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Sulfhydryl modifying reagents inhibit Q_A^- oxidation in reaction centers from *Rhodobacter sphaeroides* and *capsulatus*, but not *Rhodopseudomonas viridis*

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Abstract

The effects of various sulfhydryl-modifying reagents on reaction centers (RCs) from purple photosynthetic bacteria have been examined, with particular emphasis on the activity of the acceptor quinones, Q_A and Q_B, comprising the two electron gate. Mercurial reagents, especially p-chloromercuribenzenesulfonate (pCMBS), were effective in inhibiting Q_B function in RCs from *Rhodobacter sphaeroides* and *Rb. capsulatus*, but not in *Rhodopseudomonas viridis*. The inhibition was fully reversible by dialysis against dithiothreitol (DTT). The effect on Q_B function was not an apparent one mediated by an alteration in the redox potential of Q_A. N-ethylmaleimide (NEM) had no effect on any of the quinone functions, even at very high concentrations. Comparison of the X-ray structures of the RCs from *Rb. sphaeroides* and *Rp. viridis* and the known amino acid sequences for all three bacterial RCs suggest that a cysteine residue at position 108 in the L subunit of the *Rhodobacter* species is the most likely candidate for the site of action of the mercurial reagents. This was strongly supported by the absence of any effect of pCMBS on a site specific mutation of *Rb. sphaeroides* (L108CS) with residue L108 changed from cysteine to serine. These results imply a long distance (>20 Å) effect on the functioning of Q_B, perhaps involving a relatively gross structural alteration.

Introduction

The primary events of photosynthesis that convert light energy into the free energy of a charge separation, take place in a membrane bound pigment-protein complex called the reaction center (RC). RCs from the photosynthetic bacterium, *Rhodobacter sphaeroides*, consist of three subunits, L, M and H. L and M are partially homologous and form a heterodimer that binds all the cofactors of the photochemical and charge stabilizing reactions: four bacteriochlorophylls (BChl), two bacteriopheophytins, two ubiquinones and one atom of high spin ferrous iron (Okamura et al. 1982, Allen et al. 1988a,b). Electron transfer in the RCs is initiated from the excited singlet state of the primary donor, P, a dimer of BChl, and passes through a short chain of redox intermediates before arriving at the primary quinone, Q_A, forming the metastable state $P^+Q_A^-$ (Kirmaier and Holten 1987). The electron can then be transferred to the secondary quinone, Q_B, when available, forming $P^+Q_AQ_B^-$. In the absence of an electron

donor to P^+, these charge separation states decay by intramolecular recombination. $P^+Q_A^-$ can recombine directly but $P^+Q_AQ_B^-$ decays by equilibration with $P^+Q_A^-Q_B$ which recombines directly (Wraight 1979, Wraight and Stein 1983, Crofts and Wraight 1983, Kleinfeld et al. 1984):

$$PQ_AQ_B \underset{k_{QA}}{\overset{h\nu}{\rightleftharpoons}} P^+Q_A^-Q_B \overset{K_2}{\longleftrightarrow} P^+Q_AQ_B^-$$

In the presence of an electron donor to P^+, the state $PQ_AQ_B^-$ is formed. This can be activated by a second flash leading to the reduction of Q_B to the quinol state. This then unbinds and is replaced by another quinone (Crofts and Wraight 1983). This is the normal mechanism of exporting reducing equivalents from the RC:

First flash: $PQ_AQ_B \xrightarrow{h\nu} P^+Q_A^-Q_B \overset{D \quad D^+}{\rightleftharpoons} PQ_AQ_B^-$

Second flash: $PQ_AQ_B^- \xrightarrow{h\nu} P^+Q_A^-Q_B^- \overset{D \quad D^+}{\rightleftharpoons} PQ_AQ_B^{2-} \rightleftharpoons PQ_AQ_B$ (Q / QH_2)

(The details of H^+-ion uptake necessary for the formation of QH_2 are complex and are omitted). This behavior results in binary oscillations in the formation and disappearance of semiquinone, which are readily seen (Vermeglio 1977, Wraight 1977), and the activity is referred to as a two-electron gate.

The structures of the RCs from *Rb. sphaeroides* and *Rhodopseudomonas viridis* are now known from X-ray diffraction studies (Deisenhofer et al. 1985, Michel et al. 1986a, Allen et al. 1988a,b) and a great deal of detail can be surmised about reaction processes and electron transfer pathways. Needless to say, however, the structures are complex and the involvement of specific amino acid residues may not be obvious. We have tested various covalent modifying agents for clues to functional relationships in RC activities and have found that certain sulfhydryl-modifying agents have a rather specific effect on the activity of the two- electron gate of isolated bacterial RCs.

Methods

Reaction centers were isolated following earlier procedures, from *Rb. sphaeroides*, R26 (Wraight and Stein 1983, Maróti and Wraight 1988), *Rb. capsulatus* (Prince and Youvan 1987), and *Rp. viridis* (Prince et al. 1977). For some experiments, *Rb. sphaeroides* RCs were incorporated into unilamellar, egg phosphatidyl choline vesicles, by detergent dilution achieved by rapid injection into a suspension of preformed vesicles, followed by several freeze–thaw cycles. Unilamellar vesicles were made by repeated French pressing of multilamellar liposomes, formed by hydration of a dried lipid mixture (Barenholz et al. 1979). The lipid mixture was prepared by drying down phospholipid and ubiquinone (Q-10, in 1% mole ratio) from ether.

Routine treatment of RCs with p-chloromercuribenzenesulfonic acid (pCMBS) was carried out with 20 μM RCs, 0.06% Triton X-100, 10 mM phosphate, pH 7.0, 0.5 mM pCMBS, at 20°C. Chloride ion should be avoided in the use of mercurial agents (Riordan and Vallee 1972), and we found the effective concentration of pCMBS decreased 10-fold in 10 mM phosphate compared to 100 mM NaCl. Samples were diluted to 1 μM RCs for assay. Dialysis of samples was performed overnight against 10 mM phosphate, pH 7.0, 0.03% lauryldimethylamine-N-oxide (LDAO), with or without 50 mM dithiothreitol (DTT).

Sulfhydryl reagents were obtained as follows: p-chloromercuribenzoic acid (pCMB), p-chloromercuribenzenesulfonic acid (pCMBS), mersalyl acid (o-[(3-[hydroxymercuri]-2-methoxypropyl) carbamoyl]-phenoxyacetic acid) and N-ethylmaleimide, from Sigma; monobromobimane from Calbiochem.

The experimental apparatus was a kinetic spectrophotometer of local design. Recombination of the $P^+(Q_AQ_B)^-$ states was monitored by the decay of the P^+ absorbance signal at 430 nm. The decay kinetics were fit to a biexponential and a constant using a modified Marquardt analysis (Sebban and Wraight, 1989, Gao et al. 1990). In all cases the resulting residuals were flat. The stable semiquinone species, Q_A^- and

Q_B^-, formed in the presence of a donor to P^+, were monitored at 450 nm (Vermeglio 1977, Wraight 1977). For RCs from *Rb. sphaeroides* and *capsulatus*, the donor used was 100 μM ferrocene, with 0.5 mM potassium ferrocyanide to rereduce the ferrocinium species formed (Maróti and Wraight 1988). For RCs from *Rp. viridis*, the donor system (to rereduce the bound cytochromes) was 200 μM N, N, N', N'-tetramethyl-p-phenylenediamine (TMPD) and 200 μM 2,3,5,6-tetramethyl-p-phenylenediamine (DAD) (Shopes and Wraight 1985). Oxidation of cytochrome c, as a donor to P^+ in RCs from *Rb. sphaeroides*, was monitored at 550 nm. Cytochrome was added from a stock prereduced by addition of stoichiometric amounts of sodium dithionite.

The mutant L108CS was made by site directed mutagenesis of residue L108 (Cys→Ser), using the Kunkle method (Kunkle 1986), and expressed in a RC^- deletion strain of *Rb. sphaeroides*, strain Ga, generated earlier (Takahashi et al. 1989). The mutagenesis was performed on the 172 bp Kpn I coding region from the L-subunit gene of the *puf* operon, originally isolated on a 13 kbp BamH I genomic fragment by Williams et al. (1983), and was directed by the sense-strand oligonucleotide 5'-TCGAAATC*TCC*CGTAAGCT-3', replacing the native codon TGC (cysteine) with TCC (serine). The mutation was confirmed by sequencing, and incorporated into a 4.5 kbp Pst I restriction fragment containing the entire *puf* operon, in pUC19. The mutant *puf* operon was then cut out of the polylinker as an EcoR I-Hind III restriction digest, cloned into the broad host-range plasmid, pRK415, under the control of the lac z promotor (Takahashi and Wraight, unpublished), and transferred to the deletion strain of *Rb. sphaeroides* by diparental mating with *E. coli*, strain S17–1 (Simon et al. 1983).

Results

Charge recombination kinetics

The effect of pCMBS on the charge recombination of *Rb. sphaeroides* RCs supplemented with ubiquinone, is shown in Fig. 1A. As generally encountered, the P^+ decay kinetics were well fit

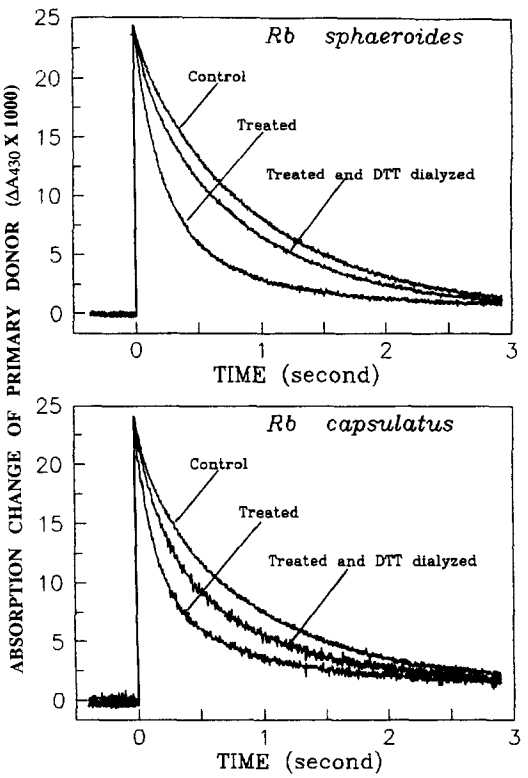

Fig. 1. The effect of pCMBS on the recovery kinetics of oxidized primary donor (P^+) in the absence of exogenous donors. Control curve: no treatment; Treated: 20 μM RCs treated with 0.5 mM pCMBS, for 1 h, followed by dialysis in the absence of DTT; Treated/Dialysed: 20 μM RCs treated with 0.5 M pCMBS, for 1 h, followed by dialysis in the presence of 50 mM DTT. All samples were assayed after dilution to 1 μM RCs in 10 mM phosphate, pH 7.0, 0.06% Triton X-100, 10 μM Q-10. **A**: *Rb. sphaeroides* RCs; **B**: *Rb. capsulatus* RCs.

by two exponential components and a small constant term (≤5%). The small fast component ($t_{1/2} \sim 90$ ms) is considered to arise from RCs lacking Q_B activity (Wraight 1979, Kleinfeld et al. 1984). The main part of the decay (90%) exhibited a halftime of about 700 ms. After treatment with 0.5 mM, the slow phase of the P^+ decay was substantially decreased (30%), but dialysis against DTT largely restored the control behavior with 88% slow phase.

Figure 1B shows the effect of pCMBS on isolated RCs from *Rhodobacter capsulatus*. The behavior is essentially identical to that seen for RCs from *Rb. sphaeroides*, although the reversibility by dialysis against DTT was less satisfactory (80% slow phase in the control, 30% after treatment with 0.5 mM pCMBS, 65% after

treatment and dialysis vs. DTT). This is consistent with the generally lower stability of RCs from this species compared to *Rb. sphaeroides* (Prince and Youvan 1987). The larger 'constant' component in the decay kinetics for RCs from *Rb. capsulatus*, compared to *Rb. sphaeroides*, is probably due to a greater susceptibility of this species to direct oxidation of Q_A^- by external oxidants, including oxygen, leaving $P^+Q_AQ_B$ as a long-lived species. This reaction has been previously documented for RCs from *Rb. sphaeroides* and *Rp. viridis* (Shopes and Wraight 1986).

Figures 2 and 3A show the dependence on pCMBS concentration and incubation time for the effect on the slow phase of P^+ decay, in isolated RCs. Based on these results, we chose 0.5 mM pCMBS and 1 h incubation time as the standard treatment. This yielded approximately 70% loss in the amplitude of the slow phase, with good reversibility. Examination of Figs. 2 and 3A indicates that the maximal effect probably approaches 100%, but the reversibility also declined slowly. Figure 3B shows that pCMBS was similarly active on RCs incorporated into phospholipid vesicles. On the other hand, this

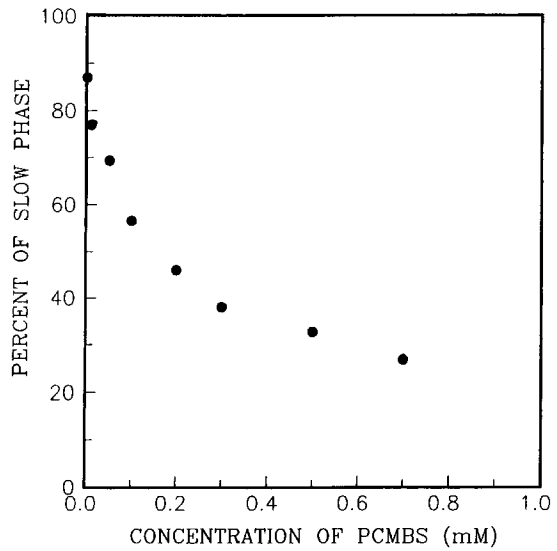

Fig. 2. The concentration dependence of the effect of pCMBS on charge recombination in isolated RCs from *Rb. sphaeroides*. Treatment with variable concentrations of pCMBS was performed as for Fig. 1, but without dialysis. RCs were removed from the incubation medium and diluted directly into the cuvette for determination of the P^+ recovery kinetics. Assay conditions as for Fig. 1.

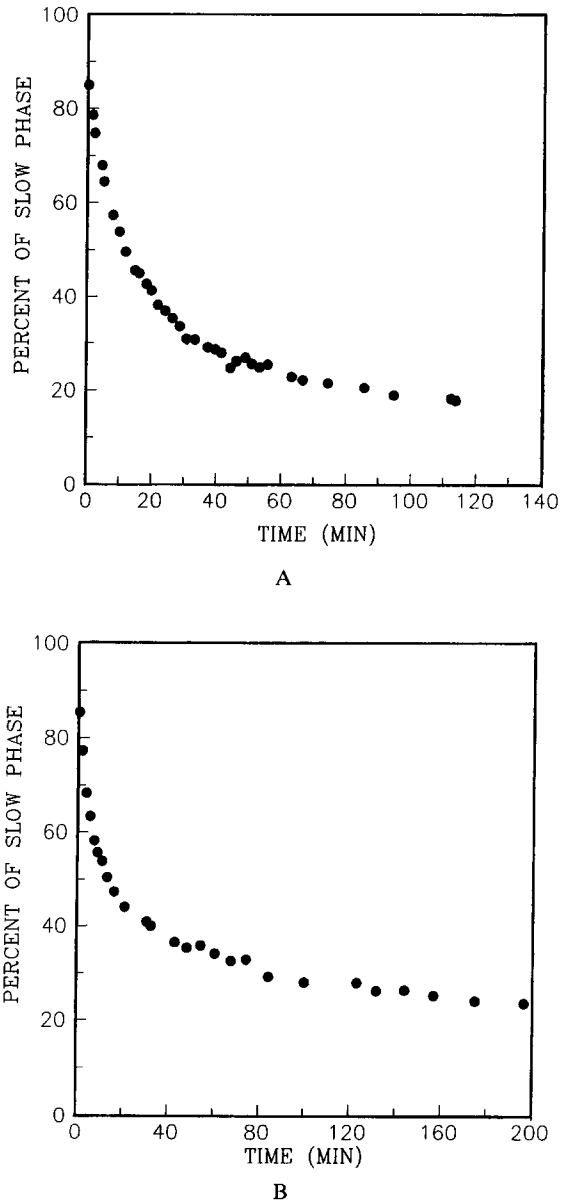

Fig. 3. The time dependence of the effect of pCMBS in isolated RCs from *Rb. sphaeroides*. RCs were treated in the cuvette, at 1 μM concentration – 0.5 mM pCMBS was present in the assay buffer. **A**: isolated RCs in assay buffer as described for Fig. 1. **B**: isolated RCs incorporated into egg phosphatidyl choline vesicles supplemented with Q-10; assay buffer: 10 mM phosphate, pH 7.0.

reagent had no effect on the decay of P^+ in chromatophores, up to 1.5 mM (not shown).

Two other mercurial reagents were tried – p-mercuribenzoic acid (pCMB) and mersalyl acid. Both acted similarly to pCMBS, but required

higher concentrations (5 mM) and had a lesser maximal effect. Neither agent had any effect on RCs in chromatophores. N-ethylmaleimide, another general sulfhydryl-modifying reagent, had no detectable effect on isolated RCs, even upto 20 mM; neither did it have any protective effect against subsequent treatment with pCMBS. Monobromobimane, a fluorescent sulfhydryl reagent, also had no inhibitory or protective effects on reaction centers. However, when monobromobimane-treated RCs were run on a polyacrylamide-SDS gel, the H subunit was clearly fluorescent.

Semiquinone oscillations

As described in the Introduction, in the presence of a donor to P^+ a series of flashes elicits binary oscillations in the formation and disappearance of semiquinone signal. Figure 4 shows these oscillations in RCs from *Rb. sphaeroides*, with ferrocene as donor. Figure 5 shows a similar measurement on RCs from *Rp. viridis*, which have a bound cytochrome *c* subunit as a rapid, endogenous donor to P^+. The cytochrome *c* absorbance changes were eliminated by the pres-

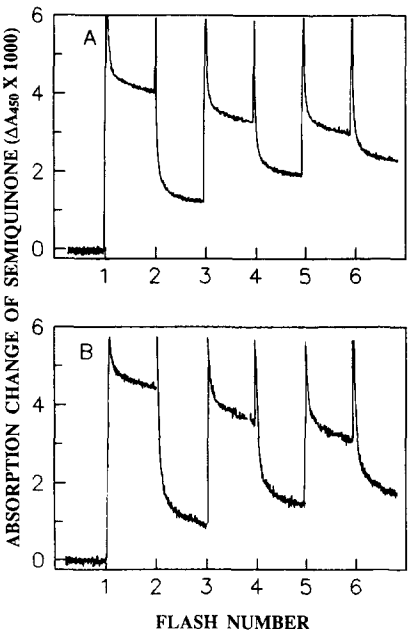

Fig. 5. The effect of pCMBS on semiquinone oscillations in isolated RCs from *Rp. viridis*. Conditions: approx. 1 μM RCs in 10 mM CHES buffer, pH 10.0, 0.06% Triton X-100, 10 μM Q-10, 200 μM TMPD and DAD as reductant of the bound cytochromes. **A**: Control – untreated RCs; **B**: RCs (20 μM) treated with 0.5 mM pCMBS, as described in Methods, followed by dialysis without DTT.

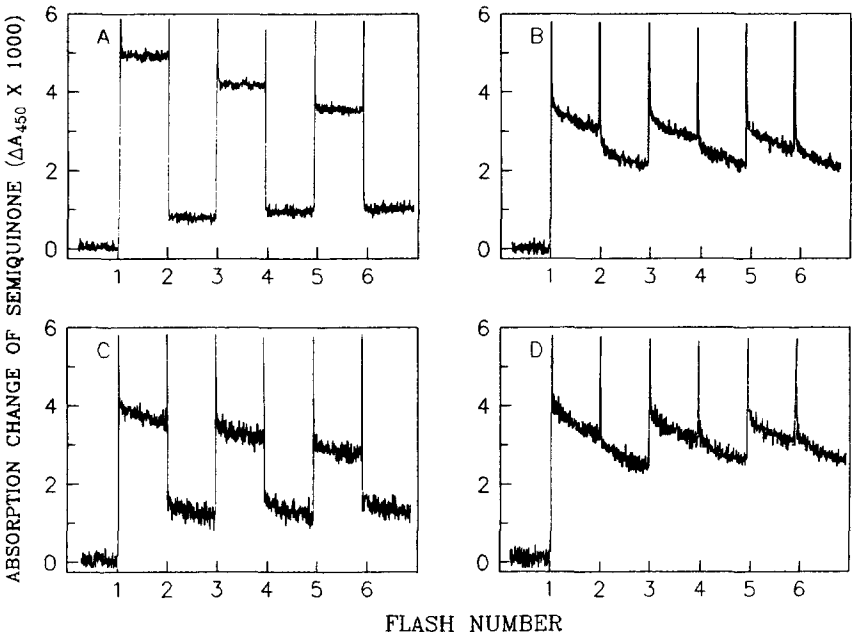

Fig. 4. The effect of pCMBS on semiquinone oscillations in isolated RCs from *Rb. sphaeroides*. Conditions: approx. 1 μM RCs in 10 mM phosphate, pH 7.0, 0.06% Triton X-100, 10 μM Q-10, 100 μM ferrocene, 0.5 mM potassium ferrocyanide.; flash period: 0.5 s. **A**: Control – untreated RCs; **B**: RCs (20 μM) treated with 0.5 mM pCMBS, as described in Methods; **C**: As for **B**, followed by dialysis against 50 mM DTT; **D**: As for **C**, but dialysed without DTT.

ence of TMPD and DAD as tertiary donors to Cyt c^+ (Shopes and Wraight 1985). It is clear that RCs from *Rb. sphaeroides* are severely inhibited by pCMBS treatment, and that this inhibition is relieved by dialysis only when DTT is present. Very similar results were obtained with RCs from *Rb. capsulatus*, although the reversibility was less satisfactory, as also seen in the charge recombination measurements described above. In contrast, the semiquinone oscillations in *Rp. viridis* appeared to be almost unaffected by pCMBS (Fig. 5). It should be noted that the *Rps. viridis* measurements were performed at pH 10.0 to slow down the rate of electron transfer from Q_A to Q_B and minimize the occurrence of double turnovers during a flash (Shopes and Wraight 1985). The effect of pCMBS treatment on *Rb. sphaeroides* RCs is not changed by incubation at this high pH.

Cytochrome oxidation

The extent of oxidation of cytochrome on the second flash, compared to that on the first, is a measure of the functionality of Q_B in reoxidizing Q_A^-. Figure 6 shows the ratio of the two, $\Delta 2/\Delta 1$, as a function of time between flashes. In the control the ratio was large (0.83) even at the shortest times measured (30 ms). The ratio slowly increased to about 0.95, with a halftime of 35 s. After treatment with pCMBS, the ratio $\Delta 2/\Delta 1$ was much smaller (0.28) at short times, but increased to 1 with a similar halftime (45 s).

Also shown in Fig. 6 is a measure of the semiquinone absorbance signal at 450 nm, in treated RCs, determined separately with ferrocene as donor. On the slow time scale represented by the insert to Fig. 6, P^+ has already decayed due to donation from ferrocene. The data are presented as the difference, or 'deficit', between the semiquinone amplitudes after the first and second flashes, relative to the first flash amplitude. This is a measure of the Q_B^- content, formed on the first flash and capable of disappearing on the second flash with the formation of quinol. The constancy of this ratio over a wide time range shows that the residual Q_B activity in treated RCs (~20%) is normal, i.e., Q_B^- is very long-lived ($t_{1/2} > 60$ s), as is well known for this redox species (Vermeglio 1977, Wraight 1977, Kleinfeld et al. 1984).

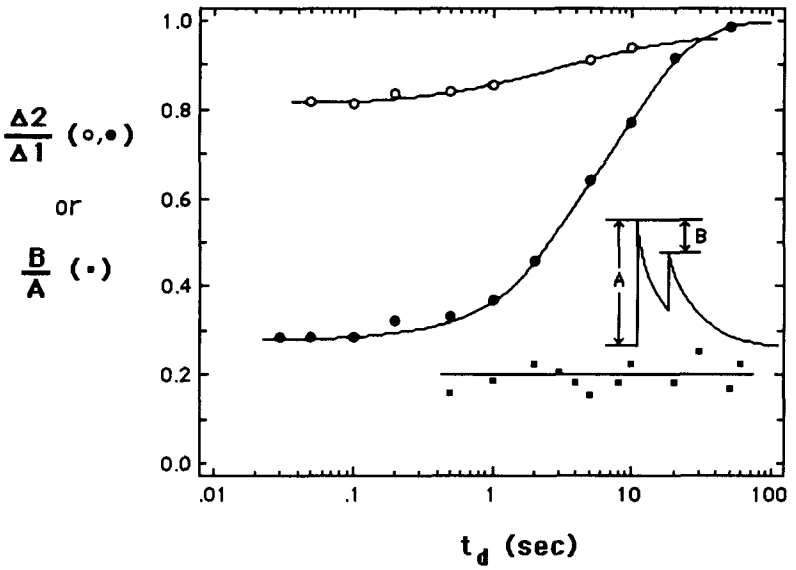

Fig. 6. The effect of pCMBS on the recovery of second flash capability in isolated RCs from *Rb. sphaeroides*. (●, ○) Cytochrome oxidation measured at 550 nm; the ratio of oxidation after flash 1 and flash 2-$\Delta A_{550}(2)/\Delta A_{550}(1)$; (○) untreated RCs; (●) pCMBS treated RCs. ■ Semiquinone turnover in pCMBS-treated RCs, measured at 450 nm; the amplitudes of the semiquinone absorbance signal (after removal of the P^+ contribution by rapid donation) after the first and second flashes are presented as the ratio of the second flash 'deficit' (B) to the first flash amplitude (A), as shown in the insert. Conditions: 1 μM RCs (or pCMBS-treated RCs) in 10 mM phosphate, pH 7.0, 0.06% Triton X-100, 10 μM Q-10, 100 μM cytochrome *c*.

Mutant studies

As described in the Discussion, we were led to consider cysteine L108 as the most likely target for the inhibitory action of the mercurial sulfhydryl reagents. We therefore constructed a mutant (L108CS), by site directed mutagenesis, in which Cys^{L108} was replaced by serine. As shown in Fig. 7, RCs from L108CS were unaffected by pCMBS, as monitored either through the recombination kinetics or through the semiquinone oscillations.

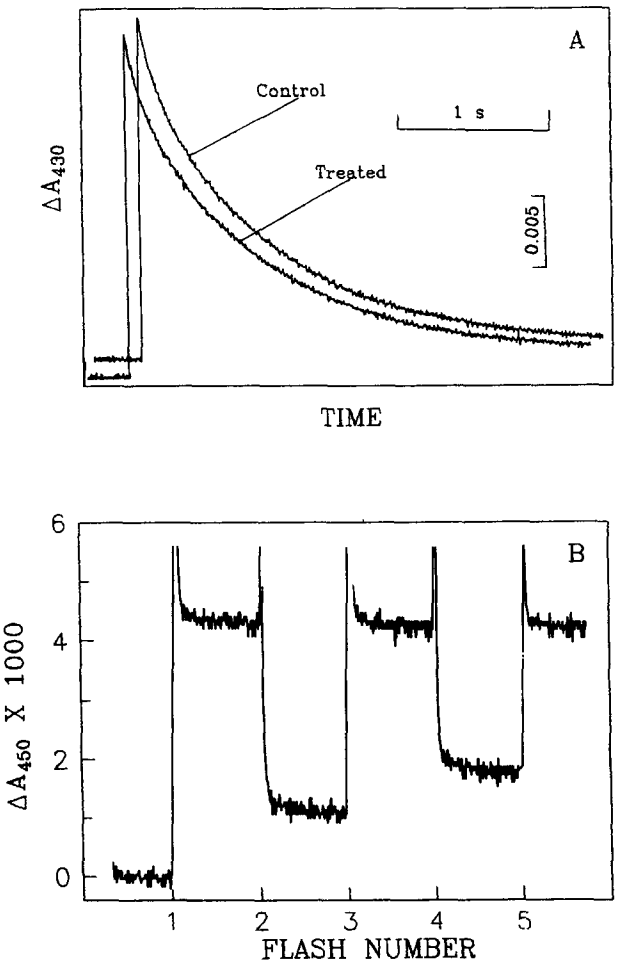

Fig. 7. The effect of pCMBS on Q_B function in isolated RCs from Rb. sphaeroides mutant L108CS, with CysL108 replaced by serine. **A.** Charge recombination kinetics in untreated and pCMBS-treated, dialyzed RCs. Conditions as for Fig. 1. **B.** Semiquinone oscillations in pCMBS-treated, dialyzed RCs. Conditions as for Fig. 4D.

Discussion

The various effects of pCMBS treatment on RCs from *Rb. sphaeroides* and *Rb. capsulatus* shown in this study, are all indicative of a primary effect on the activity of Q_B in the acceptor quinone complex: the decrease in the amplitude of the slow phase of P^+ recovery (Figs. 1–3); the loss of semiquinone oscillation amplitude in the presence of donor (Fig. 4); and the small value of the $\Delta 2/\Delta 1$ ratio for the extent of cytochrome oxidation (Fig. 6). Comparison with the long-lived semiquinone state (Q_B^-) of the remaining, normal RCs (Fig. 6) suggests that the slow increase in the cytochrome ratio was due to the direct oxidation of Q_A^-. As expected, this ratio, at short times, was much larger in the control RCs. If the acceptor quinone complex were fully operational, the ratio should be about 0.93, reflecting only the electron transfer equilibrium constant between $Q_A^-Q_B$ and $Q_AQ_B^-$ ($K_2 = 12–15$) (Wraight 1979, Wraight and Stein 1983, Kleinfeld et al. 1984). The observed control value was about 0.83, the shortfall from 0.93 being generally considered to represent RCs that are damaged in some way such that their Q_B activity cannot be reconstituted by added quinone (Kleinfeld et al. 1984). This is variable from one preparation to another, but is usually less than 10% in a good preparation. The same proportion is seen in the fast phase amplitude of the recombination kinetics in untreated RCs (Fig. 1).

A striking feature of the results presented here, is the lack of effect of pCMBS on RCs from *Rp. viridis*. This fortuitous result allows us to pinpoint the target of pCMBS, assuming it to be a cysteine residue. The number of cysteine residues in the three subunits common to all three species tested (L, M and H), varies from 9 in *Rp. viridis* (Michel et al. 1986b) to 5 in *Rb. sphaeroides* (Williams et al. 1986) and *Rb. capsulatus* (Youvan et al. 1984). *Rb. capsulatus* has no cysteine residues in the H subunit making it unlikely that the effect arises there, unless it has different origins in *Rb. sphaeroides* and *Rb. capsulatus*. Of the cysteines in L and M, three are conserved between *Rb. sphaeroides* (R.s.) and *Rb. capsulatus* (R.c.): L92 (R.s.)/L93 (R.c.), L108 (R.s.)/L109 (R.c.) and L247(R.s.)/L248(R.c.). Helpfully, L92/L93 is also conserved in *Rp. viridis* (L92), rendering it unlikely to

be responsible for the pCMBS effect. L247 is also unlikely, being located at the donor side of the RC and highly exposed.

We are left, therefore, with Cys^{L108} as the most likely candidate and this assignment was confirmed by site directed mutation of this residue to serine. RCs from the resulting mutant (L108CS) showed no effect of pCMBS on the functioning of Q_B (Fig. 7). However, although L108 is at the right end of the RC, it is by no means close to the quinones. It is closest to Q_A (~12 Å), but the sulfhydryl group points towards Q_B, about 23 Å away. The relative proximity to Q_A raised the possibility that the effects on acceptor quinone function were mediated via the redox potential of the primary quinone, i.e., if the E_m of Q_A was raised sufficiently, it would be incapable of reducing Q_B, simply on thermodynamic grounds, and the result would be an apparent failure of Q_B activity. We have tested this by extracting Q_A from treated RCs and reconstituting with anthraquinone. This substitution results in a functional Q_A with a much lower E_m than the native ubiquinone, as seen by a substantial increase in the rate of the $P^+Q_A^-$ recombination due to thermal accessibility of the P^+I^- state (Woodbury et al. 1986, Shopes and Wraight 1987). However, the rate of recombination in anthraquinone-reconstituted RCs was not significantly altered by treatment with pCMBS, effectively ruling out a primary effect on Q_A.

We conclude that if Cys^{L108} is the target for covalent modification by mercurial sulfhydryl modifiers, its distance from the Q_B site indicates that the effect is somewhat indirect due, for example, to a gross distortion of the protein. This might arise simply from the incorporation of a bulky entity, or from a tendency for the charged acidic groups of the mercurial agents to rotate towards the aqueous phase causing a substantial disturbance of the secondary and tertiary structures of the protein. It is undetermined at the present time whether the loss of Q_B function is due to total loss of quinone binding activity or to alteration of the in situ redox properties such that Q_B can no longer accept electrons from Q_A^-.

The lack of effect of NEM is not due simply to a lack of disturbance of the structure of the protein by this smaller, neutral moiety, as pretreatment by NEM did not protect against subsequent treatment by pCMBS. It may seem surprising that a relatively buried residue is susceptible to attack by pCMBS, a charged reagent, but inert to the smaller neutral NEM. This is not uncommon, however, and may be accounted for by the reactivity of NEM with the thiolate anion, whereas the mercurials are equally reactive with the protonated and deprotonated species (Strauss 1984). It is also surprising that RCs incorporated into phospholipid vesicles are still affected by pCMBS and that this reagent is more effective than its less polar analogue, pCMB. Presumably L108 is sufficiently near the membrane interface that these agents can penetrate. Finally, the lack of susceptibility of RCs in situ, in the chromatophore membrane, may indicate that complexation of the RC with light harvesting pigment proteins does confer some degree of inaccessibility.

Acknowledgements

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