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SYMMETRY-RELATED MUTANTS IN THE QUINONE BINDING SITES OF THE REACTION CENTER -- THE EFFECTS OF CHANGES IN CHARGE DISTRIBUTION

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Abstract

To probe the structural elements that contribute to the functional asymmetries of the two ubiquinone$_{10}$ binding pockets in the reaction center of *Rhodobacter capsulatus*, we targeted the L212Glu-L213Asp (near Q_B) and the M246Ala-M247Ala (near Q_A) pairs of symmetry-related residues for site-specific mutagenesis. We have constructed site-specific mutants that eliminate the sequence differences at these positions (L212Glu-L213Asp→Ala-Ala or M246Ala-M247Ala→Glu-Asp), and have reversed that asymmetry by constructing a quadruple-mutant strain, "RQ" (L212Glu-L213Asp-M246Ala-M247Ala→Ala-Ala-Glu-Asp). The mutations were designed to change the charge distribution in the quinone-binding region of the reaction center; none of the strains is capable of photosynthetic growth. In photocompetent phenotypic revertants of the RQ strain, second-site mutations which affect Q_B function are coupled to mutations in the Q_A site which restore an Ala or substitute a Tyr at the M247 site; one strain carries an additional Met→Leu substitution at M260 near Q_A. All of the RQ revertants retain the engineered M246Ala→Glu mutation in the Q_A site as well as the L212Ala-L213Ala mutations in the Q_B site. Kinetic characterization of the RQ revertants will give us an idea of what structural and functional elements are important for restoring efficiency to electron and proton transfer pathways in the RQ RC, which is far from native. To date, these preliminary results underscore the importance of an asymmetric distribution of polar amino acids in the quinone binding pockets and its influence on the functional properties of the reaction center.

Introduction

Stable, photo-induced transmembrane charge separation is achieved in the photosynthetic reaction center (RC) by the reduction of quinone species, Q_A and Q_B, which serve as the penultimate and terminal electron acceptors, respectively. In RCs of *Rhodobacter (R.) sphaeroides* and *R. capsulatus*, Q_A and Q_B are identical ubiquinone$_{10}$ molecules, whose different *in situ* chemical properties are defined by the natures of the protein environments that constitute their binding sites. Q_A serves as a one-electron gate and does not become directly protonated. Q_B

becomes doubly reduced following two successive turnovers of Q_A^-. The quinol form, Q_BH_2, is generated by the conduction of protons through the protein to the buried Q_B binding site following the second flash. The Q_B site binds the various intermediate states of the quinone with differing affinities, facilitating the retention of Q_B^- and the diffusion of Q_B and Q_BH_2 into and out of, respectively, the RC.

The functional asymmetry of the two quinone binding pockets extends to the midpoint potentials of the Q_A/Q_A^- and Q_B/Q_B^- couples, which differ by about 60 mV at neutral pH. The Q_A and Q_B binding sites share structural andsequence homology and are related by an axis of approximate twofold symmetry in the high resolution structures of the RCs of *Rhodopseudomonas viridis* (Deisenhofer et al. 1985) and *R. sphaeroides* (Stowell et al. 1997; reviewed in Ermler et al. 1994). The Q_A site is relatively more hydrophobic than the Q_B binding site. The symmetry-related residues M246Ala in the Q_A site and L212Glu in the Q_B site represent a strictly-conserved polarity difference that distinguishes the two binding sites. The adjacent residues M247Ala (at Q_A) and L213Asp (at Q_B) represent a similar symmetry-related difference that is not as strictly conserved among several species of photosynthetic bacteria whose sequences are known [residue M247 is Ala or Gln, while residue L213 is Asp or Asn]. L212Glu and L213Asp have been shown to be important components of the pathway for proton transfer to Q_B^{2-} (Paddock et al. 1989; Takahashi & Wraight 1992; reviewed in Sebban et al. 1995a), whereas their alanine counterparts near Q_A are hydrophobic and nonpolarizable.

To probe the structural elements that contribute to the functional asymmetries of the two ubiquinone$_{10}$ binding pockets, we constructed a family of site-specific mutants of the *R. capsulatus* RC that eliminated the sequence differences at these symmetry-related positions, or reversed that asymmetry (Table 1). All of the site-specific mutants are incapable of photosynthetic growth (PS^-), and pseudo-revertants have been isolated that have regained the photosynthetic phenotype (PS^+). The pathway for proton transfer to Q_B^{2-} has been shown to be blocked in the L212-L213AA mutant (Hanson et al. 1992b, 1993; Maróti et al. 1994), and we have characterized a number of phenotypic revertants of this strain that have regained this function (Hanson et al.

1993; Maróti et al. 1994), and preliminary characterization of two phenotypic revertants of the M246-M247ED strain has also been reported previously (Laible et al. 1997). Here we describe phenotypic revertants derived from the RQ quadruple mutant, an RC in which the important sequence asymmetry at these positions in the Q_A and Q_B binding sites has been reversed.

Materials and Methods

The quadruple mutant RQ was constructed by ligating L and M gene segments from the previously-constructed L212-L213AA (Hanson et al. 1993) and M246-247ED (Laible et al. 1997) site-specific mutants. Cultures derived from independent colonies of the RQ strain were grown under chemoheterotrophic conditions. Aliquots of these dark cultures were used to inoculate anaerobic tubes of RPYE medium (Hanson et al. 1992b), which were then incubated in the light for several weeks. Individual revertants were isolated by restreaking for single, photosynthetic colonies on MPYE/kan agar. Growth curves and the localization of compensatory mutations by plasmid recloning, complementation mapping, and DNA sequencing have been described (Hanson et al. 1992a). The original RQ revertants carry compensatory mutations in both the L and M genes of the RC (all compensatory mutations were plasmid-borne), thus unique restriction sites of plasmids pU29 (Bylina et al. 1986) and pU2922 (Bylina et al. 1989) were used to construct "half revertants" which coupled a revertant Q_A site with a wild-type Q_B site (the strains designated with an "A" in Table 2), and vice versa (strains designated with a "B").

Results and Discussion

The RQ mutant strain potentially carries the largest change in charge distribution of any of the mutants listed in Table 1. In RCs of *R. capsulatus*, L213Asp is ionized at neutral pH (Hanson et al. 1992b), and the apparent pK_a of L212Glu is ~10.1 (Maróti et al. 1995; Sebban et al. 1995a,b). [Other experimental evidence obtained with *R. sphaeroides* RCs suggests that L212Glu may be partially ionized in the neutral pH region (Hienerwadel et al. 1995), and theoretical electrostatic calculations predict that this residue interacts strongly with several other nearby

charged residues (Beroza et al. 1995; Lancaster et al. 1996)]. In the RQ mutant, these residues near Q_B are replaced by neutral alanines. The Glu and Asp residues at M246-M247 in the RQ mutant are polar, and their ionization states have not yet been determined. However, the effects of the mutations extend well beyond the immediate environment of the quinones. The RC of the RQ mutant is characterized by a slightly slower rate of $P^*\rightarrow P^+H_A^-$ electron transfer (P. D. Laible and D. K. Hanson, unpublished observations) and these mutations also resolve the Q_X bacteriochlorophyll transitions near 590 nm in the 4 K absorption spectrum into two distinct peaks of ~587 nm and ~602 nm (T. J. DiMagno, personal communication).

Since neither symmetrized double mutant strain (L212-L213AA or M246-M247ED) is capable of photosynthetic growth, RCs of the quadruple mutant RQ strain must compensate for engineered mutations in both quinone sites in order to regain function. Table 2 summarizes the sequences found near the Q_A and Q_B sites of RCs of the phenotypic revertants isolated from the PS^- RQ mutant strain, and Fig. 1 shows the locations of those residues. The changes at each quinone binding site are summarized below:

Revertant Q_A sites. The engineered M246Glu substitution is still present in the Q_A site of all of the RQ revertants, even though Ala at this site is strictly conserved in several species, and Ala or Gly had already been seen to substitute for M246Glu in phenotypic revertants of the M246-M247ED mutant (Table 2; Laible et al. 1997). The RQ revertants have compensated for the deleterious changes in the Q_A site by the change of M247Asp to the original Ala (RQrev4) or to Tyr (RQrev3 & RQrev5). In the case of RQrev6, a third mutation, M260Met→Leu, has been added to the M246Glu-M247Tyr sequence found in the Q_A sites of RQrev3 and RQrev5. Molecular modeling shows that the bulky M247Tyr points away from Q_A into a cavity of the structure (Fig. 1). It is likely that the M260Leu substitution relieves some of the crowding that is caused by the presence of M246Glu, which could lead to a slightly different binding site for Q_A and, thus, a change in its redox potential.

Revertant Q_B sites. None of the RQ revertants regained a wild-type Q_B site since the engineered L212Ala-L213Ala mutations at Q_B are still present. As seen previously for phenotypic

revertants of the L212-L213AA strain (Hanson et al. 1993; Maróti et al. 1994; Sebban et al. 1995b), distant suppressor mutations compensate for the loss of L212Glu and L213Asp by making the electrostatic environment of Q_B more negative, either by the addition of an acidic residue (RQrev3/6, RQrev5) or the removal of a basic residue (RQrev4). The M43Asn→Asp suppressor mutation seen in RQrev3 and RQrev6 [characterized previously in (Hanson et al. 1992a; Maróti et al. 1994)] is 9 Å from Q_B (Fig. 1). Suppressor mutations in RQrev4 (L217Arg→Cys) and RQrev5 (M5Asn→Asp) are also distant from Q_B (≈11Å and ≈10Å, respectively). All of these suppressor mutations affect members of a network of charge-charge interactions that connects the many ionizable residues in this region of the RC (Sebban et al. 1995). Distant mutation-induced shifts in these interactions propagate as the pK_as of other residues involved in the network change, resulting in an experimentally quantifiable increase in the negative potential surrounding Q_B^-(Maróti et al. 1994; Sebban et al. 1995b; Miksovska et al. 1996; Valerio-Lepiniec et al. 1997).

"Half-revertants". Since the RQ revertants carry compensatory mutations at both the Q_A and Q_B sites, we constructed plasmids that separate the amino acid substitutions which compensate for site-specific mutations near Q_A from those which compensate for site-specific mutations near Q_B. The strains designated with an "A" carry chimeric plasmids in which the Q_A-site mutations of the revertant are coupled with a wild-type Q_B site. The strains designated "B" carry a revertant Q_B site linked to a wild-type Q_A site. Uncoupling the compensatory mutations using these chimeric plasmids will allow us to determine how the electrostatic mutations near Q_B interact over a large distance with those at the Q_A site in the full revertants to restore the functions lost in the RQ mutant RC.

Preliminary result of kinetic experiments indicate that the RQrevs"X"A have rates of $P^+H_A^-Q_A \rightarrow P^+H_AQ_A^-$ electron transfer that are intermediate between those of the wild-type and RQ strains (P. D. Laible and D. K. Hanson, unpublished observations). The L217Arg→Cys mutation, which is additional to the L212Ala-L213Ala mutations in the RC of RQrev4B, is capable of restoring a nearly native free energy gap between the $P^+Q_A^-$ and $P^+Q_B^-$ states, with a similar pH

dependence (Valerio-Lepiniec et al., 1997). In contrast, the energy level of the $P^+Q_B^-$ state in the RC of RQrev5B is only slightly higher than that of the L212-L213AA double mutant. However, the native-like electrostatic environment for Q_B^- in RQrev4B does not result in a corresponding increase in the rate of transfer for the second electron since that rate is similar in both the RQrev4B and RQrev5B RCs. The L217Arg→Cys mutation does not compensate for the proton donor function of L213Asp, which is another component that is essential for rapid proton transfer to Q_B^-.

Preliminary results indicate that the symmetry-reversed RC of the RQ strain is capable of transferring two electrons to Q_B, at a rate which is not sufficient to support photosynthetic growth (M. Valerio-Lepiniec and P. Sebban, personal communication). Revertants of the RQ strain will give us an idea of what structural and functional elements are important for restoring efficiency to electron and proton transfer pathways in the RQ RC, which is far from native. Experiments are in progress that will determine whether the nature of the compensatory residues that restore function to the Q_A site influences the type of compensatory mutations that are recovered near Q_B (and vice versa). Results should be applicable to mechanistic problems of RC evolution, electron and proton transfer, and protein structure/function.

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Table 1. Symmetry-related Mutants in the Quinone Sites

	Q_A site		Q_B site		
Strain	**M246**	**M247**	**L212**	**L213**	**Phenotype**
Wild type	Ala	Ala	Glu	Asp	PS^+
L212-L213AA	Ala	Ala	Ala	Ala	PS^-
M246-M247ED	Glu	Asp	Glu	Asp	PS^-
RQ	Glu	Asp	Ala	Ala	PS^-

Table 2: Phenotypic revertants of quinone site mutants

	Q_A site			Q_B site						
Strain	**M246**	**M247**	**M260**	**L212**	**L213**	**L217**	**M5**	**M43**	**Phenotype**	**Doubling Time (h)**
Wild type	Ala	Ala	Met	Glu	Asp	Arg	Asn	Asn	PS^+	5.0
RQ	**Glu**[a]	**Asp**	Met	**Ala**	**Ala**	Arg	Asn	Asn	PS^-, **site-specific**	>100
RQrev4	**Glu**	Ala	Met	**Ala**	**Ala**	**Cys**	Asn	Asn	PS^+, spontaneous	18.5
RQrev4A	**Glu**	Ala	Met	Glu	Asp	Arg	Asn	Asn	PS^+, constructed	5.9
RQrev4B	Ala	Ala	Met	**Ala**	**Ala**	**Cys**	Asn	Asn	PS^+, constructed	18.5
RQrev5	**Glu**	**Tyr**	Met	**Ala**	**Ala**	Arg	**Asp**	Asn	PS^+, spontaneous	21.1
RQrev5B	Ala	Ala	Met	**Ala**	**Ala**	Arg	**Asp**	Asn	PS^+, constructed	8.0
RQrev3	**Glu**	**Tyr**	Met	**Ala**	**Ala**	Arg	Asn	**Asp**	PS^+, spontaneous	n.d.
RQrev3A=5A	**Glu**	**Tyr**	Met	Glu	Asp	Arg	Asn	Asn	PS^+, constructed	n.d.
RQrev3B=6B	Ala	Ala	Met	**Ala**	**Ala**	Arg	Asn	**Asp**	PS+, constructed	6.1
RQrev6	**Glu**	**Tyr**	**Leu**	**Ala**	**Ala**	Arg	Asn	**Asp**	PS^+, spontaneous	9.7
RQrev6A	**Glu**	**Tyr**	**Leu**	Glu	Asp	Arg	Asn	Asn	PS^+, constructed	15.6
M246-M247ED	**Glu**	**Asp**	Met	Glu	Asp	Arg	Asn	Asn	PS^-, **site-specific**	>100
$Q_A$rev1	Ala	**Asp**	Met	Glu	Asp	Arg	Asn	Asn	PS^+, spontaneous	6.0
$Q_A$rev2	**Gly**	**Asp**	Met	Glu	Asp	Arg	Asn	Asn	PS^+, spontaneous	20.0

[a]Bold type indicates mutant residue.

Figure 1. Molecular model of a cross-section of the *Rb. sphaeroides* reaction center (Chang et al., 1991; viewed from the periplasmic surface) near the level of the quinones, showing the α-carbon backbone of the L, M, and H chains, and the side chains of residues which are substituted in the revertant strains. All of these residues lie at approximately the same level in the membrane. Wild-type residues shown: L212Glu, L213Asp, L217Arg, M43Asn, M5Asn, M260Met. Also pictured: Mutant residues M246Glu, M247Tyr.

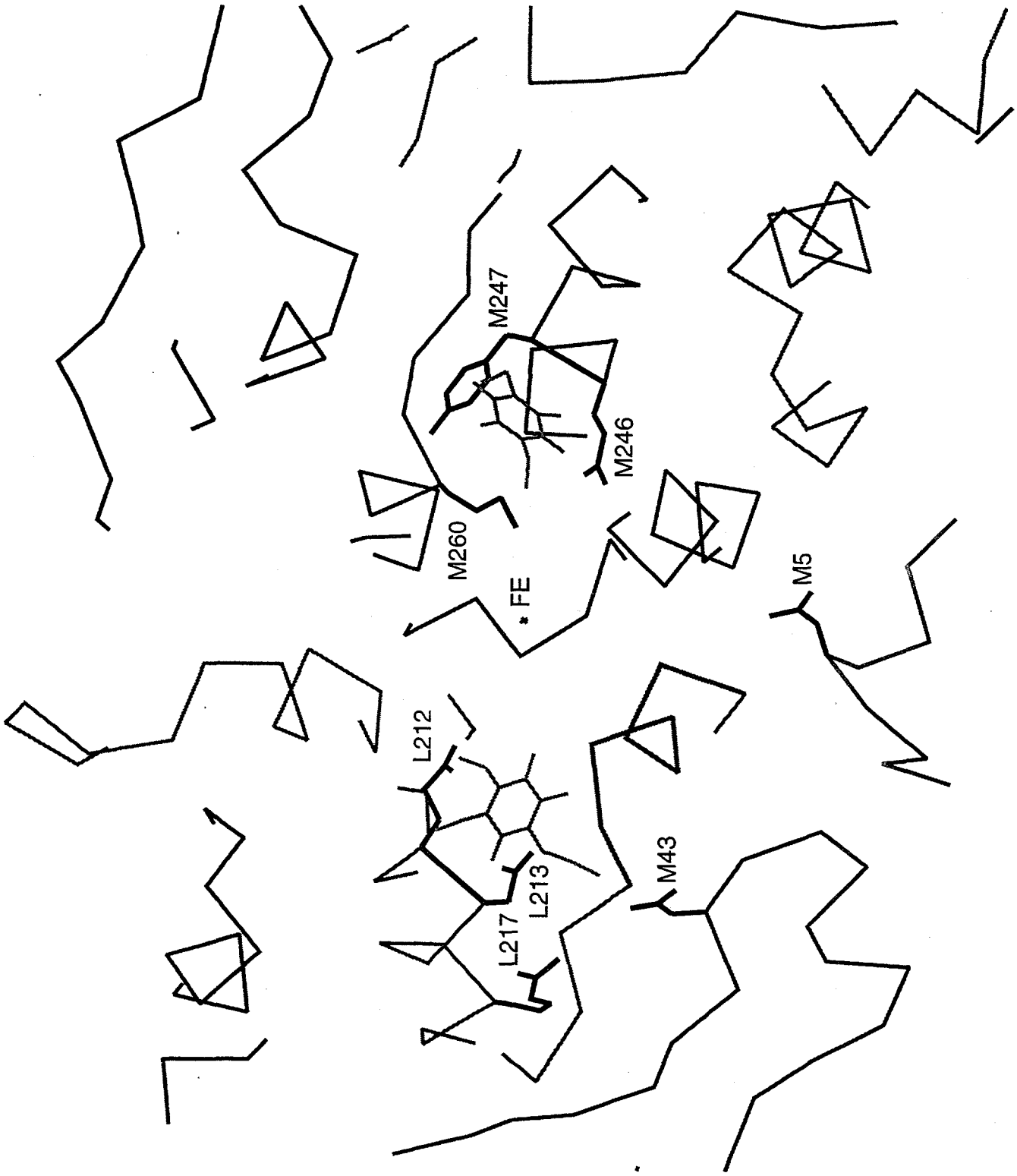

Figure 1