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Small weak acids stimulate proton transfer events in site-directed mutants of the two ionizable residues, Glu^{L212} and Asp^{L213}, in the Q_B-binding site of *Rhodobacter sphaeroides* reaction center

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Mutations of the two ionizable residues, Glu^{L212} and Asp^{L213}, in the secondary quinone (Q_B) binding site of reaction centers (RCs) from *Rhodobacter sphaeroides* cause major dysfunctions in the proton transfer processes leading to the formation of quinol. Mutant RCs with $Asp^{L213} \rightarrow$ Asn are especially severely blocked, and the rate of the proton-limited transfer of the second electron is at least 10^4 times slower than in the wild-type. Small, weak acids, such as azide/hydrazoic acid (N_3^-/HN_3; $pK \sim 4.7$) accelerated the electron transfer rate in mutant RCs in a pH and concentration-dependent manner, consistent with their functioning as protein-penetrating protonophores, delivering protons to the Q_B site in a non-specific, diffusive process. Other small weak acids acted similarly with efficacies dependent on their size and pK values. In terms of the concentration of protonated species, the relative effectiveness was: nitrite > cyanate ~ formate > azide >> acetate. The behavior of bacterial RCs containing the $Asp^{L213} \rightarrow$ Asn mutation resembles that of bicarbonate-depleted photosystem II, and the mutational block is partially alleviated by bicarbonate. The possibility is discussed that bicarbonate acts in PS II as an analogue to the carboxylic acid residues of the bacterial proton conduction pathway.

Reaction center; Proton transfer; Quinone; Site-directed mutagenesis; Weak acid; *Rb. sphaeroides*

1. INTRODUCTION

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 bind all the cofactors involved in photochemical charge separation. When a RC is activated by a brief flash of light, an electron is transferred from the primary donor, P, to the primary and secondary quinones (Q_A and Q_B, respectively). The negative charge on the quinones induces pK shifts of nearby amino acid residues, resulting in protonation of the RC and partial shielding and stabilization of the charge on Q_B^- [1–3]. However, the quinone headgroup is not directly protonated and the semiquinone is anionic. With multiple light flashes, and in the presence of an electron donor to re-reduce P^+, the quinones undergo reduction and oxidation as follows [1,4]:

first flash: $Q_AQ_B \xrightarrow{h\nu} Q_A^-Q_B \underset{(H^+)}{\overset{K_2}{\rightleftharpoons}} Q_AQ_B^-(H^+)$ (stable)

second flash:

$$Q_AQ_B^-(H^+) \xrightarrow{h\nu} Q_A^-Q_B^-(H^+) \underset{(H^+)}{\overset{K_3}{\rightleftharpoons}} Q_AQ_BH_2 \overset{Q_B,\,QH_2}{\longleftrightarrow} Q_AQ_B$$

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After the second flash, two protons are delivered to the quinone headgroup to form quinol (Q_BH_2), which leaves the RC and is replaced by quinone from the membrane pool. Binary oscillations can be seen in the formation and disappearance of semiquinone and in the uptake of protons from the medium [1,2]. In vivo, protons involved in the reduction of Q_B come exclusively from the cytosolic side of the membrane [5] and must traverse the H-subunit which lies over the quinone binding sites [6,7]. Subsequent oxidation of quinol by the b/c_1 complex releases protons to the periplasmic side of the membrane and completes the contribution to the electrochemical potential across the membrane, which is used for the synthesis of ATP [4,5]. The protonation events in the RC are an integral part of the electrochemiosmotic energy conservation processes of purple bacteria, but the RC is not a proton pump, per se.

Recent studies using site-directed mutagenesis have strongly implicated two ionizable residues in the proton transfer pathway to the Q_B site in *Rb. sphaeroides* – glutamic and aspartic acids at positions 212 and 213, respectively, of the L-subunit. Alteration of Glu^{L212} to the non-ionizable residue glutamine (mutant L212EQ), caused significant alterations in the quinone redox reactions [8]. The pH dependences of the $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$ equilibrium and electron transfer rate at alkaline pH were essentially eliminated. This identified Glu^{L212} (with an unusually high $pK \sim 9.6$) as the residue that inhibits the first electron transfer when ionized. The

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mutation also resulted in a significantly lower steady state rate of turnover when compared to the wild-type (Wt). This was attributed to a reduced rate of transfer of the second proton in the formation of quinol, i.e. $Q_BH^- \rightarrow\!\!\!\!\!\!\times\!\!\leftarrow Q_BH_2$.

Alteration of Asp^{L213} to asparagine (mutant L213DN) caused even more severe effects, including a substantial increase in the $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$ equilibrium in favor of Q_B reduction, and modification of the pH dependence of the equilibrium [9]. Most dramatic was an almost complete obstruction of the first proton transfer associated with the double reduction of Q_B after the second flash, leading to a drastic failure of the transfer of the second electron to Q_B, i.e. $Q_A^-Q_B^- \rightarrow\!\!\!\!\!\!\times\!\!\leftarrow Q_AQ_BH^-$. Following a suggestion by D. Oesterhelt, we examined the effects of azide in both types of mutant, Glu^{L213} and Asp^{L213}. We report here that azide and other small, weak acid species can substantially accelerate the rates of the severely proton limited processes. Similar effects are seen in the ion pumps of *Halobacterium halobium* - in halorhodopsin [10,11] and in certain mutants of bacteriorhodopsin [12,13] - and are interpreted as arising from passive penetration of the protein structure by small protonophores.

2. MATERIALS AND METHODS

Details of the molecular biological techniques involved in generating *Rb. sphaeroides* with mutant RCs have been described previously [14]. The L212 (Glu→Gln) and L213 (Asp→Asn) single mutations and the L212 (Glu→Gln)/L213 (Asp→Asn) double mutation (designated as mutants L212EQ, L213DN and L212EQ/L213DN, respectively) were obtained by the in vitro mutagenesis method of Kunkel [15], using oligonucleotides containing the alterations of codons for amino acid residues 212 (GAG→AAG) and 213 (GAT→AAT) of the L-subunit gene. Screening of the desired mutations was performed by DNA sequencing. A kanamycin (Km)-resistant *Rb. sphaeroides* RC deletion strain, GaKM(+) [14] was complemented *in trans* with a tetracycline (Tc)-resistant broad host range plasmid pRK404 [16,17] carrying the RC genes with the various mutations. Expression of mutant RCs was accomplished by growing the complemented deletion strain under semiaerobic conditions, in the dark, in Sistrom's minimum medium [18] 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 [19], using 220 mM NaCl, 0.1% lauryl dimethylamine-*N*-oxide (LDAO; Onyx Chemical Corp., NJ), 10 mM Tris (pH 8.0) to elute the RCs from the DEAE-Sephacel column.

The second electron transfer to Q_B was monitored by the decay of the semiquinone signal after two flashes, measured at 450 nm with 5–100 μM ferrocene as donor to P^+. The kinetic spectrophotometer apparatus utilized unchopped optics and was of local design. The assay solution was 2.5–50 mM KCl, 20 μM ubiquinone (Q-10; Sigma, St. Louis, MO), 0.03% Triton X-100, 1–5 mM buffer (MES, PIPES, Tris, glycyl-glycine, CHES or CAPS, depending on the pH) and about 1 μM RC.

3. RESULTS

Fig. 1 shows the behavior of the semiquinone species in isolated L213DN mutant RCs after two flashes. At

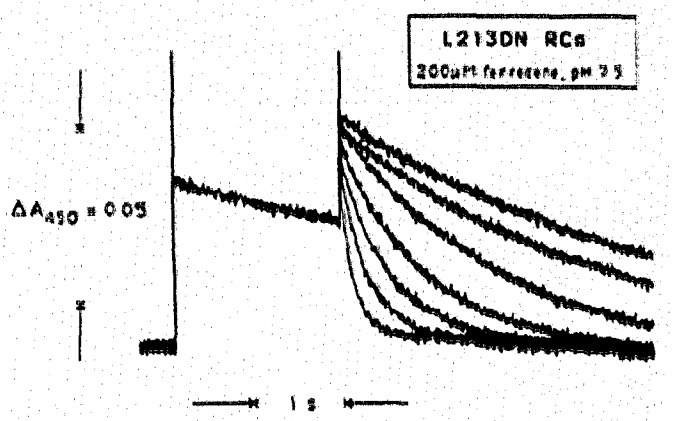

Fig. 1. The effect of azide on the decay of the $Q_A^-Q_B^-$ state in L213DN mutant RCs. Formation and decay of the semiquinone signals monitored at 450 nm (ΔA_{450}); time between flashes = 2 s. Conditions: approx ~ 1 μM RCs in 2.5 mM KCl, 20 μM Q-10, 0.03% Triton X-100, 5 mM Buffer, pH 7.5, with 200 μM ferrocene as exogenous donor. Sodium azide added at the following concentrations: 0, 10, 40, 98, 193, 374, 856 mM, increasing from the top down.

pH 7.5, the formation of the $Q_A^-Q_B^-$ state after the second flash was clearly indicated by the increased absorbance at 450 nm. In the absence of azide, the signal decayed with $t_{1/2}$ ~3 s. In Wt RCs, the disappearance of semiquinone after the second flash is too fast to resolve on this time scale ($t_{1/2}$ ~300 μs, at pH 7.5 [1]). Addition of increasing amounts of azide caused a progressive increase in the rate of the semiquinone decay after the second flash. At this pH, the rate was doubled in the presence of 24 mM and became linear with azide concentration above about 500 mM (Fig. 2). In the concentration range where the effect was linear with concentration, azide was less effective at higher pH (not

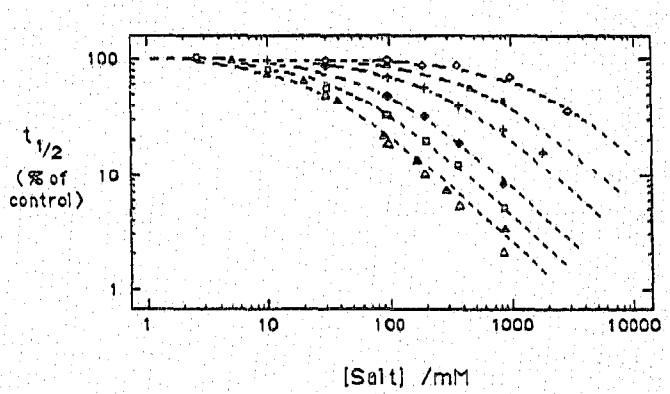

Fig. 2. The effect of various salts on the decay of the $Q_A^-Q_B^-$ state in L213DN mutant RCs. Decay times for ΔA_{450} after the second flash are plotted as a function of salt concentration. Relative half-times shown, with 100% = 2.69±0.36 s. Conditions as for Fig. 1, with various added salts; ▲, NaN_3; △, $NaNO_2$; □, NaSCN; ●, Na-formate; +, NH_4Cl; ■, NaCl; ◇, Na-acetate. The curves are theoretical fits, assuming that the effect of added salt is linear with concentration and is additive to the existing rate process.

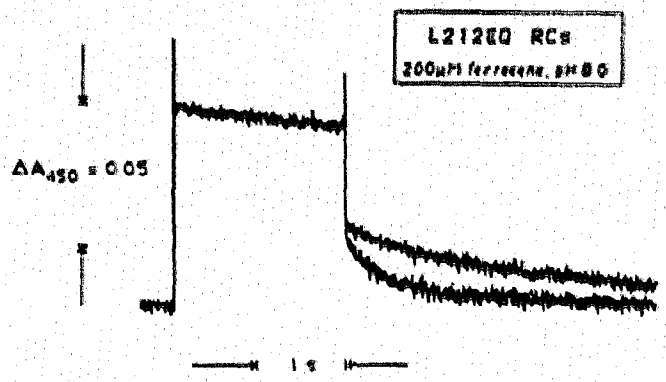

Fig. 3. The effect of azide on the decay of the $Q_A^- Q_B^-$ state in L212EQ mutant RCs. Formation and decay of the semiquinone signal was monitored at 450 nm; time between flashes = 2 s. Conditions: approx ~1 μM RCs in 2.5 mM KCl, 20 μM Q-10, 0.03% Triton X-100, 5 mM Buffer, pH 8.0, with 200 μM ferrocene as exogenous donor. Top trace, no azide; bottom trace, 856 mM sodium azide.

shown). This was somewhat masked by the fact that the control rate became faster at lower pH [9,20] and the effect of azide was not apparent until its contribution exceeded the intrinsic process.

Identical behavior was observed for RCs from the L212EQ/L213DN double mutant (not shown), and a similar effect of azide on the semiquinone behavior in isolated L212EQ mutant RCs is shown in Fig. 3. The stable semiquinone formed after one flash disappeared after the second flash with predominantly rapid kinetics, followed by a small slow phase. The amplitude and kinetics of the slow phase were pH dependent and at pH 7.5 the half-time was about 500 ms [20]. Addition of azide caused a significant acceleration of this phase, in a concentration- and pH-dependent manner.

Table I

Concentrations of various ions required for a two-fold acceleration of the second electron transfer[a]

Salt	pK[b]	$C_{1/2}^{Tot}$ (M)	$C_{1/2}^{Acid}$[c] (M)
$LiClO_4$	-	(0.09)[d]	-
NaCl	-	0.66	-
NaSCN	0.95	0.045	13×10^{-9}
$NaNO_2$	3.2	0.023	1.1×10^{-6}
KOCN	3.5	0.115	12×10^{-6}
$NaHCO_2$	3.8	0.08	16×10^{-6}
NaN_3	4.7	0.024	38×10^{-6}
CH_3CO_2Na	4.8	1.8	3.6×10^{-3}
$NaHCO_3$	6.4	0.07	5.1×10^{-3}
NH_4Cl	9.3	0.26	0.26

[a] Conditions as for Fig. 1

[b] pK values are taken from Handbook of Proton Ionization, Heats and Related Thermodynamic Quantities by J.J. Christensen, L.D. Hansen and R.M. Izatt (Wiley-Interscience Publ., 1976)

[c] Calculated from: $C_{1/2}^{Acid} = C_{1/2}^{Tot}[1 + 10^{(7.5-pK)}]^{-1}$. For pK values much less than the pH, this may be approximated by: $C_{1/2}^{Acid} = C_{1/2}^{Tot} \times 10^{(pK-7.5)}$

[d] See text

Other small anions were tested for their ability to promote the semiquinone disproportionation in L213DN mutant RCs, after two flashes (Fig. 2). Table I summarizes the results. In terms of total concentrations, the relative order of activity was nitrite ~ azide > thiocyanate > formate ~ bicarbonate > cyanate > chloride > acetate. Ammonium chloride was slightly more effective than NaCl. In the presence of 200 mM NH_4Cl, the rate of the semiquinone decay was slower at pH 8.0 than at pH 7.5, consistent with its action being independent of the concentration of the neutral base form. Lithium perchlorate also accelerated the decay rate, with a half-concentration similar to that of formate, but the concentration dependence was flatter than for any of the other salts and did not approach a linear relationship with unit slope.

4. DISCUSSION

Delivery of protons from the aqueous phase to Q_B in situ must involve some intraprotein transfer as the quinone is completely occluded by its immediate binding site in the L-subunit and by the H-subunit which lies over it. However, the protein environment over the Q_B binding site is quite polar and residues constituting possible pathways for proton transfer through the H- and L-subunits have been identified by Allen et al. [7] for *Rb. sphaeroides* and by Deisenhofer and Michel [21] for *Rps. viridis*. Examination of the X-ray structures shows the potential for H-bonded networks to be extensive, including crystallographically defined water molecules.

It has been well established in recent studies from this lab, that the L213DN and L212EQ/L213DN mutants suffer a total block in the normal proton transfer process necessary for double reduction of Q_B at alkaline pH [9,20]. At pH values above 7.5, the rate of transfer in these mutants is at least 10^4 times slower than in the wild-type. At lower pH values, the rate increases, eventually becoming linear with proton concentration. However, it is still 10^3 times slower than the Wt rate and we have suggested that the acceleration may represent passive penetration of the protein matrix by aqueous protons rather than the activation of a novel proton conduction path [9]. The stimulation of the proton limited second electron transfer event by azide and other weak acid anions is strong support for an inherent permeability of the protein structure to small molecules. If this is so, we should compare the activities of these agents in terms of the protonated species, determined from the pK and the prevailing pH (Table I). Omitting the very strong acid salts, chloride and perchlorate, the relative order of effectiveness now becomes: thiocyanate ≫ nitrite ~ cyanate ~ formate > azide ≫ acetate > bicarbonate. Thiocyanate is a fairly strong acid and calculation of its effectiveness in this way may be strongly distorted by any contributions

from other mechanisms. It is well known, for example, to have significant chaotropic activity. This is also evident in the effect of lithium perchlorate, a strong chaotrope.

For the weak acid species, the order of effectiveness is similar to that reported for reactivation of the proton pumping photocycle in certain mutants of bacteriorhodopsin [12,13]. The normal activity of this membrane protein involves a light driven *cis-trans* isomerization of a retinal cofactor, bound in a Schiff's base linkage to a lysine residue [22]. The isomerization drives the release of a proton from the protonated Schiff's base to one side of the membrane, followed by a directed reprotonation from the other side of the membrane via a specific aspartic acid residue, D96 [23,24]. From the recently published structural model derived from high resolution electron microscopy [25], the channel leading to this residue is quite hydrophobic and lacks residues that fit obviously into a proton-conducting motif. However, it probably does contain water, and an aqueous channel is likely. The native aspartic acid residue, which is protonated in the dark under all conditions examined to date, apparently provides an effective gate, acting as a unique proton donor to the deprotonated Schiff's base. Mutation of this residue to non-ionizable asparagine (mutant D96N), caused an effective block in proton delivery to the Schiff's base and a drastic inhibition of the photocycle of bacteriorhodopsin [23,24]. Addition of azide in the 0.001–1 M concentration range facilitated reprotonation of the Schiff's base in this mutant and accelerated the photocycle to rates faster than the wild-type ($\sim 800\ s^{-1}$) [12]. Furthermore, the direction of charge movement in the restored photocycle activity was the same as in the Wt, showing that the proton facilitation by azide was specifically directed from one side of the membrane [12]. Other anions also worked, in the following relative order for total concentrations: cyanate > azide > nitrite > formate > acetate. Thiocyanate had no detectable effect. When based on the concentration of protonated species, the order was: cyanate > nitrite > azide > formate ≫ acetate.

It is remarkable that proton delivery to Q_B in reaction centers is also very strongly gated by a single residue, Asp^{L213}. Mutation at this site was also partially alleviated by weak acids, although their activity was significantly smaller in RCs than in bacteriorhodopsin. For example, it is uncertain whether the rate can be completely restored in RCs, as the Wt rate is greater than $2 \times 10^3\ s^{-1}$, at pH 7.5, and in 1 M azide the restored rate was only $10\ s^{-1}$. However, for all active ions, except perchlorate, the rate was still increasing linearly at the highest concentrations. The generally lower activity of weak acids in RCs and the apparently greater discrimination on the basis of size, e.g. between formate and acetate, may indicate a rather tight structure in the protein surrounding the Q_B site. This is consistent with a highly hydrogen bonded structure for the H-subunit compared to the more apolar character of the proton donor channel in bacteriorhodopsin. Physical size limitations could also account for the surprisingly high activity of Cl^-. It has been suggested that, because of its small size and amphiphilicity, HCl can be a significant carrier of protons through membranes, accounting for the so-called 'chloride paradox' of high Cl^- exchange rates across lipid bilayers [26]. Anions also affect the photocycle of halorhodopsin, similarly to that of the D96N mutant of bacteriorhodopsin, and Lanyi has shown that fluoride is highly effective in proton donating activity; furthermore, the intrinsic rate of protonation by acid species was inversely related to the pK [11].

The behavior of the L213DN mutant is reminiscent of the so-called bicarbonate effect in photosystem II of oxygenic organisms (see [27] for review). In the absence of bicarbonate – usually depleted by treatment with various anions, especially formate [28], but also nitrite and azide (to a lesser extent) [29] and nitric oxide [30] – the functioning of the quinone electron acceptors is impaired and is restored by addition of bicarbonate. Some inhibition is seen on the first electron transfer to Q_B but it is more severe for the second electron transfer [31]. However, in spite of the strong functional resemblence between the acceptor sides of PS II and bacterial reaction centers, no effect of bicarbonate or bicarbonate depletion is seen in the turnover of bacterial reaction centers [32]. It is intriguing, therefore, that the L213DN mutant reaction centers studied here, show some similarity to bicarbonate-depleted PS II, and that bicarbonate partially alleviated their impaired function. The complete interruption in proton delivery seen in the bacterial L213DN mutant is comparable to the dramatic effects on the second electron transfer ascribed to bicarbonate depletion in PS II, and the more minor disturbances of the first electron transfer are also seen in both systems [20,30].

The obvious, but distant, homology between the L- and M-subunits of bacterial RCs and the D1 and D2 subunits of photosystem II allows some speculation concerning the structure of the Q_A and Q_B binding sites in PS II. A relatively detailed model of the Q_B binding site, proposed by Robinson and Crofts and coworkers [33,34], provides no carboxylic residues analogous to Glu^{L212} and Asp^{L213}, but places a histidine residue in a similar position. Although derived with due regard for the conservation of certain residues and for various residues involved in resistance to herbicides that compete with Q_B, the sequence alignments in this model are often arbitrary, and others are certainly possible (e.g. [35]). Nevertheless, it does suggest a possibility for bicarbonate involvement in the proton transfer pathway, acting in lieu of a fixed carboxylic acid. A role for bicarbonate in the protonation of Q_B^- and Q_B^{2-} has been proposed by Govindjee and co-workers [27,36],

and the possible significance of the specific protonation properties of bicarbonate was pointed out by Good nearly 30 years ago [37]. We suggest that a role for bicarbonate in PS II may be to function as part of the terminal proton transfer path to Q_B^- or Q_B^{2-}, analogous to Asp^{L213} in *Rb. sphaeroides* RCs [9,20]. The inhibitory nature of formate, and various other small anions, in PS II, could be due to the full involvement of their functional groups in binding, leaving nothing free to participate in proton transfer [36,37].

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