Absorption Changes Induced by the Binding of Triazines to the Q_B Pocket in Reaction Centers of *Rhodobacter capsulatus*†

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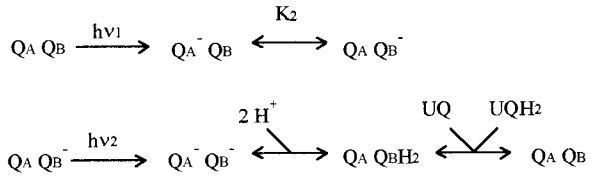
ABSTRACT: Inhibitors which block electron transfer from the primary (Q_A) to the secondary (Q_B) quinone of the bacterial reaction center are competing with the pool ubiquinones for binding at the Q_B pocket. Due to the much greater stability of the semiquinone state Q_B^- compared with fully oxidized or reduced quinone, a displacement of the inhibitors takes place after one flash from state Q_A^-I to state $Q_AQ_B^-$. This process can be monitored from near-IR absorption changes which reflect local absorption shifts specific to Q_A^- and Q_B^-. An anomalous behavior was observed when using triazines in chromatophores of *R. capsulatus*: the IR absorption change reflecting the formation of Q_B^- after one flash was absent. A normal transient decay of this signal was, however, triggered by a second flash, followed by a rapid return to the baseline. We show that this phenomenon is due to an absorption change induced by inhibitor binding (thus present in the dark baseline), with a spectrum close to that of Q_B^-, so that the Q_B^- changes are canceled out during the inhibitor displacement process. On the second flash, one monitors the destruction of the semiquinone, leading transiently to the Q_AQ_B state, followed by inhibitor rebinding. This allows a direct measurement of the binding kinetics. This behavior was observed both in chromatophores and in isolated reaction centers from *R. capsulatus*, but not in *R. sphaeroides*.

In the photosynthetic reaction centers of sulfur purple bacteria, or of photosystem II in chloroplasts, the reduction of the lipid-soluble quinone pool is catalyzed at the so-called Q_B[1] site. The electrons are transferred from the reduced primary quinone acceptor Q_A^-, according to a two-state cycle ("two-electron gate"), summarized in the following scheme [see (*1*) for a review]:



The first line describes the formation of the stable semiquinone Q_B^- following the first photochemical charge separation ($h\nu_1$). The second photoreduction step ($h\nu_2$) results in the formation of the doubly reduced and protonated quinol Q_BH_2, which is then released from the RC and replaced by a fresh quinone from the pool. The oxidized (Q_B) and fully reduced (Q_BH_2) states are weakly bound to the Q_B pocket and rapidly exchanged with pool quinones, whereas the semiquinone state Q_B^- is strongly attached.

A number of inhibitors (some of them widely used as herbicides) act by competing with quinone for binding at the Q_B site (*2*−*6*). Crystallographic data in RCs of *Rhodopseudomonas viridis* and *R. sphaeroides* are available showing detailed binding structures in the Q_B pocket for Q_B, for Q_B^- (*7*, *8*), and for several inhibitors (*9*−*12*). The inhibitor binding is markedly affected by the state of the two-electron gate, because of the much stronger binding of Q_B^- compared with Q_B or Q_BH_2. The dissociation constant of the semiquinone state is very small (*1*), so that the availability of the Q_B pocket to the inhibitor is controlled by the equilibrium constant $K'_2 = [Q_AQ_B^-]/[Q_A^-Q_B]$ (here and in the following, the prime is meant to denote apparent equilibrium constants encompassing the competition of UQ binding under the conditions present in chromatophores). In chromatophores around pH 7, K'_2 is in the range 20−100 (depending on the redox state of the primary donor P (*25*), so that one expects the effective dissociation constant of the inhibitor to be increased by a similar factor in the "odd state" of the RC (i.e., when one semiquinone is present) compared with the "even state" (when zero or two semiquinones are present). This contrast is actually enhanced by another effect due to the influence of Q_A^- on the binding properties of the Q_B pocket (*2*). A consequence of this is that, at appropriate inhibitor concentration, one can have almost total binding in the dark (even state) and almost total inhibitor displacement after relaxation of the odd state equilibria, leading to the formation of $Q_AQ_B^-$.

In previous work (*26*), we investigated these effects, monitoring directly the inhibitor displacement after one flash. The decay of Q_A^- and concomitant buildup of Q_B^- were followed from the near-IR absorption changes specific for each of these semiquinones (electrochromic shifts of the neighboring bacteriopheophytins). In the course of this study,

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[1] Abbreviations: BPhe, bacteriopheophytin; *o*-phe (or *o*-phenanthroline), 1,10-phenanthroline; Q_A, primary quinone acceptor; Q_B, secondary quinone acceptor; RC, reaction center; *R.*, *Rhodobacter*; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-1,4-phenylenediamine; UQ, ubiquinone-10.

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we observed anomalous kinetics for the absorption changes of Q_B^- when using chromatophores or isolated reaction centers from *R. capsulatus*. This phenomenon is described and analyzed in the present paper, showing that the binding of triazines induces a spectral shift similar to that caused by Q_B^-. A useful consequence is that the binding kinetics of the inhibitor can be directly observed.

MATERIALS AND METHODS

The mutant FJ2 of *Rhodobacter capsulatus* was a kind gift of F. Daldal (*13*). It is devoid of the physiological donors to the RC (cytochromes c_2 and c_y). A similar strain for *Rhodobacter sphaeroides*, CYC17, was a kind gift of T. Donohue (*14*). This mutant lacks cytochrome c_2 and iso-c_2. Growth conditions and chromatophore preparation were previously described (*15*). Isolated RCs from the green mutant MT-1122 from *R. capsulatus* were prepared as described in (*16*).

For spectroscopic experiments, the chromatophores were diluted in a medium containing 50 mM KCl and 50 mM Tris or MOPS buffer, pH 7.2. The RC concentration of the chromatophore suspension was around 100 nM, based on an extinction coefficient of 20 mM^{-1} cm^{-1} for P^+ at 600 nm. Valinomycin (3 μM) was added in order to collapse the membrane potential and associated absorption changes. TMPD (1−2 mM) was used as an exogenous donor. The suspension was kept anaerobic under an argon flow, and 5 mM KCN was added for suppressing the oxidase-mediated autoxidation of TMPD.

Absorption changes were measured using a Joliot-type spectrophotometer (*17, 18*), as previously described (*15*). This apparatus was also used for recording the ±atrazine dark difference spectra shown in Figure 7. This was done by first recording a "baseline" spectrum with uninhibited material in both the reference and measurement cuvettes. A second spectrum was then recorded with atrazine added in the sample present in the measurement cuvette and an equivalent addition of ethanol in the other sample. A third spectrum was recorded with atrazine present in both samples. Subtraction of spectra (2) − (1) or (2) − (3) (or comparison of individual experiments) yielded similar difference spectra, with a variable overall slope. This slope is due to the absorption spectrum of the samples which appears to some extent in the differential procedure due to uncontrolled small differences in the filling of the cuvettes.

In experiments using a concentration of inhibitor that saturates the dark binding, we used (when mentioned in the figure legends) the phase-resetting procedure described previously (*26*), replacing the first flash of a sequence by a pair of flashes spaced 10 ms apart. This resets nearly all RCs in the odd state and avoids the progressive mixing of the odd and even states in the course of an experiment unless a long dark time is allowed between individual runs. This does not affect the results when the sequence consists of two flashes (or one resetting pair followed by one flash), as was checked by comparing with experiments run after several minutes of dark adaptation, allowing full oxidation of Q_B^-. It causes, however, a baseline shift when a significant amount of Q_B^- is present in the dark baseline. Such is the case in the multiple flash experiment of Figure 2.

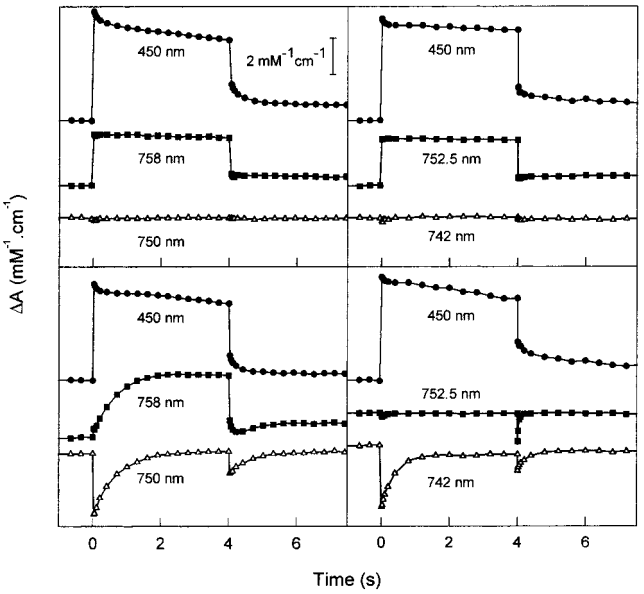

FIGURE 1: Absorption changes caused by two saturating flashes spaced 4 s apart, at three wavelengths, as indicated. The results obtained with chromatophores of *R. sphaeroides* are displayed in the left-hand panels; those obtained with chromatophores of *R. capsulatus* are in the right-hand panels. Top panels, no inhibitor present. Bottom panels, 20 μM terbutryn (in *R. sphaeroides*) or 150 μM atrazine (in *R. capsulatus*) was present.

RESULTS

Figure 1 compares the absorption changes induced by two flashes, spaced 4 s apart, in chromatophores of *R. sphaeroides* (left-hand panels) and *R. capsulatus* (right-hand panels), in the absence (top) or presence of a saturating concentration of a triazine inhibitor (bottom; terbutryn was used for *R. sphaeroides* and atrazine for *R. capsulatus*, for reasons explained later). One millimolar TMPD was present, ensuring rapid reduction of P^+ ($t_{1/2} \approx 1$ ms). The rapid transient due to P^+ formation and reduction is not resolved in this figure. We discuss first the *R. sphaeroides* results (left-hand panels). Three wavelengths were used; 450 nm is a peak of the ubisemiquinone minus ubiquinone difference spectrum, where Q_A^- and Q_B^- have roughly similar extinction coefficients (some contribution of TMPD changes is also present at this wavelength). The observed binary pattern reflects the semiquinone formation caused by the first flash and its destruction by the second flash. In the infrared region, 750 nm is an isosbestic wavelength for Q_B^-, while Q_A^- displays a negative absorption change (see the spectra in Figure 4). Conversely, 758 nm is isosbestic for Q_A^- changes and lies in the positive region of the Q_B^- spectrum. In the absence of inhibitor (top), the rapid formation of Q_B^- is observed following the first flash and its rapid destruction following the second flash. No change is observed at 750 nm because the transient formation of Q_A^- does not appear on this time scale. In the presence of terbutryn (bottom), while no Q_B^- is initially present after the first flash (758 nm), a large amount of Q_A^- is seen at 750 nm. Displacement of the inhibitor is then observed in the 100 ms range ($t_{1/2} = 400$ ms), reflected by the concomitant decay of Q_A^- and formation of Q_B^-. The 450 nm change is neutral with respect to this process, so that the trace is essentially unmodified by the presence of the inhibitor. Similar results were obtained when using atrazine, although the large K'_i (≈100 μM) for

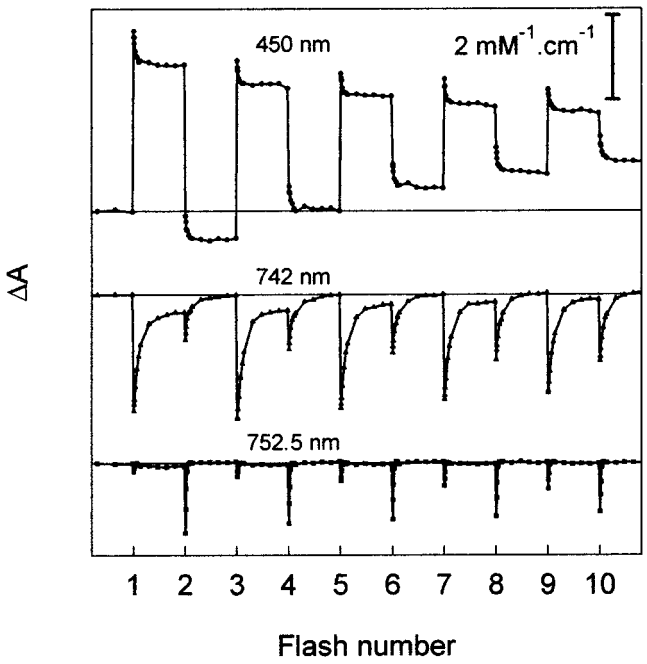

FIGURE 2: Absorption changes at 450, 742, and 752.5 nm on a series of 10 flashes, spaced 3 s apart. Chromatophores of *R. capsulatus* FJ2 in the presence of 150 μM atrazine. The resetting procedure descibed under Materials and Methods was used; i.e., "flash 1" was actually a pair of flashes spaced 15 ms apart. Some Q_B^- is present in the dark baseline, but nearly all centers are reset to Q_B^- following the flash pair: this explains why more semiquinone is destroyed on "flash 2" than formed on "flash 1" in the 450 nm trace.

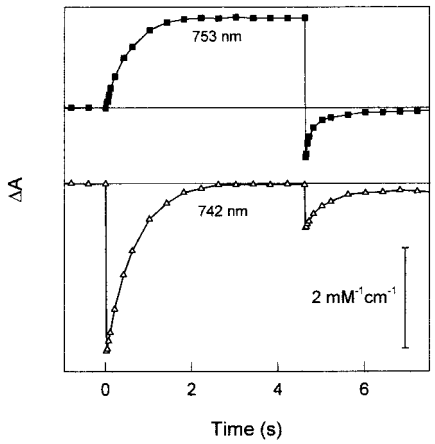

FIGURE 3: Absorption changes at 753.5 and 742 nm on a series of two flashes spaced 4.5 s apart. Chromatophores of *R. capsulatus* FJ2 in the presence of 4 mM *o*-phe. The resetting procedure described under Materials and Methods was used.

this inhibitor in chromatophores of *R. sphaeroides* precluded measurements under saturating conditions.

The IR spectra of Q_A^- and Q_B^- are somewhat different in *R. capsulatus* (right-hand panels) from those of *R. sphaeroides* (see Figure 4). Q_A^- can be monitored at 742 nm (isosbestic for Q_B^-) and Q_B^- at 752.5 nm (isosbestic for Q_A^-). The pattern of absorption changes observed for *R. capsulatus* resembles that of *R. sphaeroides*, except for the 752.5 nm trace (expected to monitor Q_B^- kinetics) in the presence of atrazine (bottom). Whereas no change is observed at this wavelength after the first flash, a negative spike appears after the second flash, followed by a relatively rapid ($t_{1/2} \approx 30$ ms) relaxation toward the baseline. These kinetics

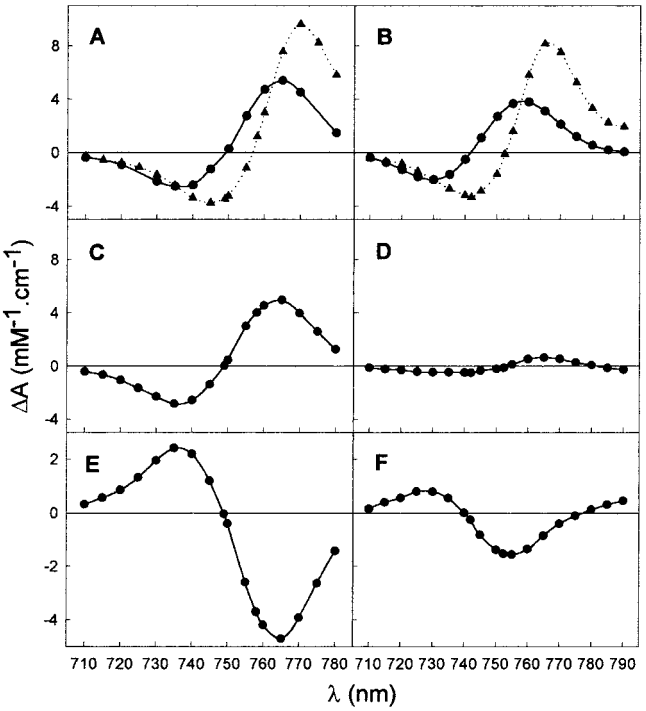

FIGURE 4: Absorption changes in the near-IR region in chromatophores of *R. sphaeroides* CYC17 (left-hand panels) and chromatophores of *R. capsulatus* FJ2 (right-hand panels). Panels A and B: spectra of Q_B^- (circles) measured in the 100 ms range after the first flash in the absence of inhibitor; spectra of Q_A^- (triangles) measured at 15 ms after the first flash in the presence of 150 μM atrazine. Panels C and D: spectra of the change measured at 3 s after the first flash (before firing flash 2). Panels E and F: spectra of the change induced by the second flash (absorption at 15 ms after flash 2 minus absorption before flash 2).

(rate constant denoted k_{sp}) are faster than those of the inhibitor displacement (rate constant k_{dis}; the dependence of k_{sp} and k_{dis} on the atrazine concentration is shown below in Figure 5). It is clear, however, that the functioning of the two-electron gate is essentially the same as observed in *R. sphaeroides*. The inhibitor displacement and formation of Q_B^- occur in the same way, as evidenced by the 450 and 742 nm traces. The binary oscillation is sustained during a long train of appropriately spaced flashes, as shown in Figure 2. At 752.5 nm, the negative spike occurs recurrently on even-numbered flashes: it thus appears somehow as a marker of the $Q_A^-Q_B^- \rightarrow Q_AQ_BH_2$ reaction. Similar results were obtained with terbutryn. The spike is, however, more difficult to resolve due to faster kinetics, and a complicating feature, described below in Figure 8, is observed when using fully saturating concentrations. This is why we chose atrazine as a more illustrative case in Figure 1. At variance with triazines, the pattern observed with *o*-phe (Figure 3) in *R. capsulatus* is, in first approximation, similar to the "normal" (*R. sphaeroides*) case. On closer examination, however, this pattern appears intermediate between the two situations, showing both the Q_B^- rise at 753 nm (with slightly diminished extent) and a small negative spike on even-numbered flashes. We also assayed the inhibitor stigmatellin, which is, however, more difficult to handle in such experiments because of its very slow release rate (*26*). It clearly appeared (not shown) that the Q_B^- change at 753 nm was present after inhibitor displacement, but our data do not allow

us to discriminate between a 100% normal pattern or an intermediate one like that obtained with *o*-phe.

Figure 4 shows spectral information for *R. sphaeroides* (left panels) and *R. capsulatus* (right panels). In the top panels are shown the spectra of the absorption change induced by the first flash in the absence of inhibitor (i.e., the Q_B^- spectra, circles) or the 15 ms change in the presence of inhibitor (Q_A^- spectra, triangles). The middle panels show the spectra observed 4 s after the first flash (or resetting flash pair), i.e., after completion of the inhibitor displacement process. In *R. sphaeroides* (left panels), the spectrum is identical to that of Q_B^- recorded in the absence of inhibitor. In *R. capsulatus* (right panels), very small absorption changes are recorded under such conditions. The spectrum of these changes does not resemble that of Q_B^-, but rather that of Q_A^-. The bottom panels show the changes induced by the second flash, measured at a short time after the flash (i.e., absorption level 15 ms after the second flash minus absorption level before this flash). These spectra were corrected for the contribution of Q_A^-, estimated from the relative extent of the 742 nm trace (749 nm in *R. sphaeroides*) on the second flash, using the spectrum of the top panels. One expects to obtain in this manner (for *R. sphaeroides* at least) the change reflecting the destruction of Q_B^-. Indeed, the corrected spectrum is similar to the Q_B^- change observed in the absence of inhibitor. Interestingly, this is also true for *R. capsulatus* chromatophores, although the Q_B^- spectrum was not detected after the first flash. Thus, in this material, the IR changes associated with Q_B^- look like they are distorted by some high-frequency passing filter: the rapid transient reflecting Q_B^- destruction is conserved, but the steady signal reflecting Q_B^- formation after the first flash is canceled out. These effects are specific of *R. capsulatus* and were observed in chromatophores, in whole cells (not shown), and in isolated RCs (not shown). The "normal" behavior observed with *R. sphaeroides* was also obtained with *Rubrivivax gelatinosus* (not shown). No other bacterium was tested as yet.

Our first attempt for interpreting these results was to postulate a second, noncompetitive, binding site for triazines in *R. capsulatus*. Indeed, our reasoning was that the inhibitor appears to still affect the RC *after* it has been displaced from the Q_B pocket. We thus speculated that the presence of the inhibitor on the second site could modify local electrochromic effects, e.g., by affecting proton binding. This model, however, is not consistent with a number of observations, summarized below:

(a) The rate of the second electron-transfer reaction $Q_A^-Q_B^- + 2H^+ \rightarrow Q_AQ_BH_2$, as observed at 450 nm, was not modified (with $t_{1/2} = 70$ μs at pH 7.3, not shown), which is not consistent with a drastic modification of proton binding, according to current views [see (*19*)].

(b) When varying the inhibitor concentration, the suppression of the IR absorption changes associated with Q_B^- after the first flash closely followed the amount of inhibited RCs in the dark (an example is shown below in Figure 6). In other words, there cannot be an independent second site (unless the binding on this site and on Q_B is strongly cooperative).

(c) The relaxation kinetics (rate constant k_{sp}) of the spike depend on the inhibitor concentration, [I]. A linear dependence of k_{sp} vs [I] was observed (Figure 5) on a range extending from subsaturation (below the K'_i) to oversaturation

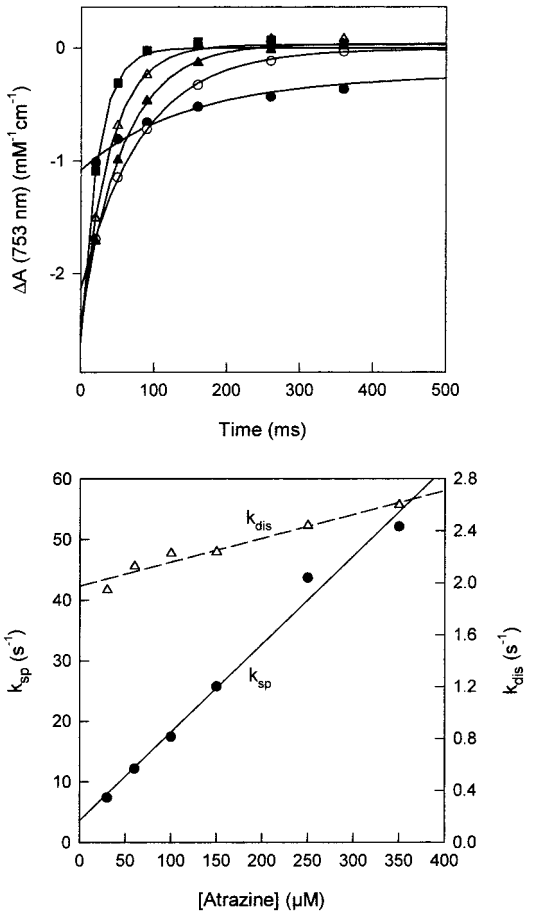

FIGURE 5: Top panel: kinetics of the spike induced by the second flash at 753 nm. Chromatophores of *R. capsulatus* FJ2 in the presence of 30 (solid circles), 60 (open circles), 100 (solid triangles), 150 (open triangles), and 250 (solid squares) μM atrazine. The lines are one-exponential fits of the data. Bottom panel: solid circles (left-hand scale), rate constant k_{sp} of the spike relaxation after the second flash (estimated from the fits shown in the top panel) against atrazine concentration; open triangles (right-hand scale), rate constant k_{dis} for the inhibitor displacement obtained by fitting the 741 nm kinetics following the first flash.

(several times the K'_i). This clearly indicates that the spike relaxation is not controlled by some first-order rearrangement within the RC. On the other hand, this is strongly suggestive of binding kinetics.

We are thus led to reject the hypothesis of a second binding site and to propose the following interpretation. We assume that the inhibitor binding to the Q_B pocket causes a BPhe shift which resembles the shift observed in the presence of Q_B^-. This spectral feature is thus present in the dark, so that the formation of Q_B^- due to inhibitor displacement causes no aborption change with respect to the dark baseline. On the other hand, when the semiquinone is destroyed by the second flash, quinol leaves the Q_B pocket and is rapidly replaced by an oxidized quinone; eventually, the inhibitor rebinds. The destruction of semiquinone is accompanied by the usual absorption changes (appearing as a negative transient at 752.5 nm), and the subsequent binding process ("spike relaxation") restores the baseline (see Figure 9).

The experiment of Figure 6 was designed to test some aspects of this model. It shows the 752.5 nm (Q_B^-), 741 nm (Q_A^-), and 450 nm ($Q_A^- + Q_B^-$) changes induced by a sequence of two flashes at a subsaturating concentration of

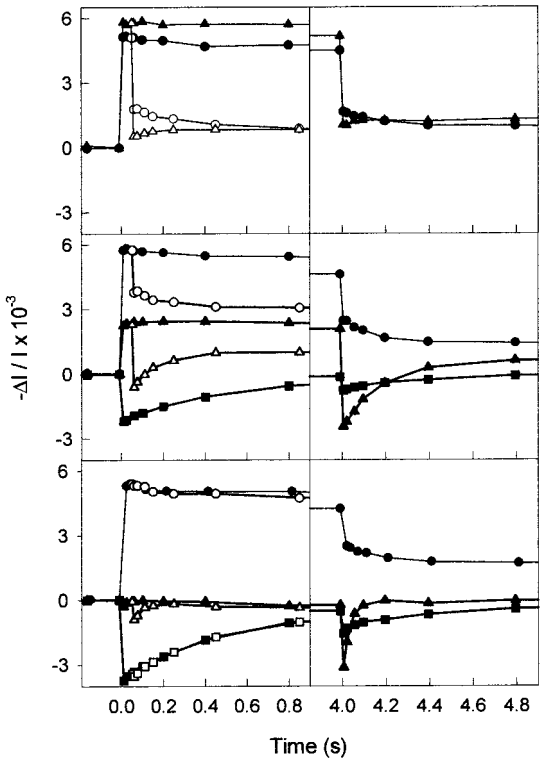

FIGURE 6: Absoprtion changes induced by a series of two flashes in chromatophores of *R. capsulatus* FJ2, at 450 nm (circles), 752.5 nm (triangles), and 741 nm (squares). Top panels, no inhibitor present. Middle panels, 20 μM atrazine. Bottom panels, 120 μM atrazine. The right-hand panels show the traces obtained on the first flash (closed symbols) and on the second flash (open symbols) when fired 50 ms after the first one. The right-hand panels show the traces obtained on the second flash when fired 4 s after the first. The 741 nm data in the absence of inhibitor were a flat line omitted in the top panels. The vertical scale for the 752.5 nm traces was expanded 2-fold for clarity.

atrazine (middle panels, to be compared with traces obtained in the absence of inhibitor, top panels, and in the presence of saturating inhibitor, bottom panels). About 60% of the RCs have bound atrazine in the dark. As mentioned above, the relative extent of the 752.5 nm change (triangles) following the first flash corresponds to the fraction of uninhibited centers. The initial (downward) change induced by the second flash fired 4 s after the first one (right-hand panels) has the same extent as in the control and thus monitors the destruction of all the Q_B^- present, including the "hidden part" missing in the signal induced by the first flash. Rising kinetics are then observed (spike relaxation), with a relative extent similar to the inhibited fraction. Also shown in the left-hand panels are the traces observed with a short interval ($\Delta t = 50$ ms) between the first and the second flash. This spacing is short with respect to the inhibitor displacement kinetics ($t_{1/2} \approx 350$ ms). Thus, the second flash should almost only affect the uninhibited RCs since the inhibited ones are essentially in the closed Q_A^- state. The initial downward change induced by this flash monitors the destruction of Q_B^- in uninhibited centers. A rising phase is then observed (open triangles in the left-hand middle panel), similar to the spike relaxation occurring in the experiment with long Δt (closed triangles in the right-hand middle panel), although its extent is smaller. It is noteworthy that the change thus observed is not a mixture of the traces recorded when running the experiment (at short Δt) in the absence of

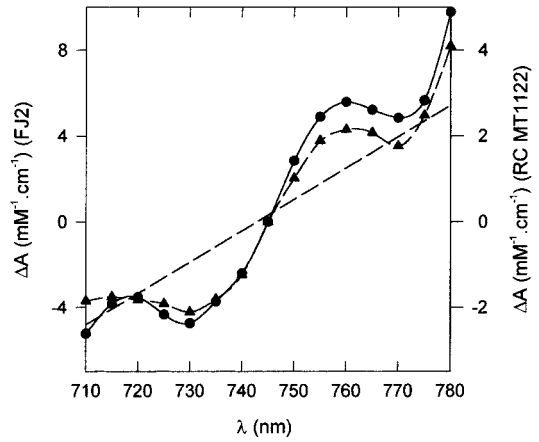

FIGURE 7: Spectra of the absorption change induced in the dark by addition of 120 μM atrazine to chromatophores of *R. capsulatus* FJ2 (circles, left-hand scale) or isolated RCs from *R. capsulatus* MT1122 (triangles, right-hand scale) in the presence of 150 μM atrazine. The RCs were in a medium containing 10 mM MOPS buffer, pH 7.7, and 0.05% Triton X-100, with 40 μM UQ-6.

inhibitor and in the presence of saturating inhibitor, none of which displays a significant spike. This apparent paradox is well explained by our model. Let us denote f the fraction of inhibited RCs in the dark. The uninhibited centers ($1 - f$) are converted to Q_B^- after the first flash and back to Q_B after the second one. Equilibration with the inhibitor then takes place so that a fraction f of these RCs eventually binds the inhibitor. This process thus involves $f(1 - f)$ centers. The initially inhibited centers are converted to Q_A^- on the first flash, are left unchanged by the second one, and are subject to inhibitor displacement at later times, which causes no absorption change at this wavelength. We thus expect a rising phase monitoring inhibitor binding on $f(1 - f)$ centers, consistent with the observed trace.

The present hypothesis accounts with nice parsimony for the experimental features described so far. Moreover, it yields a testable prediction, viz., that a Q_B^- -like spectrum should be obtained for the difference of absorption spectra in the dark recorded in the presence and absence of triazine. This is a delicate experiment, especially in chromatophores, because we are searching for small relative changes, but it is not beyond the resolution of the Joliot spectrophotometer. The difference spectra obtained as described under Materials and Methods are shown in Figure 7, for chromatophores (circles) or isolated RCs from *R. capsulatus* (triangles). The similar rising slope in both experiments is coincidental, because large fluctuations of the slope were observed when running control experiments where the reference and measurement cuvettes were refilled with samples taken from the same suspension. Although the slope was fluctuating, such control spectra were otherwise flat, so that the S-shaped feature appearing in the ±inhibitor spectra is significant. Clearly, the inhibitor binding causes an absorption change with similar features to that associated with Q_B^-.

The case of terbutryn is illustrated in Figure 8. Similar to atrazine, the binding of terbutryn in *R. capsulatus* chromatophores suppresses the regular Q_B^- change in the IR and induces a negative spike on even-numbered flashes. The k_{off} and k_{on} rates of terbutryn are greater than those of atrazine (*26*), and, accordingly, both the displacement and spike kinetics are faster. A new feature appears, however, at high

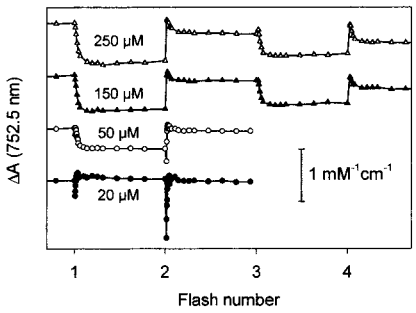

FIGURE 8: Absorption changes at 752.5 nm in the presence of increasing concentrations of terbutryn, as indicated, during a series of two or four (for the two higher concentrations) flashes, spaced 2.5 s apart. Chromatophores of *R. capsulatus* FJ2.

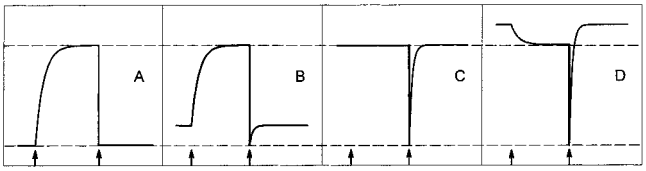

FIGURE 9: Scheme summarizing our interpretation for the various patterns obtained when monitoring the IR changes of Q_B^- in various circumstances. Two flashes (arrows) are shown, and the damping is assumed negligible. The bottom dashed line indicates the absorption level of state Q_AQ_B and the top dashed line that of $Q_AQ_B^-$. Panel A, "normal case" observed with *R. sphaeroides*. Panels B−D refer to *R. capsulatus*, with *o*-phe, atrazine, or a high concentration of terbutryn, respectively.

terbutryn concentrations: the formation of Q_B^- is accompanied by a negative change at 752.5 nm, destroyed on even-numbered flashes, so that one observes an oscillating pattern inverted with respect to the normal Q_B^- changes. The spectrum of the change induced by the first flash after inhibitor displacement is complete, has the same shape as that of Q_B^-, with inverted sign. A similar trend was actually also observed with atrazine at high concentrations, although to a smaller extent.

DISCUSSION

The present results show that the binding of terbutryn or atrazine to the Q_B pocket in chromatophores or isolated reaction centers from *R. capsulatus* induces an absorption change in the dark. The spectrum of this change resembles that of the BPhe shift associated with Q_B^-. This phenomenon does not occur in *R. sphaeroides*, nor in *Rubrivivax gelatinosus*. The close equivalence of the inhibitor-induced and Q_B^- -induced changes, judging from the disappearance of the Q_B^- spectrum after the first flash (Figure 4D), was only observed for the two triazines assayed. With *o*-phenanthroline, a similar effect is present at a much smaller extent. With stigmatellin, it is also clear that the cancellation of the Q_B^- spectrum does not occur, but the low accuracy of our data in this case (due to the slow release rate of this inhibitor) does not rule out that some intermediate effect might be present, as for *o*-phenanthroline.

The scheme of Figure 9 illustrates our interpretation. The absolute absorption levels corresponding to states Q_AQ_B and $Q_AQ_B^-$ of the RC are indicated by the thin horizontal dashed lines. The boldface line before flash 1 in each panel indicates the absorption level corresponding to state Q_AI, with bound inhibitor. This is the observed experimental baseline when the inhibitor is bound in the dark. The first flash induces inhibitor displacement reflected by an absorption change starting from this baseline and ending up at the $Q_AQ_B^-$ level. The second flash destroys Q_B^-, and the RCs are rapidly converted to the Q_AQ_B state (lower thin dashed line). Then the inhibitor rebinds, and the absorption level returns to the initial baseline. The "normal" case (that of *R. sphaeroides* for the assayed inhibitors and, possibly, that of *R. capsulatus* for stigmatellin) is illustrated in the left-most panel, where the Q_AI and Q_AQ_B absorption levels are identical. The second panel describes the *o*-phe case, where the Q_AI level is slightly above Q_AQ_B. The third panel describes the atrazine case, where the Q_AI level is close to the $Q_AQ_B^-$ level. This also applies to terbutryn in the low concentration range (roughly, up to 4 times the K'_i). The pattern observed with terbutryn at higher concentrations is depicted in the rightmost panel: the Q_AI level is now above the $Q_AQ_B^-$ level, accounting for the inverted oscillating pattern of the Q_B^- changes.

It is surprising that terbutryn and atrazine, which are expected to be neutral molecules, cause a similar bandshift as that associated with the anionic Q_B^- and believed to be an electrochromic effect. Several possibilities may be envisaged. First, the inhibitor binding might dislodge a proton normally present in the Q_B pocket, perhaps for steric reasons. This would result in a negative charge change, causing an equivalent electrochromic shift to that caused by the anionic Q_B^-. The energy for breaking this bond would be taken from the overall binding energy (the dissociation constant for inhibitors in *R. capsulatus* is slightly larger than in *R. sphaeroides* see ref *26*). Alternatively, the inhibitor might induce a reorganization of the H-bond network that would be propagated (possibly through a structural rearrangement) as far as the $BPhe_B$, accounting for the spectral shift which would thus not be electrochromic in nature. In the same line, one may wonder whether the Q_B^- -induced change is strictly electrochromic or also caused by a modification of the H-bond network. Absorption shifts due to ligand binding are of course not unprecedented: for instance, in the cyt bc_1 complex, antimycin (*20*, *21*) induces a red-shift of reduced cytochrome *b*, and myxothiazol induces a blue-shift (*21*, *22*).

It is remarkable that despite the large degree of homology between the RCs of *R. capsulatus* and *R. sphaeroides*, an all-or-none difference is found regarding the spectral shift induced by triazines. Among the amino acids involved in Q_B or inhibitor binding, the only difference is found at L224, where Ile (in *R. sphaeroides* or *Rhodopseudomonas viridis*) is replaced by Val in *R. capsulatus*, which is not expected to cause a big change. At variance with *R. sphaeroides*, no crystallographic structure of the *R. capsulatus* RC is available. However, significant differences in the orientations of the bacteriochlorophyll monomer and of the bacteriopheophytin in branch B were reported by Foloppe et al. (*23*) from a modeling study of the *R. capsulatus* RC. This provides a structural lead that may be related with the present results.

The effect of terbutryn at high concentration is surprising. In the titration range for inhibition of electron transfer (around $K'_i = 6\ \mu M$), its effect is similar to that of atrazine. It is thus unexpected that higher concentrations (above 4 times the K'_i) still increase the baseline shift, giving rise to the inverted oscillation pattern shown in Figure 8. This suggests the involvement of a second binding site with lower affinity, which exerts an additional shift of $BPhe_B$. Moreover, the inhibitor bound at this site must be released upon

formation of Q_B^- (it is subject to the displacement process, as for the higher affinity site). Thus, the secondary site is also in competition with quinone binding. Previous indications of a second site for terbutryn binding have been reported (*6*, *24*). There may actually be no real difference between terbutryn and atrazine in this respect, except a lower relative affinity of the latter for the putative second site. At high atrazine concentration (350 μM, i.e., 17 $\times$ K'_i), small inverted oscillations were indeed present, comparable to the case of terbutryn around 30 μM (i.e., 5 $\times$ K'_i).

Although small, the spectrum observed after displacement of atrazine (or terbutryn in the low concentration range) is not flat (see Figure 4, panel D), but has the shape of the Q_A^- spectrum. The presence of some amount of Q_A^- is actually expected, resulting from the overall equilibrium $Q_AQ_B^- + I \leftrightarrow Q_A^-I + UQ$. The equilibrium constant is $K''_2 = K'_2/(1 + [I]/K'_i)$, where K'_2 is the equilibrium constant for electron transfer and K'_i the inhibitor dissociation constant (*26*). Using the value of $K'_2 \approx 20$ (in the presence of reduced P) estimated from oscillation damping in ref *26* and the value of $K'_i \approx 30$ μM (estimated from the recombination rate in ref *26*, thus a value relating to the center in the $P^+Q_A^-$ state), one expects $K''_2 \approx 3.3$ while the value estimated from the extent of Q_A^- appearing in Figure 4D is about 9. It is possible that the amount of Q_A^- measured in this manner is underestimated, if the spectral change induced by atrazine binding is not a pure Q_B^--like change, but also includes some Q_A^--like contribution. Another possibility is that the dissociation constant of atrazine in the RC state PQ_A^- is larger than estimated in the $P^+Q_A^-$ state. This view is actually supported by the analysis of the displacement rate discussed below.

An interesting consequence of the spectral change caused by the binding of triazines on the Q_B pocket of *R. capsulatus* is that the kinetics of inhibitor binding can be directly observed ("spike" relaxation kinetics). One thus has access both to the binding and to the release (through the decay of Q_A^- during inhibitor displacement) kinetics of the inhibitor. The expected dependence of the rate of the spike relaxation on [I] is $k_{sp} = k_{on}[I] + k_{off}$. A linear dependence was indeed observed (Figure 5, circles), yielding a slope $k_{on} \approx 0.152$ s^{-1} μM^{-1} and, with lesser relative accuracy, an intercept $k_{off} \approx 2$ s^{-1}. The ratio of these values is $k_{off}/k_{on} \approx 13$ μM, in reasonable agreement with the apparent dissociation constant $K'_i \approx 20$ μM (which relates to the PQ_A state of the RC) estimated with better accuracy from titration of inhibitor binding in ref *26*. The other plot in Figure 5 (triangles) shows the dependence on [I] of k_{dis}, the rate constant for inhibitor displacement observed at 741 nm after the first flash. The theoretical expression (eq 27 in ref *26*) predicts a linear dependence, with intercept k_{off} and slope $k_{off}/K'_i(1 + K'_2)$. The intercept of the regression line yields a value of $k_{off} \approx 2$ s^{-1}, similar to that estimated from k_{sp}. The slope is 0.0018 s^{-1} μM^{-1}, so that one obtains $K'_i(1 + K'_2) \approx 1080$, where K'_i and K'_2 both relate to the RC state PQ_A^-. Taking $K'_2 \approx 20$, this results in $K'_i \approx 52$ μM, significantly larger than the value of 30 μM that we estimated (*26*) for state $P^+Q_A^-$. A similar trend, with $K'_i(P^+Q_A^-) < K'_i(PQ_A^-)$, was observed for terbutryn although less marked (*26*). Altogether, our results [extending previous conclusions from Wraight's group (*2*)] suggest that the RC state modulates the apparent affinity of the inhibitor (triazines or *o*-phe) so that $K'_i(PQ_A) < K'_i(P^+Q_A^-) < K'_i(PQ_A^-)$. It is noteworthy that the values of k_{off} estimated (for atrazine) in the various states, 2 s^{-1}, 3.4 s^{-1} (*26*), and 2 s^{-1}, respectively, are quite similar, so that the modulation of K'_i is due to the apparent k_{on}, reflecting the competition with UQ binding on the Q_B pocket. This is consistent with our suggestion (*26*) that these effects could be predominantly due to a modulation by the RC state (in the opposite direction) of the affinity for UQ binding.

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