, which back-reacts much like $P^+Q_A^-$ because the $Q_A^-Q_B^- \rightarrow Q_AQ_B^{2-}$ electron transfer rate is very slow, as shown above. Proper adaptation of a previously flashed sample required more than 1 h to recover slow-phase amplitudes of 85–90%. The very slow relaxation of RCs in the $PQ_AQ_B^-$ state is probably due to the generally poor redox equilibration of the quinone region [20], the lack of any added mediators and the high E_m for the Q_B/Q_B^- couple in L213DN RCs. The existence of the $PQ_AQ_B^-$ state is also evident in the small component of decay of the 450 nm absorbance signal, after the first flash in the presence of ferrocene as donor (Fig. 3A). This component constitutes about 10% of the total semiquinone signal. However, the extinction coefficient of $Q_A^-Q_B^-$ is only 1.6-times that of $Q_AQ_B^-$ [15], so this actually represents about 15% of the RCs. Unless exceptionally long dark-adaptation times are used, subsequent activation by flash trains show progressively more of this decaying component on the first flash.

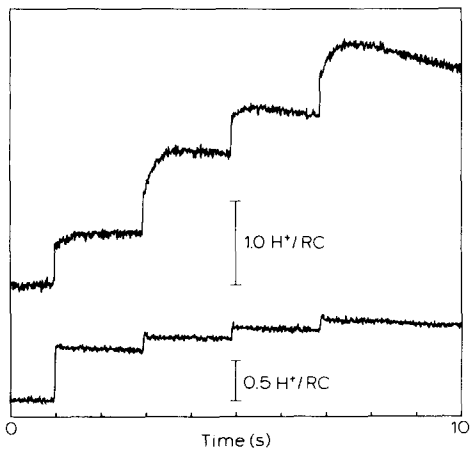

Fig. 4. Proton uptake by RCs from L213DN, monitored at 586 nm. Conditions: 1 μM RC, 20 μM Q-10, 50 mM NaCl, 0.03% Triton X-100, 100 μM ferrocene. (A) pH 6.0, 40 μM Chlorophenol red (10 mM Mes subtracted). (B) pH 8.0, 40 μM Cresol red (10 mM Tris subtracted).

The observed behavior of L213DN RCs leads us to suggest that the primary lesion is a severe obstruction of the normal uptake of the first proton associated with, or necessary for, the double reduction of Q_B after the second flash, leading to a failure of the transfer of the second electron to Q_B. The process is almost completely blocked at pH ≥ 7.0, but is facilitated at lower pH. Protonation of Q_B is likely to involve a number of protonatable residues which cooperate to transfer protons from the aqueous phase to the Q_B site, and some residues which may be involved in such a scheme have been indicated by Allen et al. [8]. The side-chain of Asp^{L213} is located close to a Q_B carbonyl group (about 6 Å) and to the hydroxyl group of Ser^{L223} (about 4.5 Å), which appears to hydrogen bond to the quinone carbonyl [7]. Either of these groups could act as the direct proton-donor to Q_B. Even if Ser^{L223} serves as the immediate proton donor to the quinone, Asp^{L213} may play a crucial role in the reprotonation of Ser^{L223} to permit net proton transfer to the quinone. From the pH dependence of the $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$ equilibrium (K_2) in Wt RCs, we suggest that ionization of Asp^{L213} may determine the behavior below pH 6.0, in which case this residue would be fully ionized in the $Q_AQ_B^-$ state above

pH 6.0. In order to participate in proton donation on the second transfer, therefore, it would have to be rapidly reprotonated, perhaps by internal transfer, in the $Q_A^- Q_B^-$ or $Q_A Q_B^{2-}$ states. The exact order of events cannot be established from available data, and two possibilities must be considered [3]:

$$Q_A^- Q_B^- \overset{K_{H(1)} \ll 1}{\longleftrightarrow} Q_A^- Q_B^- (H^+) \overset{K_{E(2)} \gg 1}{\longleftrightarrow} Q_A Q_B H^- \quad (A)$$

$$Q_A^- Q_B^- \overset{K_{E(2)} \ll 1}{\longleftrightarrow} Q_A Q_B^{2-} \overset{K_{H(1)} \gg 1}{\longleftrightarrow} Q_A Q_B H^- \quad (B)$$

where $K_{H(1)}$ and $K_{(E(2)}$ are equilibrium constants for the transfer of the first proton (involving Asp^{L213}) and the second electron, respectively. In either case, an unfavorable equilibrium is followed by a very favorable one, thereby pulling the net electron transfer over. Both situations have been widely encountered in the electrochemistry of quinones in solution [21]. The first case (A) is attractive because the electron transfer to the neutral semiquinone is easily imagined to proceed rapidly. The second case (B) is attractive because the dianion, Q_B^{2-}, is expected to have a very high pK (> 13), probably capable of obtaining a proton from Ser^{L223} with the help of the protonated Asp^{L213} to reprotonate the serine hydroxylate.

Turnover in the acceptor quinone complex is normally completed by the transfer of a second H^+-ion to form Q_BH_2, which is then released from the Q_B site as quinol and replaced by an oxidized quinone [2,3,17,22]. From the behavior of the Glu^{L212} mutation reported by Paddock et al. [9], it is likely that Glu^{L212}, located about 5 Å from the other carbonyl group of Q_B, is involved in donation of the second proton, i.e., mutation to a non-ionizable residue permits three turnovers of the RC leading to accumulation of the state $PQ_A^- Q_B H^-$.

This overall description implies two slightly different pathways for donation of the first and second protons in the formation of Q_BH_2 in RCs of *Rb. sphaeroides*, such as has been suggested by Allen et al. [8]. However, in *Rps. viridis* [23] and *R. rubrum* [24], the residue L213 (or equivalent) is naturally asparagine. This is a perplexing fact, especially in view of the very dramatic effects of the mutation in *Rb. sphaeroides* – a decrease in proton and electron transfer rates of 10^4-fold. Presumably other compensating differences allow the participation of other residues in this function and it is noteworthy that a relatively nearby residue (about 8 Å) is Asn in *Rb. sphaeroides* (M44), but Asp in *Rps. viridis* (M43) and *R. rubrum* (M43). It may also be significant to the notion of a transiently protonated Asp^{L213} participating in proton transfer in *Rb. sphaeroides*, that Asn^{L213} in *Rps. viridis* is much closer, and is directly hydrogen-bonded, to Ser^{L223}.

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