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EFFECT OF INHIBITORS, REDOX STATE AND ISOPRENOID CHAIN LENGTH ON THE AFFINITY OF UBIQUINONE FOR THE SECONDARY ACCEPTOR BINDING SITE IN THE REACTION CENTERS OF PHOTOSYNTHETIC BACTERIA

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Quinone and inhibitor binding to *Rhodopseudomonas sphaeroides* (R-26 and GA) reaction centers were studied using spectroscopic methods and by direct adsorption of reaction centers onto anion exchange filters in the presence of ^{14}C-labelled quinone or inhibitor. These measurements show that as secondary acceptor, Q_B, ubiquinone (UQ) is tightly bound in the semiquinone form and loosely bound in the quinone and quinol forms. The quinol is probably more loosely bound than the quinone. *o*-Phenanthroline and terbutryn, a triazine inhibitor, compete with UQ and with each other for binding to the reaction center. Inhibition by *o*-phenanthroline of electron transfer from the primary to the secondary quinone acceptor (Q_A to Q_B) occurs via displacement of UQ from the Q_B binding site. Displacement of UQ by terbutryn is apparently accessory to the inhibition of electron transfer. Terbutryn binding is lowered by reduction of Q_B to Q_B^- but is practically unaffected by reduction of Q_A to Q_A^- in the absence of Q_B. UQ-9 and UQ-10 have a 5- to 6-fold higher binding affinity to the Q_B site than does UQ-1, indicating that the long isoprenoid chain facilitates the binding to the Q_B site.

Introduction

The reaction center of photosynthetic bacteria is the site in which light energy drives the transmembrane primary electron transfer of the photosynthetic electron transport chain. The reaction center of *Rhodopseudomonas sphaeroides* is composed of three polypeptides of 28, 32 and 36 kDa (L, M and H, respectively, [1]) in a ratio of 1 : 1 : 1. The reaction center also contains 4 BChl, 2 BPh [2], 1 Fe^{2+} [3] and 1 or 2 UQ-10 [4]. Analogous components are also found in the reaction centers of Photosystem II and we now know that the mechanism of electron transfer among the quinone acceptors is practically identical in both. The primary quinone acceptor, Q_A, participates in only a single one-electron redox reaction (Q_A/Q_A^-). The secondary quinone electron acceptor, Q_B, acts as a two-electron gate, undergoing reduction in two one-electron steps from the quinone (Q_B) to the quinol (Q_BH_2) [5–8]. Of the reduced forms, only the semiquinone (Q_B^-) is stable (half-lifetime, 3–4 min). Q_BH_2 is unstable and is transformed to Q_B on the millisecond time scale, with the release of two electrons and two protons from the reaction center. Recent biophysical evidence [9–13] has

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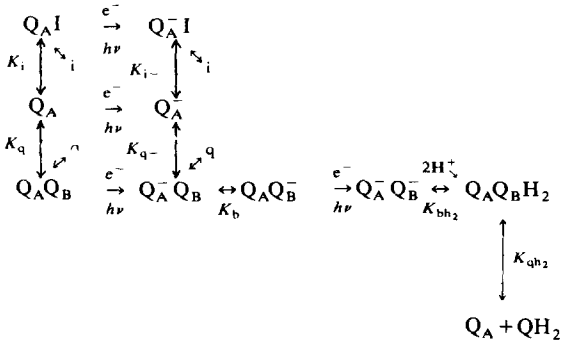
Abbreviations: Atrazine, 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine; azido-atrazine, 2-azido-4-ethylamino-6-isopropylamino-*s*-triazine; BChl, bacteriochlorophyll; BPh, bacteriopheophytin; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; LDAO, lauryldimethylamino *N*-oxide; RC, reaction center; terbutryn, 2-thiomethyl-4-ethylamino-6-*t*-butylamino-*s*-triazine; UQ-*n*, ubiquinone, *n* corresponds to the number of isoprenoid units.

favoured a mechanism for this latter reaction in which the quinol dissociates from its binding site and is replaced by a quinone, rather than a transfer of the pair of electrons to a tertiary acceptor as was envisaged earlier. Additional features of this model are: (a) a reversible binding of quinone to the Q_B site and (b) inhibition of electron transfer from Q_A to Q_B through displacement of the latter by certain herbicides.

The general features of the model are summarized by Scheme I (see also Ref. 14):



Scheme I.

where q = quinone, i = inhibitor, and K_{qh_2}, K_q and K_i are the dissociation constants for quinol, quinone and inhibitor, respectively, in the dark, K_{q-} and K_{i-} are the dissociation constants for quinone and inhibitor following a light flash (i.e., upon reduction of Q_A to Q_A^-).

We have sought by direct biochemical means to test this model on bacterial reaction centers. With the aid of ^{14}C-labeled ubiquinone-9 and terbutryn, a triazine herbicide, we show that the quinone is reversibly bound to the Q_B site and competes with electron transport inhibitors for binding. The isoprenoid side chain is also shown to facilitate quinone binding.

Materials and Methods

Reaction centers from *Rhodopseudomonas sphaeroides* strains R-26 and GA were isolated according to the procedures of Schenck et al. [15] and Cogdell et al. [16], respectively.

Terbutryn, a triazine herbicide known to inhibit Q_A to Q_B electron transfer in photosynthetic bacteria [9], was a gift of Dr. Charles Arntzen. [^{14}C]terburyn (specific activity 4.92 μCi/μmol) was a gift of Ciba-Geigy, Switzerland.

Spectrophotometric measurements were performed in a flash detection spectrophotometer similar to that described by Joliot et al. [17]. Actinic flashes (complete in 600 ns) were provided by a dye laser (Phase-R model DL-1100) containing Rhodamine 6G. Ubisemiquinone was detected at 450 nm.

Rates of reduction of the oxidized primary donor, P-870$^+$, by charge recombination with the acceptor-side electron were measured by detecting P-870$^+$ at 425 nm at various times following a saturating actinic flash. These recombination rates were used to determine the extent of electron transfer from Q_A^- to secondary acceptor, Q_B, in the presence and absence of electron transport inhibitors. The basis of this technique is explained below.

Recombination kinetics

Charge recombination following light-induced charge separation in reaction centers has a limiting value of 80–100 ms, $t_{1/2}$(fast), corresponding to recombination between P-870$^+$ and Q_A^-. These kinetics will be slowed by a sharing of the electron with Q_B in an equilibrium governed by dissociation constant, K_{q-}, and equilibrium constant, K_b. If binding and dissociation of the secondary quinone is slow on the time scale of the charge recombination [14], then these kinetics will be biphasic. Those centers having bound secondary UQ in the dark will recombine with a $t_{1/2}$ of 1.2 s. Those without Q_B will recombine with $t_{1/2}$(fast). The fraction of centers recombining rapidly is given by:

$$1-\frac{[Q_AQ_B^-]}{[RC]}=1-\frac{K_b\frac{q}{K_{q-}}}{1+\frac{i}{K_{i-}}+\frac{q}{K_{q-}}(1+K_b)} \qquad (1)$$

As we will see this situation is observed for UQ-9 and UQ-10 in the presence of LDAO.

If on the other hand quinone binding and dissociation are rapid on the time-scale of the charge recombination [14] then the recombination kinetics should be monophasic with a $t_{1/2}$ determined

by the dissociation and the equilibrium constants which prevail following a flash, as indicated below:

$$t_{1/2}(\text{slow}) = t_{1/2}(\text{fast}) \cdot \left(1 - \frac{[Q_A Q_B^-]}{[RC]}\right)^{-1}$$

where

$$\frac{[Q_A Q_B^-]}{[RC]} = \frac{K_b \frac{q}{Kq-}}{1 + \frac{i}{K_{i-}} + \frac{q}{K_{q-}}(1 + K_b)} \quad (2)$$

Isolation of [^{14}C]UQ-9

[^{14}C]UQ was isolated from ^{14}C-labelled *Chlorella pyrenoidosa* by extraction with methanol. The methanol-water extract was itself extracted into petroleum ether. The pretroleum ether was evaporated off and the residue taken up in benzene. Following adsorption to and elution from a silica gel column using the same solvent, the UQ was purified on preparative 1 mm thick silica gel thin-layer plates (Whatman PK6F) eluted with benzene. The band containing the UQ was extracted with ethanol, respotted on a reversed-phase silica gel plate (Whatman KC18F, 0.2 mm thick) and eluted with 95% acetone/5% water. Comparison with UQ isoprenylogs (courtesy of Hoffman-LaRoche) indicated the ubiquinone to be UQ-9. Autoradiograms indicated a purity on the order of 95%. The specific activity was 600 μCi/μmol, as determined by liquid scintillation counting and oxidized minus reduced (by borohydride) difference spectra (extinction coefficient 13.7 $mM^{-1} \cdot cm^{-1}$ at 275 nm, Ref. 19).

UQ-9 and 10 (added from concentrated LDAO solution) were incorporated into the Q_B site by incubation of 10 μM reaction centers, about 90% depleted of secondary acceptor, with UQ in 1% LDAO, 10 mM Tris, pH 8.0 for 1 h at room temperature. To incorporate labeled UQ-9 into the Q_B site, 15 μM [^{14}C]UQ-9 was used. Reaction centers incubated with this concentration of UQ-9 showed approx. 30% of the secondary quinone binding sites occupied, as estimated by charge recombination between P-870$^+$ and the acceptor side, UQ-1, DBMIB, and the inhibitors were all added from ethanol solutions. The final concentration of ethanol never exceeded 1%.

Binding studies

These studies involved measuring binding to reaction centers of labelled quinone in the presence and absence of inhibitors and of labelled inhibitor as a function of the redox state of Q_B.

Secondary quinone binding to reaction centers was studied following flash illumination in the presence of diaminodurene (DAD) (to reduce P-870$^+$ rapidly and prevent charge recombination) or following incubation in the dark with inhibitors or quinone analogues. In the binding experiments, 25–100 pmol reaction center were suspended in 1% LDAO/10 mM Tris (pH 8.0). Further additions, their concentrations and those of the reaction centers are specified in the experiments that follow. Following the indicated pretreatments, the reaction centers were separated from unbound quinone or inhibitor by centrifuging at $1000 \times g$ through positively charged nylon filters (Pall Process Filtration Ltd., model NRZ) mounted in centrifugal microfilters (Bioanalytical Systems Inc., model MF-1). Reaction centers are bound quantitatively to the NRZ filters. In quinone binding experiments, the NRZ filters were overlaid by reconstituted cellulose filters (Schleicher and Schull, model RC58) which did not bind reaction centers, but which improved the reproducibility of the measurements. Using this technique, reaction centers could be separated from unbound quinone or inhibitor in 15–30 s. Following centrifugation, the NRZ filter was removed from the microfilter, suspended in 2 ml of Aqua Luma (Kontron Analytique) and counted in a scintillation counter. Control experiments were performed to determine binding of quinones or inhibitors to the filters in the absence of, or with heat-denatured reaction centers. These gave the same result. The presence of 1% LDAO was required to minimize such binding to the NRZ filters. All the experiments reported are corrected for this non-reaction center-related binding. In a typical experiment with [^{14}C]UQ-9, background radioactivity was less than or equal to 35% of that bound to reaction centers.

Results and Discussion

Binding of secondary quinone to reaction centers

Condition (a): 10 μl *R. sphaeroides* R-26 reaction centers at a concentration of 5 μM in 10 mM

Tris (pH 8.0)/1% LDAO/7.5 μM [^{14}C]UQ-9/200 μM DAD were given no, one or two saturating flashes at 1 s intervals. These were then incubated for 2 min in the dark, diluted to 210 μl with 10 mM Tris (pH 8.0)/1% LDAO and immediately centrifuged through the RC58 and the NRZ filters.

Condition (b): 10 μl reaction centers at a concentration of 5 μM in 10 mM Tris (pH 8.0)/1% LDAO/7.5 μM [^{14}C]UQ-9 were diluted, in the dark, to 210 μl with 10 mM Tris (pH 8.0)/1% LDAO/100 μM DAD and immediately given no, one or two flashes at 1 s intervals. These reaction centers were then incubated for 2 min in the dark followed by centrifugation as above.

The essential difference between these two conditions is that the reaction centers were 21-times less concentrated, during and for two min following, flash excitation in (b) than in (a). This was to minimize UQ-9 uptake following flash excitation in condition (b) (see below).

Scintillation counting of the NRZ membranes indicated that in condition (a) a single flash increased the labeling of the reaction centers (28 ± 13% greater, 5 expts.) relative to the dark-incubated centers.

This result indicates that (1) only a fraction of the reaction centers in the dark contains the secondary acceptor, (2) the association of the quinone with the Q_B site is in dynamic equilibrium and (3) the dissociation rate of the semiquinone (formed after one flash) is slower than that for the quinone.

Point (3) could also be explained by a higher association rate of UQ-9 upon reduction of Q_A. This is unlikely, however, as Wraight [9] has shown, in lipid vesicles, that the binding affinity of the secondary quinone (in the quinol form) is lowered by the reduction of Q_A ($K_{q-} > K_q$). Thus, the increase in labeling, after the flash, is driven by equilibrium constant K_b (10–20, pH 7, Ref. 18) and by the higher binding affinity of the semiquinone anion of UQ-9.

Direct spectrophotometric measurements confirm points (1) and (2). Under condition (a) above, but before dilution and in the absence of DAD, 30% of the centers recombine with slow 1.2 s kinetics following a single saturating flash (not shown). Thus, approximately this percentage of the reaction centers contain both Q_A and Q_B, the remainder containing only Q_A. Both spectrophotometric and radioactive binding measurements indicated that dilution of the centers with 1% LDAO decreased the amount of UQ-9 associated with the secondary binding site ($t_{1/2} \simeq 1.5$ min), as expected for reversible binding.

In both conditions (a) and (b) above, two flashes consistently gave less binding (18 ± 11% less, 5 expts.) than one flash. This result indicates that the dissociation rate of UQH_2 is more rapid than for UQ^-.

In cases of condition (b) where little or no uptake of [^{14}C]UQ-9 occurred after one flash, then two or more flashes gave less labeling (25 ± 12% less, 3 expts.) than in centers maintained in the dark. This result suggests that the off-rate of UQH_2 is greater than that of UQ.

Addition of sodium dithionite to reaction centers in condition (a) in the dark decreased [^{14}C]UQ-9 binding by 32 ± 3% (2 expts.). These results indicate that in state $Q_A^-Q_BH_2$ the secondary quinone is less tightly bound than in state Q_AQ_B. This result could arise from a lowered affinity for UQH_2 as opposed to UQ and/or a lowered affinity for the secondary quinone (UQ or UQH_2) upon reduction of Q_A, as suggested by Wraight [9]. This result is consistent with the observation of Okamura et al. [4] that secondary quinone extraction is facilitated by reduction.

The above-mentioned binding measurements can only give a qualitative picture of quinone binding to the Q_B site as they are limited by the equilibration times for [^{14}C]UQ-9 between micelles with and without reaction centers. Nonetheless, they do give evidence for a dynamic equilibrium for such binding and are consistent with a mechanism in which UQH_2 is readily replaced by UQ.

Inhibitor binding

Reversible binding of secondary quinone suggests that a simple mechanism to explain inhibition of Q_A^- oxidation by Q_B in the presence of urea and triazine herbicides might be by dissociation of Q_B from its binding site. Indeed, chemical arguments of Okamura et al. [4] and kinetic arguments put forward by Velthuys [10], Rich [11], Wraight [9] and Lavergne [12] favour such a model. Furthermore, Vermaas et al. [20] have shown that covalent binding of an azido-quinone decreases the binding affinity of atrazine in Photosystem II,

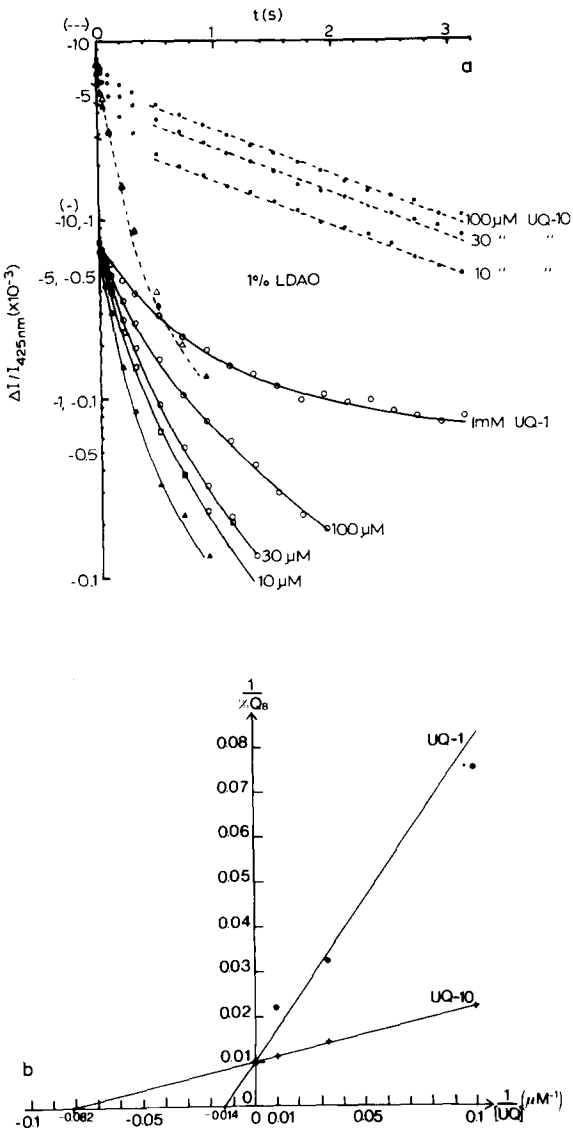


Fig. 1. (a) Semi-log plot of the kinetics of reduction of P-870$^+$ by charge recombination following a saturating actinic flash in the presence of the indicated concentrations of UQ-1 (○, □) and UQ-10 (●), GA reaction centers (90 nM) were suspended in 10 mM Tris, pH 8.0 containing 1% LDAO, (▲) no additions, (△) 100 μM UQ-10 plus 2.4 mM *o*-phenanthroline. The inner ordinate scale (- - - - - -) refers to the experiments involving UQ-10, the outer scale (———) those involving UQ-1. (b) Double reciprocal plot of the concentration of UQ-1 or UQ-10 [UQ] versus the percentage of centers containing Q_B (% Q_B) as determined from Fig. 1a. This percentage is given by $(A(q)-A(q=0))/(A(q=\infty)-A(q=0))\times 100$, where A is the amplitude of the 1.2 s phase of charge recombination in the case of UQ-10 and the amplitude of $\Delta I/I_{425\,nm}$ at 0.7 s after the flash in the case of UQ-1. q is the concentration of quinone.

suggestive of a competition between quinone and inhibitor for binding. While likely, it has not yet been shown that the reaction center alone is capable of showing such competition. We have looked at the mechanism of inhibition in the most direct way, through measurements of quinone-inhibitor competition for binding in bacterial reaction centers.

Reaction centers treated with detergent to extract Q_B (Fig. 1) show charge recombination between P-870$^+$ and Q_A^- with a half-time of 80–100 ms. Addition of high concentrations of the inhibitors *o*-phenanthroline (2.4 mM, Fig. 1) and terbutryn (50 μM, Fig. 2) to reaction centers containing Q_B show similar recombination times, indicating that back-reaction occurs from Q_A^-. These measurements show that oxidation of Q_A^- by Q_B is not competitive with oxidation by P-870$^+$ in the presence of these inhibitors. However, independent experiments on reaction centers (10UQ-10/RC, 0.025% LDAO, not shown) in the presence of the artificial donor, DAD, which blocks the back reaction, indicate a half dissociation rate for terbutryn of close to 200 ms. This means that in this time half of the centers transfer an electron from Q_A^- to Q_B, a reaction which requires a dissociation of terbutryn. This dissociation explains the small slow phase of charge recombination in the presence of this inhibitor (Fig. 2).

If quinone and inhibitor compete for binding to the reaction center, then increasing concentrations

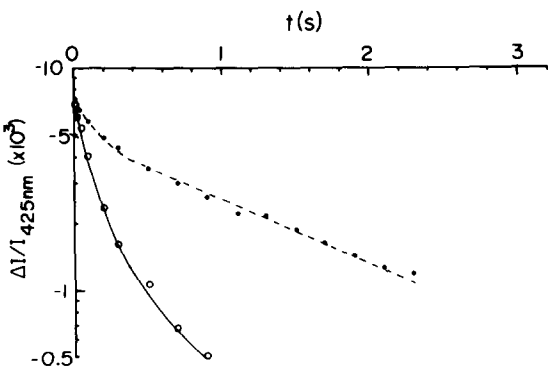


Fig. 2. Semi-log plot of the kinetics of reduction of P-870$^+$ by charge recombination following a saturating flash in the presence (○) and absence (●) of 50 μM terbutryn. Conditions as in Fig. 1, except that the R-26 reaction centers (100 nM) were equilibrated with 50 μM UQ-9.

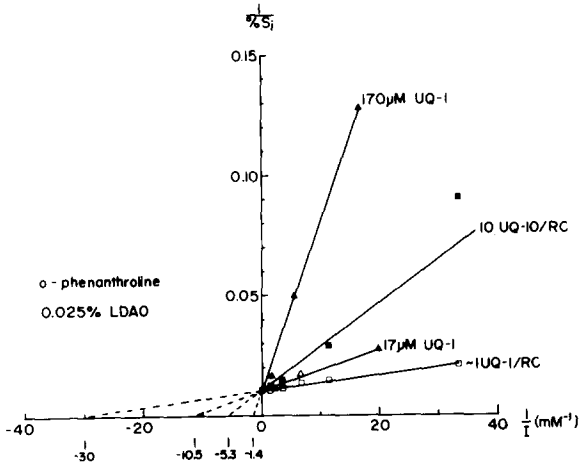


Fig. 3. Double reciprocal plot of the concentration of *o*-phenanthroline (I) versus the percent of R-26 reaction centers binding inhibitor (% Si) at different quinone concentrations. Inhibitor binding was measured as the percent loss of the slow phase of charge recombination following a saturating laser flash. This percentage is given as $(A(i=0)-A(i))/(A(i=0)-A(i=\infty))\times 100$, where A is the amplitude of the 1.2 s phase of charge recombination in the case of UQ-10 and the amplitude of $\Delta I/I_{425\,nm}$ at 0.9 s after the flash in the case of UQ-1. R-26 reaction centers (100 nM) were suspended in 0.025% LDAO 10 mM Tris (pH 8.0).

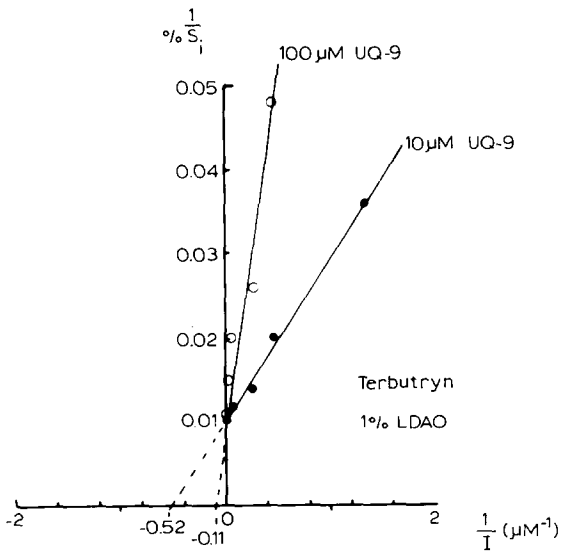


Fig. 4. Double reciprocal plot of the concentration of terbutryn versus the percent of reaction centers binding inhibitor (measured as in fig. 3 for UQ-10), R-26 reaction centers (100 nM) were suspended in 1% LDAO/10 mM Tris (pH 8.0) in the presence of 10 or 100 μM UQ-9.

of quinone should increase the apparent dissociation constants for inhibitor binding. Using the slow charge recombination rate as an assay for inhibitor action, we show (Figs. 3 and 4) that this is indeed the case for the inhibitors *o*-phenanthroline and terbutryn, respectively. In these double reciprocal plots I is the concentration of inhibitor and %Si is the percent of centers, binding inhibitor. The higher the concentration of UQ, the greater the apparent dissociation constant for inhibitor binding. If we assume a simple model of competitive inhibition, then by extrapolating the inhibitor binding dependence to $1/\%\mathrm{Si}=0$ and solving for $1/I=[-1/K_{i-}][1/(1+q/K_{q-})]$ at the two quinone concentrations we can calculate K_{i-} and K_{q-}. The data of Fig. 3 indicate a $K_{i-}=27$ μM for *o*-phenanthroline and a $K_{q-}=7$ μM for UQ-1 in 0.025% LDAO. A range of 27–45 μM was observed for K_{i-} (*o*-phenanthroline) and of 7–12 μM for K_{q-} (UQ-1) in different experiments under these conditions. The data of Fig. 4 indicate a $K_{i-}=1.1$ μM for terbutryn and a $K_{q-}=14$ μM for UQ-9 in 1% LDAO.

These data and those that follow show that inhibition by *o*-phenanthroline is satisfactorily explained by a model in which the secondary quinone and the inhibitor compete for binding to the reaction center. The case of terbutryn is, however, more complicated and requires a model in which both quinone and inhibitor can be bound together, but where the binding of one decreases the binding affinity of the other. Binding of the inhibitor blocks Q_A^- to Q_B electron transfer without necessarily displacing Q_B.

Displacement of the secondary quinone by inhibitors

Reaction centers equilibrated with [^{14}C]UQ-9 were mixed with saturating concentrations of unlabelled quinones known to occupy the secondary binding site. Measurements of the loss of labelled quinone from the reaction centers (Table I) permitted us to determine how much of the label was associated with the Q_B site. Comparison with the loss of label, induced by the addition of inhibitors, indicated how effective these were in releasing Q_B.

Figs. 1, 8b and 5 show that both UQ-1 and DBMIB act as secondary electron acceptors in reaction centers. They induce a slow phase in the

TABLE I

Ten μl R-26 reaction centers (5 μM) in 1% LDAO/10 mM Tris (pH 8.0) 7.5 μM [^{14}C]UQ-9 were diluted 1 : 1 with 1% LDAO/10 mM Tris (pH 8.0) containing unlabelled quinone and/or inhibitor such that the final concentrations were those indicated in the Table. After 10 min at room temperature, 190 μl of 1% LDAO/10 mM Tris (pH 8.0), containing the same concentrations of quinones and/or inhibitors, were added followed by immediate centrifugation through RC58 and NRZ filters. About 45 s elapsed between the final dilution and the separation of reaction centers and unbound [^{14}C]UQ-9. The loss of bound [^{14}C]UQ-9 is expressed relative to the amount of [^{14}C]UQ-9 bound in the absence of added quinones and/or inhibitors.

Inhibitor of quinone analogue	% loss of [^{14}C]UQ-9
150 μM terbutryn	−10 to −15%
10 mM *o*-phenanthroline	−33%
3 mM UQ-1	−27%
500 μM DBMIB	−30%
10 mM *o*-phenanthroline + 500 μM DBMIB	−40%

charge recombination between P-870$^+$ and the acceptor side electron. DBMIB has previously been shown to act as secondary acceptor in chromatophores of *R. rubrum* [21]. Electron transfer from Q_A^- to DBMIB is inhibited by *o*-phenanthroline (acceleration of charge recombination, not shown),

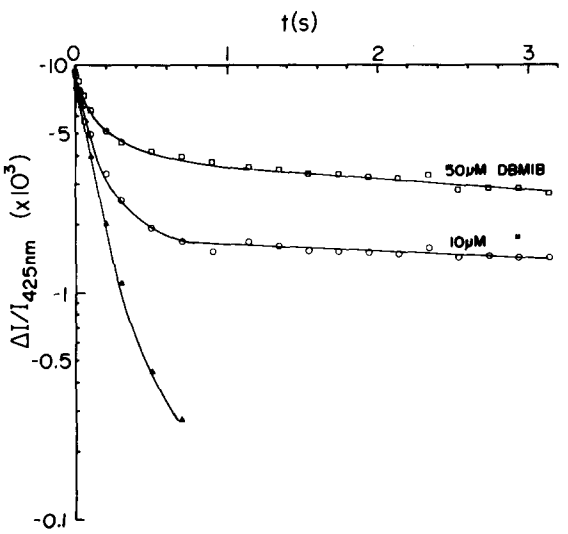


Fig. 5. Semi-log plot of the kinetics of reduction of P-870$^+$ by charge recombination following a saturating flash in the presence (10 μM (○), 50 μM (□)) or absence of DBMIB. GA reaction centers (150 nM) were suspended in 1% LDAO/10 mM Tris (pH 8.0). (▲), No additions.

but is insensitive to 150 μM terbutryn, unlike electron transfer to UQ-1 and UQ-10.

Direct measurements of the release of Q_B in the presence of saturating concentrations of DBMIB and UQ-1 are indicated in Table I, where 500 μM DBMIB and 3 mM UQ-1 release 30 and 27%, respectively, of the counts bound to the reaction centers. Comparison with saturating concentrations (for inhibition of electron transfer) of *o*-phenanthroline (10 mM) and terbutryn (150 μM) shows that the first dissociates 33% of the bound counts, while the latter releases only 10–15%. Addition of 500 μM DBMIB to 10 mM *o*-phenanthroline increases the counts released by the latter by only 20%, indicating that these two species dissociate the same bound quinone. We conclude that saturating concentrations of *o*-phenanthroline release all the UQ-9 bound to the Q_B site, while terbutryn dissociates at most one-third to one-half that amount. This observation is consistent with *o*-phenanthroline inhibition occurring via displacement of Q_B. The result with terbutryn, however, indicates that full inhibition of Q_A^- to Q_B transfer can occur without complete dissociation of Q_B.

We note that neither the inhibitors nor the quinone analogues alone or in combination release more than 40% of the labelled quinone bound to the reaction center. It is possible that the remaining counts arise from [^{14}C]UQ-9 which is either bound non-specifically or which may have exchanged with the primary quinone acceptor. It is also possible that the remaining counts correspond to continued partial binding of the secondary quinone via the isoprenoid chain, an hypothesis that will be discussed further in the conclusion.

While *o*-phenanthroline is capable of releasing all of the secondary quinone, this does not mean that such release is the mechanism of inhibition. Fig. 6 shows, however, that inhibition of Q_A^- to Q_B electron transfer and release of 70% of the [^{14}C]UQ-9, dissociable by *o*-phenanthroline, show the same dependence on the concentration of the inhibitor ($I_{50} \simeq 60$ μM), indicating that they both arise from the same origin – i.e., inhibitor-induced release of secondary quinone. The remaining counts released by *o*-phenanthroline occur at high concentrations at which there is also some release of primary quinone, as indicated by a decrease in

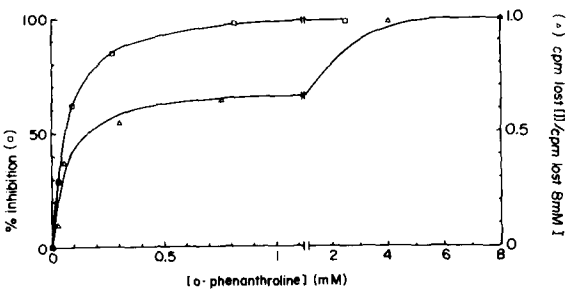


Fig. 6. Comparison of inhibition of Q_A^- to Q_B electron transfer (□) by *o*-phenanthroline with displacement of ^{14}C-labelled UQ-9 (△) at the same concentrations of inhibitor. Inhibition of electron transfer was measured as in Figs. 3 and 4 using 100 nM R-26 reaction centers in 1% LDAO/10 mM Tris (pH 8.0) containing 10 μM UQ-10. In the binding study, 5 μl of 5 μM R-26 reaction centers in 1% LDAO/10 mM Tris (pH 8.0) 7.5 μM [^{14}C]UQ-9 were incubated for 10 min with the indicated concentrations of *o*-phenanthroline, then diluted with 100 μl 1% LDAO/10 mM Tris, (pH 8.0) containing the same concentration of inhibitor. This mixture was then spun through RC58 and NRZ filters within 40 s following dilution. The loss of counts at each *o*-phenanthroline concentration is normalized to those dissociated by 8 mM inhibitor.

flash generated-P-870$^+$. As pointed out above, some of these may have become labelled through exchange. In a similar experiment using terbutryn (not shown), half of the counts releasable by the inhibitor (5% of the total bound counts) were dissociated at 1 μM terbutryn, which is also the concentration of half-inhibition of electron transfer (see also Fig. 4).

Thus, *o*-phenanthroline and UQ compete for binding to the reaction center and this competition is sufficient to cause inhibition of secondary electron transfer. In the case of terbutryn, quinone dissociation appears to be accessory to inhibition of electron transfer. Fig. 4, however, indicates a competition between the two. *o*-Phenanthroline probably inhibits by binding to the Q_B site, though we cannot exclude an allosteric mechanism. Terbutryn binding increases the Kq by a factor of 1.5–2 either through partial overlap with the quinone binding site or through a non-exclusive allosteric mechanism. Lack of inhibition by terbutryn of DBMIB reduction, suggests that this inhibitor may compete with the isoprenoid chain, present in ubiquinone, but absent in DBMIB.

Inhibitor binding depends on the redox state of Q_B

The competition observed between inhibitor and quinone in the inhibitor studies of Figs. 3 and 4 and the high affinity observed for the semiquinone form of Q_B earlier (see also Ref. 13) together suggest that inhibitor binding should depend on the redox state of the secondary quinone.

Reaction centers at a concentration of 10 μM were equilibrated with 100 μM UQ-9 in 1% LDAO. These are conditions where the Q_B site is 75–80% occupied according to spectroscopic measurements analogous to those of Fig. 1. Reaction centers were preilluminated by a variable number of saturating flashes at 1 s intervals in the presence of DAD, then mixed with 3 μM ^{14}C-labelled terbutryn and centrifuged through NRZ filters within 15 s after the last flash. Fig. 7 shows that terbutryn binding

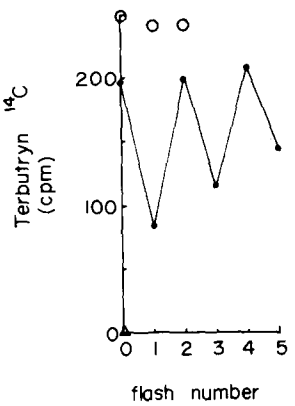


Fig. 7. Dependence of terbutryn binding to reaction centers on the number of preilluminating flashes preceding addition of the inhibitor. (●) 10 μl 10 μM R-26 reaction centers were equilibrated with 100 μM UQ-9 in 1% LDAO/10 mM Tris (pH 8.0). DAD (300 μM) was added in the dark and the sample was illuminated with the indicated number of saturating laser flashes. The sample was immediately diluted with 100 μl of 3 μM [^{14}C]terbutryn/10 mM Tris (pH 8.0)/1% LDAO and centrifuged through an NRZ filter. Approx. 40 s elapsed between the last flash and the complete separation of reaction centers from unbound terbutryn. Controls, without reaction centers were subtracted (approx. 300 cpm) to correct for terbutryn adsorption to the NRZ filters. (○), R-26 reaction centers (1 μM) were suspended in 110 μl of 3 μM [^{14}C]terbutryn in 10 mM Tris (pH 8.0)/1% LDAO – condition which depletes the centers of Q_B (see Fig. 1). 300 μM DAD was added in the dark and the sample given no, one or two saturating flashes (1 Hz). The reaction centers were separated from unbound terbutryn as above. The separation was complete within 45 s after the last flash. Controls without reaction centers were as above. (△), Zero flash experiment as above (●) except that 5 mM *o*-phenanthroline was also present. *o*-Phenanthroline did not modify the correction for adsorption of terbutryn to the NRZ filters in the absence of reaction centers.

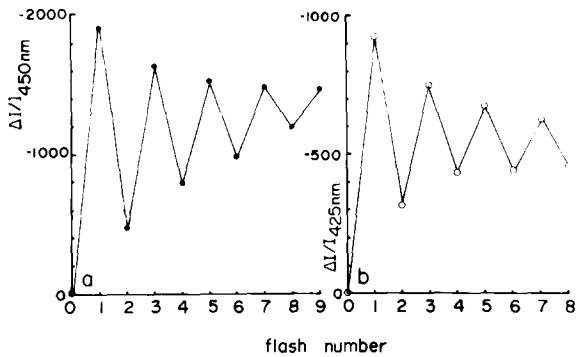


Fig. 8. (a) Oscillation of the redox state of Q_B with flash number. GA reaction centers (60 nM) with 10 UQ-10 per center were suspended in 0.025% LDAO, 10 mM Tris (pH 8.0) 250 μM DAD was added in the dark and the sample given a series of saturating flashes (1 Hz). The absorbance changes ($\Delta I/I \cdot 10^6$) at 450 nm were detected between 200 and 800 ms. (b) Similar conditions to (a) except that 175 μM UQ-1 was present instead of UQ-10. The DAD concentration was 200 μM and the absorbance changes ($\Delta I/I \cdot 10^6$) were detected at 425 nm.

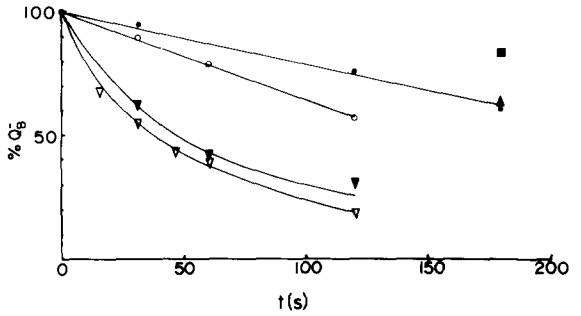


Fig. 9. Lifetime of Q_B following a saturating actinic flash. GA reaction centers (120 nM) were suspended in 0.025% LDAO/10 mM Tris (pH 8.0) in the presence of varying concentrations of UQ-1 and UQ-10, 300 μM DAD was added in the dark. One saturating preflash was given followed by a variable dark time. At the end of the dark period a sequence of saturating flashes was given (0.5 Hz) and the absorbance changes detected between 0.4 and 1.6 s after each actinic flash. The concentration of Q_B^- following the preflash was determined by analysis of the phase of the subsequent oscillation. (■), 50 UQ-10/center; (▲), 10 UQ-10/center; (●), 10 UQ-10/center + 17 μM UQ-1; (○), approx. 1 UQ-10/center + 17 μM UQ-1; (▼) 10 UQ-10/center + 100 μM UQ-1; (▽), approx. 1 UQ-10/center + 100 μM UQ-1.

is elevated on even-numbered flashes and minimal on odd-numbered flashes. $[Q_B^-]$, which is maximal on odd-numbered flashes, Fig. 8, thus lowers the binding affinity of terbutryn. A similar oscillation of period two for atrazine binding in pea chloroplasts has been reported by Jursinic and Stemler [31]. The result of Fig. 7 is consistent with the competition experiments between inhibitor and quinone (Fig. 4), and further indicates the higher affinity of UQ^- than UQ for the Q_B site. Such high binding affinity explains the long lifetime (3–4 min) of Q_B^-, which is thus not free to dismutate with other semiquinones [13]. These observations are also consistent with the results of Vermeglio [22] and Wraight and Stein [23], who showed that *o*-phenanthroline inhibited electron transfer in reaction centers, illuminated in the Q_AQ_B state, but was a poor inhibitor upon illumination in the $Q_AQ_B^-$ state.

We note the upward slope of the oscillations of Fig. 7, indicating that inhibitor binding gradually increases during the flash sequence. As UQH_2 accumulates during the flash sequence, these results suggest that UQ competes more effectively than UQH_2 for inhibitor binding. In other words, UQ has a somewhat higher binding affinity than UQH_2, consistent with the higher dissociation rate for UQH_2 than for UQ, observed under certain conditions in the [^{14}C]UQ-9 binding experiments. This conclusion is consistent with a calculated $Kq < Kqh_2$ [9] based on literature $E_{m,7}$ values for Q_B/Q_BH_2 [24] and for UQ/UQH_2 in the ubiquinone pool [25], where the former is more negative than the latter.

When reaction centers are depleted of secondary acceptor, illumination in the presence of DAD, only results in the formation of Q_A^-. Spectroscopic measurements under these conditions (not shown) indicate that, following the first and second flashes, 75% and 96% of previously dark-adapted centers, respectively, have stabilized Q_A^-. Fig. 7 indicates that, during flash illumination in the presence of DAD, there is practically no modulation of the binding of inhibitor, indicating that the binding affinity of terbutryn is not affected by the redox state of Q_A.

If 5 mM *o*-phenanthroline is added in the dark to Q_B-depleted reaction centers, then terbutryn binding is completely eliminated (Fig. 7).

Comparison of UQ-1 and UQ-10

If we look at the saturation properties of the

UQ-1 and UQ-10 (Fig. 1, double reciprocal plot), we find that $Kq-$ for UQ-1 is 71 μM. We noted earlier (Fig. 3) that the $Kq-$ for UQ-1 was on the order of 12 μM in the presence of 0.025% LDAO. These results indicate that the measured dissociation constants are dependent on the detergent concentration. This dependence arises from a dilution of quinone in detergent micelles, including those containing reaction centers, upon increasing the detergent concentration.

We can, however, make a meaningful comparison of the relative dissociation constants of different quinones at the same detergent concentration. Such a comparison requires that we look at a partition between the same phases for these quinones – ideally the secondary quinone binding site and the detergent micelles. The arguments below indicate that this is the case for UQ-1 and UQ-10.

UQ-10 is insoluble in water and is exclusively associated with detergent micelles. A saturated solution of UQ-1 in water is 1 mM at 25 °C, as determined by spectroscopic measurements (Diner, B., unpublished data), UQ-1 can readily be made 50 mM in a solution of 3% LDAO (Diner, B., unpublished data). In this case, over 98% of the UQ-1 is located within the detergent micelles. If we now lower the concentration of LDAO to 1%, then at least 94% of the UQ-1 should still be located within the detergent micelles. We conclude that we are indeed comparing the same partition for UQ-1 and UQ-10 in Fig. 1. That the K_{q-} for UQ-10 and UQ-1 are 12 and 71 μM, respectively, in 1% LDAO (Fig. 1), means that the affinity of UQ-10 for the secondary quinone binding site is some 5- to 6-fold greater than for UQ-1.

We note that 1% LDAO corresponds to 580 μM in micelles (micellar molecular weight 17 300 [26]), 40 times the concentration of UQ-10 at half saturation of the Q_B site. Thus, a reaction center, included in a micelle of approx. 50 kDa [32], increases the probability 7-fold for that micelle to contain a quinone, indicating that the quinone is bound 85% of the time. Thus, under the conditions of Fig. 1, micelles, containing reaction centers, have either no or one UQ-10, excluding Q_A. In the latter case the quinone is bound. If the micelle-micelle quinone exchange rate is slow with respect to the charge recombination rate then, as Wraight and Stein [14] have pointed out, reaction centers can recombine with only one of two half-times, either 80–100 ms or 1.2 s (without and with Q_B, respectively). This is indeed what is observed (Fig. 1). The recombination kinetics with UQ-10, in the presence of LDAO, are always biphasic. Only the amplitude and not the kinetics of the slow phase are dependent on the concentration of the quinone. Thus, for those centers located in micelles containing a UQ-10, $(q/K_{q-})(1 + K_b) \gg 1$, $t_{1/2}(\text{slow}) = t_{1/2}(\text{fast}) \cdot (1 + K_b)$, and $K_b = 12$–14 (see Materials and Methods).

In the case of UQ-1 (Fig. 1), the rate of the slow phase of charge recombination decreases with increasing quinone concentration. It is likely that with UQ-1, movement in and out of the micelles is rapid on the time-scale of the charge recombination. This mobility combined with a lower binding affinity ($K_{q-} = 71$ μM, $(q/K_{q-})(1 + K_b) \approx 1$) produces a slow recombination rate which is now dependent on the quinone concentration.

If we load up reaction centers with UQ-1 as secondary acceptor we note that the lifetime of Q_B^- goes down (Fig. 9). Loading of reaction centers with UQ-10 has no effect or if anything increases the lifetime. Probably, either a lower affinity of UQ-1 allows the semiquinones to dissociate, upon which they disproportionate, or a looser fit allows another UQ-1 to remove the electron from Q_B^- followed by disproportionation.

Conclusion

We have shown in the course of this paper that the isolated reaction center, composed of three polypeptide subunits, contains all the elements required for secondary electron transfer and its inhibition by *o*-phenanthroline and triazine inhibitors.

The secondary quinone acceptor, Q_B, is reversibly bound in the quinone or quinol form and is tightly bound as the semiquinone. Such reversible binding suggested a simple mechanism for action of electron transport inhibitors, outright displacement of the secondary quinone from its binding site [4,8–13]. While such a hypothesis is confirmed here in the case of *o*-phenanthroline, it appears to be only partially correct for inhibition by terbutryn. This latter inhibitor displaces no more than a third

to a half of the secondary quinone at saturating inhibitor concentrations (K_b increased by a factor of 1.5–2), despite competition between terbutryn and quinone for binding to the reaction center. This observation would suggest that bound tertutryn only partially overlaps with the quinone binding site. Alternatively, terbutryn may directly compete with equilibria linked to electron transport between Q_A and Q_B (e.g., conformational or ionic) *o*-Phenanthroline, however, fully displaces terbutryn, indicating full overlap with both the quinone and terbutryn binding sites. Such a model for terbutryn would also agree with the observation of Vermaas et al. [20] in pea chloroplasts. Covalent binding of an azido-quinone (presumably to the Q_B site) increased the K_i for atrazine, another triazine herbicide, but did not eliminate its binding site.

The lack of inhibition, by terbutryn, of Q_A^- to DBMIB electron transfer, may indicate that this inhibitor interacts principally with the isoprenoid side chain, present in ubiquinone, but absent in DBMIB. This observation is consistent with the absence of competition between benzosemiquinone and atrazine for binding to Photosystem II in pea chloroplasts [31]. That azido-atrazine and UQ-1 compete for binding to bacterial reaction centers [27], would suggest that the first isoprenoid unit of the quinone is already sufficient for inhibition by triazine inhibitors.

We have recently [32] demonstrated that the triazine inhibitor, [^{14}C]azido-atrazine labels principally the L subunit and, only slightly, the M subunit of the reaction center. If the arguments for involvement of subunits M [28] and, to some extent, H [29] in secondary quinone binding are correct, then inhibition is either governed by an allosteric mechanism or quinone binding is at a contact point bteween the L and M subunits. To resolve this question it would be desirable to identify, by photoaffinity labelling, the binding sites of quinones and inhibitors. Such a study would permit us to learn if the binding sites are located in the same or different regions of a particular subunit or even on different subunits.

The antagonistic binding of quinone and inhibitor is demonstrated through the increase in apparent inhibitor dissociation constants with increased quinone concentration, and upon formation of the semiquinone with its tighter binding to the reaction center. An antagonism between Q_A^- and *o*-phenanthroline [9] or terbutryn [30] has also been proposed. The transition from Q_A to Q_A^- was suggested to induce an increase in the dissociation constants of these inhibitors. Direct measurements of terbutryn binding (Fig. 7) show, however, practically no modulation of binding upon reduction of Q_A.

We also show that the isoprenoid side chain facilitates secondary quinone binding, increasing the binding affinity by a factor of at least 5–6 upon going from UQ-1 to UQ-10. The long isoprenoid chain also stabilizes the semiquinone, Q_B^-, either by excluding other quinones from picking up the unpaired electron from Q_B^- or by preventing dissociation of Q_B^- from its binding site. A free semiquinone would normally be expected to disappear through dismutation.

These observations suggest the existence of a binding site for the isoprenoid chain in addition to one for the head group. Indeed the arguments mentioned above implicating two or possibly even all three subunits in secondary quinone binding would be consistent with more than one binding site for UQ-10 (isoprenoid chain length, approx. 50 Å).

Measurements of the release of [^{14}C]UQ-9 from reaction centers following addition of 5 mM *o*-phenanthroline indicate a half-dissociation time of about 1.5 min (not shown). This slow time contrasts with the demonstration by Vermeglio [8] and Wraight [7] of oscillations of period 2 for ubisemiquinone during a long sequence of flashes (0.2 Hz) where the micellar concentration of LDAO was equal to or greater than that of the added ubiquinone. The observation of such oscillation requires that Q_BH_2 be replaced by a quinone on every even-numbered flash. The small damping parameter ($\approx 10\%$ per flash) of these oscillations indicates that the micelle-micelle exchange of quinone had to be occurring on the seconds time scale. The difference in these times may reflect the slow release of a quinone bound to the reaction center by the isoprenoid chain as opposed to the much faster transfer of a free quinone from a micelle lacking a reaction center to one containing a reaction center. We note in Table I that the various inhibitors or quinone analogues release no

more than 40% of the counts from the reaction centers. As all of these compete with the head group alone of the UQ-9, it is possible, that the counts remaining simply reflect the binding affinity of the isoprenoid chain of the UQ-9 once the head group binding site has been blocked.

One of us (C.S.) has recently isolated herbicide-resistant and herbicide-sensitive mutants of *R. sphaeroides* and has been able to show that in several cases the altered phenotype can be ascribed to a point mutation in the reaction center. This suggests that these mutants may have altered binding equilibria for quinone or herbicide, making photosynthetic electron transport hypo- or hypersensitive to inhibition. Experiments are in progress to understand the origin of the altered herbicide sensitivity.

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