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FEBS Letters 341 (1994) 10–14

FEBS 13724

FEBS LETTERS

Flash-induced electrogenic reactions in the SA(L223) reaction center mutant in *Rhodobacter sphaeroides* chromatophores

Sergei I. Bibikov[a,b], Dmitry A. Bloch[a], Dmitry A. Cherepanov[c], Dieter Oesterhelt[b], Alexey Yu. Semenov[a,*]

[a]*A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, 119899 Moscow, Russian Federation*
[b]*Max-Plank-Institut für Biochemie, München, Germany*
[c]*Institute of Electrochemistry, Russian Academy of Sciences, Moscow, Russian Federation*

Received 12 January 1994

Abstract

The charge transfer events in the SA(L223) reaction center mutant *Rhodobacter sphaeroides* chromatophores were investigated by direct electrometry. Besides the primary charge separation, the small stigmatellin-sensitive electrogenic reaction due to the electron transfer from the primary to the secondary quinone acceptor in the reaction center complex was observed after the first flash. The second flash-induced electrogenic phase of the secondary quinone protonation and subsequent electrogenic reactions of the cytochrome bc_1 complex were much slower than those in chromatophores of the wild type. It is suggested that replacement of Ser-L223 by Ala impairs both specific proton-conducting pathways leading to the secondary quinone Q_B.

Key words: Bacterial chromatophore; Reaction center complex; Electrogenic reaction; Ubiquinone; Site-specific mutation; *Rhodobacter sphaeroides*

1. Introduction

The photosynthetic bacterial reaction center (RC) is an integral membrane pigment–protein complex in which primary photochemical reactions occur. Following flash excitation of RC, an electron is transferred from a special pair bacteriochlorophyll (P) via the primary quinone acceptor (Q_A) to the secondary quinone (Q_B), producing a tightly bound semiquinone Q_B^-. A second turnover of the RC results in the formation of ubiquinol at the Q_B-binding site. A second electron transfer is coupled to the uptake of two protons from the aqueous phase and subsequent release of ubiquinol from the Q_B-site (for review see [1]).

During the last years, significant progress has been achieved in clarifying which amino acid residues in the photosynthetic RC form the proton-conducting pathway(s) from the boundary of the hydrophobic layer to Q_B. It was shown in *Rhodobacter sphaeroides* RCs that Glu-L212, Asp-L213 and Ser-L223 are crucial for the proton transfer to Q_B [2–5]. According to the X-ray diffraction data of *Rb. sphaeroides* RCs, Ser-L223 forms a hydrogen bond with one of the carbonyl oxygens of Q_B [6]. Replacement of Ser-L223 by the nonprotonatable residue Ala inhibited the second electron transfer from Q_A^- to Q_B^- and it was concluded that this residue serves as a donor of the first proton to Q_B [5].

As previously shown [7,8] the reduction of secondary ubiquinone is accompanied by several electrogenic reactions, including the electron transfer in the reaction $Q_A^- \rightarrow Q_B$ and the proton uptake by Q_B^{2-} (for review see [9]). Recently, the electrometric technique has been used for the studies of electrogenic protonation reactions in chromatophores of the EQ(L212) RC mutant in *Rb. sphaeroides* [10]. In this study we examined the electrogenic reactions in chromatophores of the SA(L223) RC mutant, where Ser-223 of the L-subunit was replaced by Ala.

2. Materials and methods

Site-specific mutagenesis of the reaction center gene was done by the gapped-duplex method [11]. The 5.4 kb *Bam*HI-*Hin*dIII DNA fragment encoding *puf*BALM and cloned into the pMa/c vector [12] was digested with the restriction enzymes *Pvu*II and *Aat*II to produce a 100 bp gap in *puf* L, hybridized to a synthetic oligonucleotide GTCGGCTACGCCATCGGGAC carrying mutation and filled in with the DNA polymerase Klenow fragment. Mutated clones were selected by the hybridization procedure on nitrocellulose filters. The entire filled-in gap region was sequenced to ensure that no additional mutations had occurred and the *puf*BALM fragment was ligated into the broad host range mobilizable plasmid pRK404 [13]. Conjugation with *Rb. sphaeroides* using a helper *E. coli* strain S17-1 and the diparental filter mating procedure was described in [14]. *Rb. sphaeroides* strain GA *pufΔ*LMX 21/3 carrying the plasmid with the wild-type (WT) RC, or the plasmid carrying

*Corresponding author. Fax: (7) (095) 939 03 38.

SSDI 0014-5793(94)00108-8

the SA(L223) mutant RC were grown semi-aerobically at 30°C in the dark as described previously [12]. Chromatophores were isolated by French press disruption from the cells grown to the late log-phase. Measurements of membrane potential ($\Delta\psi$) generation by chromatophores adsorbed onto the surface of asolectin-impregnated collodion film were done and the kinetic data were processed as in [8,15].

3. Results

Fig. 1 illustrates the electric potential generation by dark-adapted chromatophores of the WT and the SA(L223) mutant induced by two laser flashes, 2 s apart. It is evident that the first flash predominantly causes a fast electrogenic phase ($\tau < 0.2$ μs) due to the charge separation between the special pair bacteriochlorophyll P and primary quinone acceptor Q_A (Fig. 1A, curve 1). After the second flash (Fig. 1A, curve 2) two additional phases appear: a faster phase due to Q_B protonation [7,8] ($\tau \simeq 100$ μs) and a slower phase due to electrogenic ubiquinol oxidation by the cytochrome bc_1-complex [15] ($\tau \simeq 20$ ms). The difference between the flashes is presented in Fig. 1A, curve (2-1).

A totally different pattern of the flash-induced $\Delta\psi$ generation is observed for the chromatophores of the SA(L223) mutant (Fig. 1B). While the first flash-induced kinetics of $\Delta\psi$ generation is similar to that observed for the chromatophores of the WT, the second flash kinetics were complex and could be fitted as at least a 3-exponential process with two negative and one positive electrogenic phase (cf. curves 1 and 2 from the Fig. 1A and 1B). The complexity of the second flash-induced process is clearly seen in the form of the difference between the flashes (Fig. 1B, curve 2–1).

Fig. 1C and D show the flash-induced $\Delta\psi$ generation in the presence of cytochrome bc_1-complex inhibitors, antimycin A and myxothiazol, by chromatophores from WT and the SA(L223) mutant, respectively. As we have previously shown [15], the bc_1-complex inhibitors have hardly any effect on the second flash-induced quinone electrogenic phase but cause a total disappearance of the slowest phase ascribed to the turnover of the bc_1-complex (Fig. 1C). In the case of SA(L223) mutant chromatophores these inhibitors do not significantly affect the second flash-induced negative phases but essentially inhibit the slowest positive phase (Fig. 1D). Fig. 1E shows the difference between traces (2–1) from Fig. 1A and 1C for the WT chromatophores and from Fig. 1B and 1D for the mutant chromatophores. These differences reflect the total electrogenesis provided by the functioning bc_1-complex. It is evident that the phase observed in mutant chromatophores is smaller and much slower compared with that of WT chromatophores.

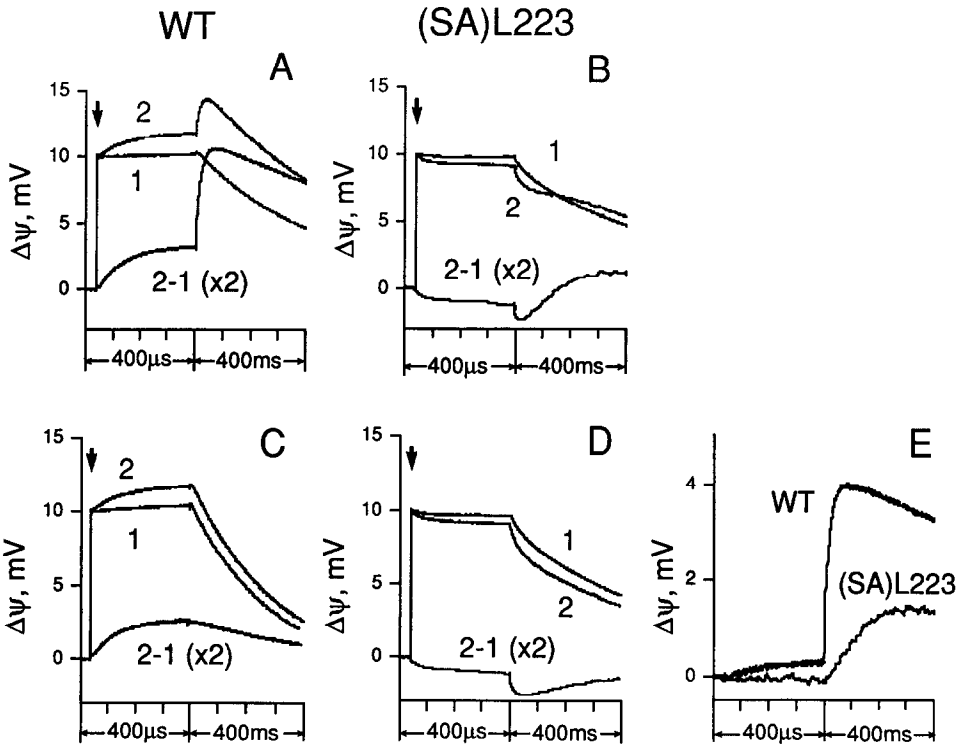

Fig. 1. The photoelectric responses of the dark-adapted chromatophores of the wild type (WT) and SA(L223) mutant upon the 1st and 2nd laser flashes and the difference between the photoelectric responses after the 2nd and 1st flash (2–1). (A,B) without inhibitors: (C,D) 3 μM myxothiazol and 3 μM antimycin A were added; (E) result of substracting difference (2–1) curves in the absence and presence of myxothiazol and antimycin. The WT curve corresponds to [A(2–1)–C(2–1)], SA(L223) curve – [B(2–1)–D(2–1)]. Curves (1) and (2) were normalized to 10 mV amplitude of the fastest charge separation phase. Incubation medium: 25 mM MOPS (pH 7), 50 mM KCl, 100 μM TMPD, 2 mM potassium ferrocyanide, E_h +300 mV. The dark interval between 1st and 2nd flashes – 2 s. Dark adaptation period – 10 min. Arrows indicate when the sample was illuminated.

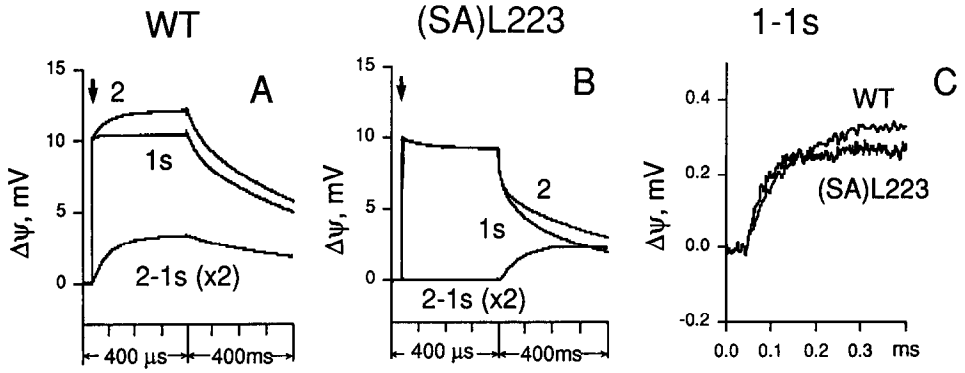

Fig. 2. The effect of stigmatellin on the photoelectric responses of dark-adapted chromatophores. (1s) indicate the first flash induced signals in the presence of stigmatellin, (2–1s) and (1–1s) are the difference between the response after the 2nd flash or 1st flash in the absence of stigmatellin and (1s), respectively. 50 nM or 20 μM stigmatellin was added to WT and SA(L223) mutant chromatophores, respectively. Conditions: same as for Fig. 1, except that 3 μM myxothiazol and 3 μM antimycin A were added to inhibit bc_1-complex-related electrogenic reactions.

Fig. 2 shows the photoelectric responses of the WT and mutant chromatophores (bc_1-complex inhibited) in the absence and presence of stigmatellin, a bc_1-complex inhibitor, which was also shown to specifically inhibit Q_B reduction (our recent unpublished observation). Fig. 2A and B show the second flash-induced signals without stigmatellin (curves 2) and the first flash-induced signals in the presence of inhibitor (curves 1S) for the WT and mutant chromatophores, respectively. As was previously shown [8], the difference between the second flash-induced kinetics in the absence of inhibitor and the first flash-induced kinetics in its presence reveals the second flash-induced electrogenic phase (further designated as phase BII) due to the protonation of doubly reduced Q_B. The important characteristic of the mutant chromatophores is the lack of difference between curves 2 and 1S in the submillisecond time scale. This difference is quite clearly observed at a much slower time scale, thus reflecting the great retardation of the BII phase in the mutant compared to the WT (cf. curves 2–1S in Fig. 2A and 2B).

According to our results (not shown), neither terbutryn nor atrazine affected the functioning of mutant chromatophores, while *o*-phenanthroline and stigmatellin were effective at higher concentrations compared with WT. It is noteworthy that the mutant T1 of *Rhodopseudomanas viridis* with SA(L223) and RH(L217)

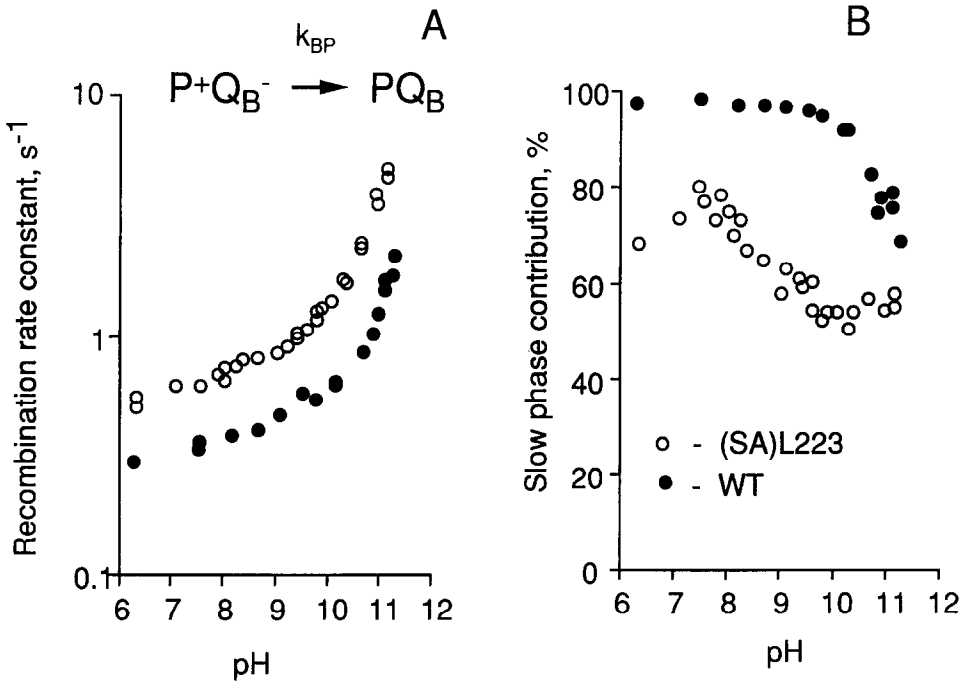

Fig. 3. The pH dependencies of (A) the charge recombination rate constant (k) of the state $P^+Q_B^-$ and (B) the contribution of $P^+Q_B^-$ decay phase amplitudeto the P^+ re-reduction signals after a flash without exogenous donors. Absorption changes at 603 nm were monitored in chromatophore suspension and the signals were treated as a sum of 2 exponentials.

substitutions was resistant to terbutryn and atrazine, but sensitive to *o*-phenanthroline [16].

It was shown earlier [8], that a small but measurable electrogenic phase could be detected by subtracting the first flash-induced $\Delta\psi$ kinetics in the presence of Q_B inhibitor from those in its absence. This phase (designated as phase BI) was ascribed to the first electron transfer from Q_A^- to Q_B [8,9]. Fig. 2C illustrates the comparison of the differences between the first flash-induced $\Delta\psi$ kinetics in the presence and absence of stigmatellin (1–1S) for the WT and SA(L223) mutant chromatophores. It can be seen that the relative magnitudes of the phases BI are similar, but the kinetics are faster for the mutant chromatophores.

The pH dependence of the rate constant of the charge recombination of the back reaction: $P^+Q_B^- \rightarrow PQ_B$ (k_{BP}) and the occupancy of the Q_B-site were measured in the suspension of chromatophores by monitoring the kinetics of P^+ reduction at 603 nm. As can be seen in Fig. 3A that at pH 7.5 the k_{BP} value is almost twofold higher for the mutant (0.6 s^{-1}) compared to the WT chromatophores (0.33 s^{-1}). This result is in contradiction to the data obtained by Paddock et al. [5] on SA(L223) RCs, who found that k_{BP} for the mutant RCs was somewhat lower than in the native RCs. The reason for the discrepancy is not clear, though it might be explained by the difference in the preparations used, i.e. RCs [5] and chromatophores (this work). It is also noteworthy that in mutant T_1 of *Rps. viridis* with SA(L223) and RH(L217) substitutions, the kinetics of $P^+Q_B^-$ recombination was faster compared to the WT [17], which our results corroborate.

Fig. 3B demonstrates the pH dependence of the occupancy of the Q_B-site, which was determined from the portion of the slow phase of the P^+ recovery monitored at 603 nm. It is seen that the occupancy rate was highest (80%) at about pH 7.5 for the SA(L223) chromatophores and decreased with alkalinization. In comparison, the occupancy rate is > 95% at pH < 9.5 for the WT chromatophores.

4. Discussion

For the mutant RC's carrying the SA(L223) mutation, both electron transfer and proton uptake rates in the reaction $Q_A^-Q_B^- + 2H^+ \rightarrow Q_AQ_BH_2$ were greatly reduced, whereas the rate constant for the first electron transfer $Q_A^-Q_B \rightarrow Q_AQ_B^-$ was found to be even higher than in the WT RC's [5]. It was also shown that the kinetics of the proton uptake by SA(L223) RC's was clearly biphasic with the rapid $k > 500$ s^{-1}) and slow ($k \cong 4$ s^{-1}) components, reflecting the rapid uptake of 1.0 H^+ per RC and additional slow uptake of 1.0 H^+ per RC [5].

Our results on the measurements of the flash-induced $\Delta\psi$ generation confirm the crucial role of the Ser-L223 in the Q_B protonation. A small first flash-induced electrogenic phase BI is sensitive to stigmatellin ($\tau \cong 20$ μs, magnitude ≅ 3% of $P^+Q_A^-$ phase in SA(L223) chromatophores at pH 7) and seems to occur due to the electrogenic character of the first electron transfer from Q_A^- to Q_B. It is noteworthy that the BI phase is faster in the mutant than in WT chromatophores ($\tau \cong 80$ μs at pH 7.0). This observation is in agreement with the results obtained for the isolated SA(L223) mutant RC from *Rb. sphaeroides* [5] and whole cells ofthe *Rps. viridis* T1 mutant [18]. The absence of the BI phase in the kinetics of a second flash-induced $\Delta\psi$ response in mutant chromatophores is obviously due to the retardation of the second electron transfer $Q_A^-Q_B^- \rightarrow Q_AQ_B^{2-}$ [5].

Besides the BI phase an additional slower negative phase was revealed in the difference between the second and the first flash-induced photoelectric signals generated by SA(L223) mutant chromatophores (Fig. 1 B,D). We suppose that this phase is due to the recombination of the primary dipole $P^+Q_A^-$ in the portion of RC's, in which the photooxidized P^+ was not reduced by TMPD. This phase was not observed with WT chromatophores since the second electron transfer to Q_B is much faster than in the mutant, and the portion of RC's in which the $P^+Q_A^-$ recombination occurs is negligible. The occurrence of the negative phase impedes a quantitative analysis of the kinetic data. This may be resolved by subtracting the first flash-induced signal in the presence of stigmatellin from the second flash-induced signal in its absence (Fig. 2B). Since a negative phase was observed on both curves, subtraction eliminates this component.

The second flash-induced quinone electrogenic phase BII is about 1000 times slower in the mutant SA(L223) in comparison to that in WT chromatophores ($k = 10$ s^{-1} in the mutant versus $k = 10^4$ s^{-1} in the WT at pH 7.0). Our value of rate constant ($k = 10$ s^{-1}) is about 2.5 times smaller than the k value for the $Q_A^-Q_B^- \rightarrow Q_AQ_B^{2-}$ transfer in the *Rps. viridis* mutant T1 [18] and about 2.5 times faster than that for the SA(L223) mutant RC in *Rb. sphaeroides* [5]. Electrogenic phase BII appears as a result of the $2H^+$ uptake by Q_B^{2-}. The slow kinetics in the mutant may be explained either by slow uptake of both H^+ or only one, in the latter case the fast uptake of a single proton per RC, observed by Paddock et al. [5] should be non-electrogenic.

It seems reasonable to compare the electrogenic data on SA(L223) mutant chromatophores with those of EQ(L212) chromatophores obtained recently by Shinkarev et al. [10]. Ser-L223 and Glu-L212 are thought to be the immediate donors of the first and second protons to Q_B, respectively [1]. In the EQ(L212) mutant [10] the BII phase relative magnitude was about two times smaller in comparison to that in WT chromatophores but was characterized by similar kinetics. A phase, attributable to the bc_1-complex turnover, was not observed at pH 7.6 since the Q_BH^- generated in EQ(L212) RC did

not receive the second proton within a reasonable time period and, hence, was not able to leave the RC to be oxidized by the bc_1-complex. Only at pH 5 did the electrogenic reactions of the bc_1-complex appear [10], probably due to the acceleration of the Q_BH^- protonation by passive penetration of H^+ ions through the protein matrix [19].

In contrast to the results obtained with the EQ(L212) mutant, we did not observe any second flash-induced electrogenesis in the millisecond time scale even at pH 5.0 (not shown). In the SA(L223) mutant both phase BII and the phase attributed to the bc_1-complex turnover were characterized by τ values of $\cong$ 100 ms. One might speculate that this observation reflects the difference in the specific pathways for the first and second protons leading to the distal and proximal carbonyls of the Q_B quinone ring. Actually, the replacement of the protonatable residue Glu-L212 by Gln does not interfere with the first proton transfer through Ser-L223, while the substitution of Ser-L223 by Ala totally prevents specific transfer of both protons to Q_B. Thus, the fast binding of a H^+ in the RC in the SA(L223) mutant [5] might be due to the protonation of an unidentified residue, located on the water–membrane–protein surface. This residue serves as a secondary proton donor either to Ser-L223 or to Glu-L212, or to both. The rates of the BII phase and subsequent electrogenic oxidation of QH_2 by the bc_1-complex in the SA(L223) mutant are probably limited by the passive penetration of protons through the protein matrix [19] or by some alternative nonspecific proton-conducting pathway.

A characteristic feature of the SA(L223) mutant is the faster recombination rate in comparison with that of the WT even though the pH dependency is essentially the same as in the WT (Fig. 3a). A second feature is that the apparent pK calculated here as the pH value at the point of the slope change was the same both for SA(L223) and WT (around 9.5). The change of the slope is related to the ionization of the reacting group (here Glu-L212 [1]). We can assume that the pK of this group was not changed in a mutant SA(L223). Electrostatic estimations support this assumption. Indeed, the distance between the OH of the Ser-L223 and the COO^- of the Glu-L212 as seen from the crystallographic data is more than 8 Å [6] and the direct electrostatic effect of the OH dipole is less than 10 mV. On the other hand, Ser-L223 seems to form a hydrogen bond with the quinone, so that the OH dipole is oriented to the center of the quinone and the distance between the OH of the Ser-L223 and the center of the quinone ring is less than 5 Å. The result is a shift in E_m at Q_B of approximately 20–30 mV in the absence of Ser-L223 which corresponds to a twofold increase of the k_{BP} in the SA(L223) mutant.

Acknowledgements: We wish to thank Prof. V.P. Skulachev and Prof. L.A. Drachev for valuable discussions and support of the project, Dr. A. Mulkidjanjan for participation in some experiments and Dr. Ju. Churin and Dr. M.V. Bibikova for the help in genetical analysis of the bacterial mutant strains. Dr. J. Shiozawa is greatfully acknowledged for the help in preparation of the paper. This work was supported by a grant from the Max-Planck-Society (Germany) and from the Russian State Programm 'Frontiers in Genetics'.

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