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Herbicide-Quinone Competition in the Acceptor Complex of Photosynthetic Reaction Centers From Rhodopseudomonas sphaeroides: A Bacterial Model for PS-II-Herbicide Activity in Plants

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A select group of herbicides that inhibit photosystem II also act at the acceptor side of the reaction center (RC) from the photosynthetic bacterium Rhodopseudomonas sphaeroides, with much the same relative specificity as in plants. These include the triazines and some phenolic compounds. The proposal that herbicides inhibit the electron transfer from the primary quinone (Q_A) to the secondary quinone (Q_B) by competing for the secondary quinone binding site—the B-site—[5], is tested here with terbutryn, the most potent of the triazines. Competition between terbutryn and ubiquinone (Q-10) was observed using the kinetics of the back-reaction as a measure of inhibition. The model includes binding equilibria before and after flash activation. The binding constants for the preflash (dark) equilibria, for reaction centers in 0.14% lauryl dimethylamine-N-oxide (LDAO), were $K_i^D = 0.8\ \mu M$ terbutryn, $K_q^D = 2\ \mu M$ Q-10; both are detergent-concentration dependent. After flash activation, binding equilibrium is not fully restored on the time scale of the back-reaction because terbutryn unbinds slowly. This gives rise to biphasic decay kinetics from which k_{off} for terbutryn was estimated to be 3 sec^{-1}. Titrations of the rate of the slow back reaction indicated that the post-flash equilibrium is less sensitive to inhibitor, in a manner that is independent of the much stronger binding of the semiquinone, Q_B^-, and indicative of a direct effect of the redox state of Q_A on the affinity of the B-site for ligands. However, the effects on K_i^L and K_q^D could not be separated: either $K_i^L > K_i^D$ or $K_q^L < K_q^D$. Some triazine-resistant mutants have been isolated and are described. All appear to be herbicide binding site mutants. Whole cells and photosynthetic membrane vesicles (chromatophores) exhibit a 10–50-fold increase in resistance to triazines due, in large part, to an increase in the rate of unbinding (k_{off}). The modifications of the binding site appear to diminish the affinity of the B-site for ubiquinone as well as terbutryn. It is concluded that bacterial RCs are a useful model for the study of herbicide activity and specificity.

Key words: reaction center, Rhodopseudomonas sphaeroides, ubiquinone, herbicide activity, herbicide resistance, herbicide specificity

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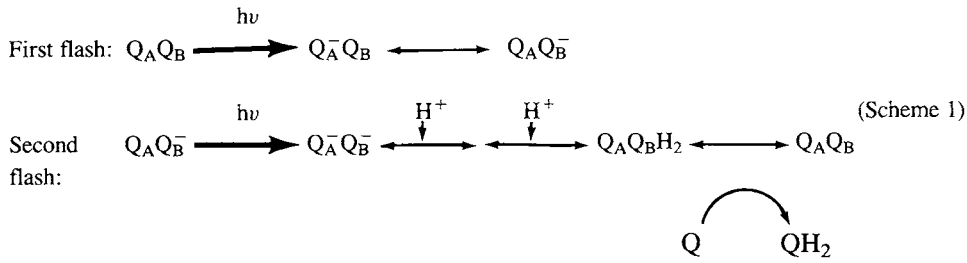
The electron acceptors of photosynthetic reaction centers must function not only to stabilize the primary charge separation but also to facilitate the subsequent transfer of reducing equivalents out of the reaction center. In the purple bacteria (Rhodospirillineae) and in photosystem II (PS II) of plants, the reaction center (RC) must interface with the two electron events involving the large pool of quinone. The electron is first stabilized on a primary acceptor quinone (Q_A) and is then transferred to a secondary quinone (Q_B), forming a semiquinone that can be stable for many minutes. A second turnover of the RC results in the full reduction of Q_B to the quinol. From there the electron leaves the RC and is passed to the quinone pool and the electron transport chain. Each RC, acting in isolation, functions as a two-electron source, exporting reducing equivalents only in pairs [see 1–3 for review]:



(Scheme 1)

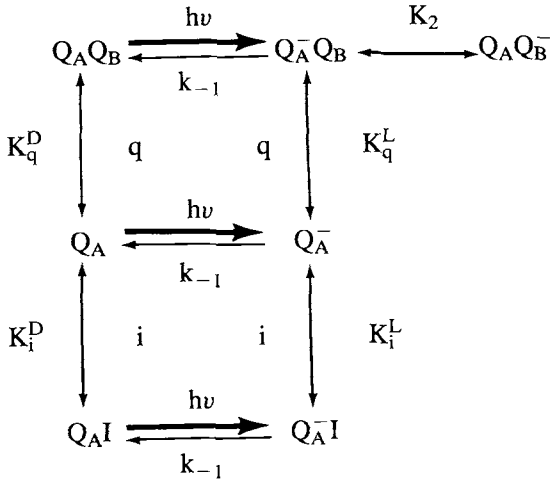
(The primary donor, P/P^+, is rereduced after each turnover and is omitted from this scheme.)

The primary and secondary quinones and their respective binding sites constitute the "acceptor quinone complex" [3]. The activity of the complex as a two-electron gate requires specific and dramatic modifications of the basic redox physical chemistry of quinones. This is brought about by binding interactions with the RC protein that specifically stabilize the semiquinone forms relative to the quinone and quinol species [4]. On the basis of the observed redox properties of Q_B, it can be estimated that the anionic semiquinone binds at least 10^6 times more strongly to the secondary quinone binding site (B-site) than does the quinone form [2]. Furthermore, the quinol appears to bind somewhat more weakly than the quinone [4,5].

Many compounds are known to inhibit the reoxidation of Q_A^- by Q_B. In plants, such inhibitors are often potent and effective herbicides. Although the structures are very diverse, binding competition between different chemical classes is commonly observed [6]. This has led to a model of an inhibitory site with several partially overlapping binding domains, each specific for a different herbicide type [8]. This idea has been strongly supported by the discovery of herbicide-resistant, mutant weed species that are desensitized to some structures, eg, triazines, but retain their sensitivity to other [see 7,9 for review]. In bacteria, the most widely used inhibitor of secondary electron transport is o-phenanthroline, but it is a relatively weak inhibitor in both plants and bacteria (I_{50} = 50–300 μM). We have found that many potent herbicides are also very effective in bacteria [5, and this work]. Interestingly, not all classes of PS II-active herbicides are effective and we consider the bacterial system to manifest some degree of herbicide selectivity. We have undertaken to characterize

the model of action of herbicides in bacteria as a possible, and experimentally convenient, model for photosystem II.

Working with isolated reaction centers from Rhodopseudomonas sphaeroides, our previous studies on the redox properties of Q_A indicated that quinone, as Q_B, and o-phenanthroline interact competitively. This led us to suggest that the general mechanism of action for inhibitors of Q_A^- reoxidation, including herbicides, is by competition with quinone for the secondary acceptor binding site, the B-site [5]. A similar proposal has been made by Velthuys [10]. The implication is that a binding equilibrium exists between free quinone and Q_B in the B-site (Scheme 2). This is entirely consistent with earlier suggestions [11,12] that unbinding of QH_2 and rebinding of Q might represent the mode of transfer of reducing equivalents to the electron transport chain.



(Scheme 2)

Binding of quinone (q) and inhibitor (i) is described here by dissociation constants. Assuming attainment of binding equilibrium, the position of the electron transfer equilibrium following a flash given by

$$\frac{Q_AQ_B^-}{\text{all } Q_A^- \text{ species}} = \frac{Q_AQ_B^-}{Q_A^- + Q_A^-I + Q_A^-Q_B}$$

$$= \frac{K_2(q)/K_q^L}{1 + (i)/K_i^L + (q)/K_q^L} = K_2^{app}. \quad \text{(Equation 1)}$$

In the absence of any electron transfer to P^+, the charge separation recombines in a manner that reflects the Q_A^-/Q_B^- electron transfer equilibrium:

$$PQ_AQ_B \underset{k_{-1}}{\overset{h\upsilon}{\rightleftharpoons}} P^+Q_A^-Q_B \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} P^+Q_AQ_B^- . \qquad \text{(Scheme 3)}$$

When electron transfer to Q_B is completely blocked, the state $P^+Q_A^-$ back reacts with $t_{1/2}(Q_A) = 0.69/k_{-1} = 65$ msec for isolated RCs from Rp sphaeroides. If electron transfer to Q_B reaches equilibrium rapidly compared to k_{-1}, the observed back-reaction slows down according to [12,13]

$$t_{1/2}(\text{slow}) = t_{1/2}(Q_A)(1 + K_2^{app}). \qquad \text{(Equation 2)}$$

This expression has been used to estimate K_2, and values of 15–25 have been reported for isolated RCs in the presence of saturating amounts of quinone [12,13]. We have recently demonstrated the expected quinone dependence of the slow component of the back-reaction and of the submillisecond electron transfer from Q_A^- to Q_B in isolated RCs suspended in Triton X-100 (0.01–1.0%) [13; and Wraight CA, Stein RR: in preparation]. The quinone dependence of the Q_A^- to Q_B electron transfer confirms the significance of the quinone binding reactions on the time scale of turnover in the acceptor complex. However, for RCs in detergent suspension, the overall kinetics of recombination are generally biphasic and the coexistence of a rapidly back-reacting population of RCs shows that quinone accessibility is heterogeneous. The rapid binding and electron transfer to Q_B and the slow phase of the back-reaction arise from RCs with secondary quinone available in the same detergent micelle at the time of the flash. If no quinone is present in a RC-containing micelle, the state of $P^+Q_A^-$ recombines directly because quinone transfer between micelles is slow (50–500 msec depending on detergent type [13; and Wraight CA, Stein RR: in preparation]. The distribution of quinone to the RCs at the time of the flash is determined by the dark binding equilibrium and is affected by any competitive interactions at the B-site, ie, by K_q^D and K_i^D. Thus, the populations of Q_A^-, Q_A^-I, and $(Q_A^-Q_B + Q_AQ_B^-)$ are fixed at the corresponding values for Q_A, Q_AI, and Q_AQ_B, and the fraction of rapidly back-reacting RCs is given by

$$\Delta F = \frac{Q_A + Q_AI}{\text{Total RCs}} = \frac{1 + (i)/K_i^D}{1 + (i)/K_i^D + (q)/K_q^D} \qquad \text{(Equation 3)}$$

Although the rate of intermicelle quinone transfer is too slow to allow reestablishment of the binding equilibrium on the time scale of the fast back-reaction (< 100 msec), it is significant compared to the slow back-reaction. Thus, RCs that have a secondary quinone available at the time of the flash manifest a quinone-dependent slow back-reaction, influenced by the binding equilibrium after the flash, ie, K_q^L and K_i^L, to whatever extent that quinone transfer occurs on this time scale. Quantitatively the rate of the slow phase is not an accurate reflection of K_q^L because the rate of quinone transfer in detergent suspensions is such that equilibria is not reached. Qualitatively, however, the slow phase provides a clear indication of the quinone inhibitor binding competition after the flash.

The expressions for the binding and electron transfer equilibria clearly indicate that inhibitors should also affect the turnover of the acceptor complex in a manner

complementary to that described for quinone. We report here some results of our studies on the effects of PS II-active herbicides on the acceptor complex of Rp sphaeroides. A major advantage of the bacterial system, as a model of PS II, is the ease with which mutants may be obtained. We have been able to obtain herbicide-resistant mutants of Rp sphaeroides by a number of means, including positive selection for spontaneous mutation. Resistance to triazines and benzonitriles, eg, ioxynil, arises readily. The preliminary characterization of five triazine-resistant mutants, generated by nitrosoguanidine mutagenesis, is described.

MATERIALS AND METHODS

Cells

Various strains of the photosynthetic bacterium Rhodopseudomonas sphaeroides were used in these studies. Reaction centers were prepared from the carotenoidless, blue-green mutant R-26. However, we encountered difficulty in preparing stable mutants from R-26, and whole cell and mutant studies were performed with the green mutant Ga, which has a substantial, but different, carotenoid complement compared to the wild type (strain 2.4.1).

All strains were grown phototrophically on a modified Hutner's medium containing succinate as the only carbon source. The R-26 mutant, which is sensitive to oxygen in the presence of light, was grown chemotrophically in the dark for 6 h following each transfer, allowing respiration to remove oxygen from the medium. The effects of inhibitors on cell growth were determined by measurement of cell density using a Klett-Summerson colorimeter with a red filter. I_{50} values were taken to be equal to the inhibitor concentration at which the growth rate of logarithmic phase cells was halved. The light intensity, provided by 25-W tungsten lamps, was roughly 30% saturating.

Mutants

Herbicide-resistant mutants of Rp sphaeroides, strain Ga, were prepared by treatment with N-methyl-N′-nitro-N-nitrosoguanidine [14]. Mutagenesis was followed by growth in liquid medium in the presence of 30 μM ametryn before plating on agar containing 30 μM ametryn. Five mutants were selected, which grew vigorously in liquid culture containing 100 μM atrazine. The mutants appeared to revert after growing for several generations in the absence of herbicide and were maintained in atrazine-containing medium. Mutagenesis of strain R26 produced only feeble, quickly reverting mutants with many pleiotropic effects.

Preparation of Photosynthetic Particles

Vesicles of the photosynthetic membrane system (chromatophores) were prepared by French-pressing cells (18,000 psi) in 100 mM NaCl, 10 mM Tris, pH 8.0; and cells were washed by centrifugation (160,000 g [av], 60 min). Reaction centers were prepared by LDAO detergent fractionation of R-26 chromatophores as described in [12].

Herbicides and Other Chemicals

Ametryn, atraton, atrazine, cyanazine, prometon, simeton, and simetryn were the generous gifts of Dr Homer LeBaron, Ciba-Geigy. o-Phenanthroline was from

Fisher. Azidoatrazine was from Pathfinder Laboratories, Missouri. All other herbicides were purchased from Chemical Services, Pennsylvania. Herbicide common names are used as defined in [15].

Tris-hydroxymethylaminomethane (Tris), Triton X-100 (TX-100), and horse heart ferricytochrome *c* were obtained from Sigma. Lauryl dimethylamine-N-oxide (LDAO, trade name Ammonyx LO) was purchased from Onyx Chemical Company, New Jersey.

Kinetic Measurements

Kinetic absorption measurements were made as previously described [12]. All measurements were made anaerobically under argon at 20°C unless otherwise noted. Both pH and E_h were monitored continuously. Data were digitized with 12-bit accuracy and stored in a minicomputer. Exponential analysis was performed by a modified Marquadt, nonlinear least-squares technique, which was checked against the eigenfunction expansion method of Provencher [16].

Measurement of Secondary Acceptor Activity

The back-reaction was measured as the disappearance of P^+ at 430 nm in isolated RCs and 541 nm in chromatophores. It reflects the availability of secondary acceptor in two ways [13]. If the inhibitor/quinone binding equilibrium is established on the time scale of the slow back-reaction, or faster, the slow phase of the back-reaction is accelerated by inhibitor and the slow phase rate reflects the binding constants after the flash, ie, K_q^L, K_i^L. If the binding equilibrium is slow compared to the fast back-reaction, the proportion of the fast phase is increased and the fraction fast phase is a measure of the binding constants before the flash, ie, K_q^D, K_i^D. Electron transfer from Q_A^- to Q_B was also measured directly, utilizing the fact that Q_A^- and Q_B^- cause slightly different electrochromic bandshifts in the bacteriochlorophyll pigments [12, 17]. We have used either 750 nm, where the absorbance changes due to P^+ and Q_A^- cancel each other, or the region 398–402 nm, where there is a peak in the $Q_A^-Q_B/Q_AQ_B^-$ difference spectrum at 398 nm and a P/P^+ isosbestic at 402 nm.

A further measure of secondary acceptor activity is the ability of RCs to oxidize ferrocytochrome *c* (cyt c) in a series of flashes [18]. Multiple turnovers of the RC require active secondary electron transfer. Measured at 550 nm, the amount of cyt c oxidation on a second flash relative to that on the first flash indicates the fraction of RCs that are successful in transferring an electron to Q_B.

RESULTS

Selectivity of Herbicide Activity in Bacteria

After our initial observation of the inhibitory effect of triazines on Q_A^- to Q_B electron transfer in isolated RCs [5], we screened several herbicides, mostly of the PS II-active variety, for effects in whole cells of Rp sphaeroides. Of the large number of compounds known to act in plants, only a few were active in bacteria (Table I). Effective inhibitors of the RC acceptor reactions were limited to two major classes of herbicides—*s*(symmetrical)-triazines and benzonitriles—and were bacteriostatic rather than bacteriocidal. The asymmetric heterocycle, metribuzin, an economically important PS II-active herbicide, was without effect on the bacterial system. Several

TABLE I. Herbicide Inhibition (Dissociation) Constants (μM)*

Herbicide name	Rhodopseudomonas sphaeroides R-26 RCs I	II	III	R-26 Cells	Ga Cells	Plants: chloroplasts
Triazines						
Ametryn	20	30	30	6	8	0.04
Atraton	95	80	80	50	42	0.60
Atrazine	120	90		20	47	0.36
Simeton	>500			65	81	1.0
Simetryn	75			9	40	3.5
Terbutryn	3		6		8	0.03
Cyanazine	>400			130	235	0.16
Prometon	>400			85	78	4.0
Azidoatrazine	3					0.1
Azidoterbutryn	4					
Ureas						
DCMU	>800				208	0.06
Chloroxuron	>300					
Benzonitriles						
Bromoxynil	30				110	
Bromxynil oct. ester	8				190	
Ioxynil		240	180		55	0.70
Nitrophenols						
Dinoseb	>200				58	2.5
2,4-DNP					120	
2,6-DNP					85	
Phenanthrenes						
o-phenanthroline	215	100	190	30	50	50.0
Quinones						
Chloranil					220	
Inactive herbicides						
Alachlor					>500	
Bromacil	>300	>300	>300		>500	0.25
Butylate					>500	
N-diallyl chloroacetamide					>500	
Dicryl	>300	>300	>300		>300	
Maleic a. hydrazide					>500	
Metribuzin	>300	>600	>600		>750	0.21
Pyrazon					>500	
Quinolin					>750	
Trifluralin					>500	

*Samples contained 1.0 μM RCs, 20 μM Q-10, 0.06% TX-100, 100 mM NaCl, 10 mM Tris (pH 8.0). Three techniques were used for RCs: I, extent of cytochrome c photooxidation, with 22 μM cyt c; II, extent of Q_B reduction; III, fraction fast back-reaction phase. See Methods. Plant inhibition constants were taken from the literature from measurements on isolated chloroplasts.

nitrophenols were effective inhibitors of cell growth, but on examination with subcellular preparations—isolated RCs and chromatophores—it was clear that their effect was not at the RC. In fact, they are known to be potent uncoupling agents. 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU), perhaps the most widely used experimental PS II inhibitor, was also without effect on isolated RCs, but it did inhibit the cytochrome b-c_1 complex in chromatophores (not shown). Sensitivity to DCMU has also been reported for yeast mitochondria [19]. The cytochrome b-c_1 complex in Rp sphaeroides is active in both photosynthetic and respiratory electron transport. Consistent with these conclusions, when tested against cell growth on agar plates, nitrophenols and DCMU were as effective in arresting aerobic, chemotrophic growth as they were on anaerobic, phototrophic growth. The *s*-triazines, on the other hand, only inhibited phototrophic growth.

All the RC studies described here were done with the carotenoidless R-26 strain, while most of the whole cell and chromatophore studies utilized the Ga strain as a control for the derived mutants. Table I shows that cell growth in both strains was inhibited by very similar levels of the effective herbicides. Equivalent behavior was also seen for chromatophores from the two strains (data not shown). Comparison of herbicide effects on chromatophores of Ga with RCs from R-26 seems, therefore, to be reasonably well founded, and preliminary studies with RCs from Ga and mutant cells fully support this.

Table I shows the sensitivity of isolated RCs to various herbicides. As noted in Methods, three different assays of acceptor activity were used, with generally good agreement. In some cases the I_{50} for RC-acceptor activity was higher than that for cell growth. Some discrepancy is to be expected in such a comparison, but lower values for inhibition of growth may reflect a degree of accumulation by the cells or perhaps another site of action in the cell, such as inhibition of the cyt b-c_1 complex. Bromoxynil and its octanoate ester appear to be significantly more potent in isolated RCs than in whole cells. However, the inhibition of acceptor activity in RCs was only partial, even at maximal levels of these agents. This may reflect a solubility limitation in the aqueous detergent suspension.

Competition Between Quinone and Herbicide

The competitive nature of the action of herbicides on secondary electron transfer, proposed earlier [4,5,10], should be reflected in a quinone dependence for the inhibition. The exact expectation, on the basis of Scheme 2, depends on which parameter is chosen as a measure of inhibition, as discussed in the introduction. Figure 1 shows the inhibition of isolated RCs by terbutryn, as indicated by the relative fraction of fast back-reaction and the half-time of the slow phase. The inhibition is normalized (I_n) to account for the arbitrary initial (uninhibited) values, $I(i = 0)$, which are determined by the degree of reconstitution of Q_B. The end-points of the inhibitor titrations, $I(i = \infty)$, were taken to be equivalent to the complete absence of secondary quinone, as measured in RCs containing only Q_A, for which $\triangle F = 1.0$ and $t_{1/2} = t_{1/2}\,(Q_A) = 65$ msec. In Figure 1, the concentration of total inhibitor is used as we have no direct assay of free inhibitor levels. This could introduce some deviations at low inhibitor concentrations.

If the choices for $I(i = \infty)$ are appropriate, these plots should pass through 1 on the ordinate, but it is clear that the fraction fast phase (Fig. 1a) does not. In fact, the intercept indicates that $\triangle F$ levels out at 75–80%. This will be discussed below. Nevertheless, the quinone dependence of the inhibition is definite, and the general

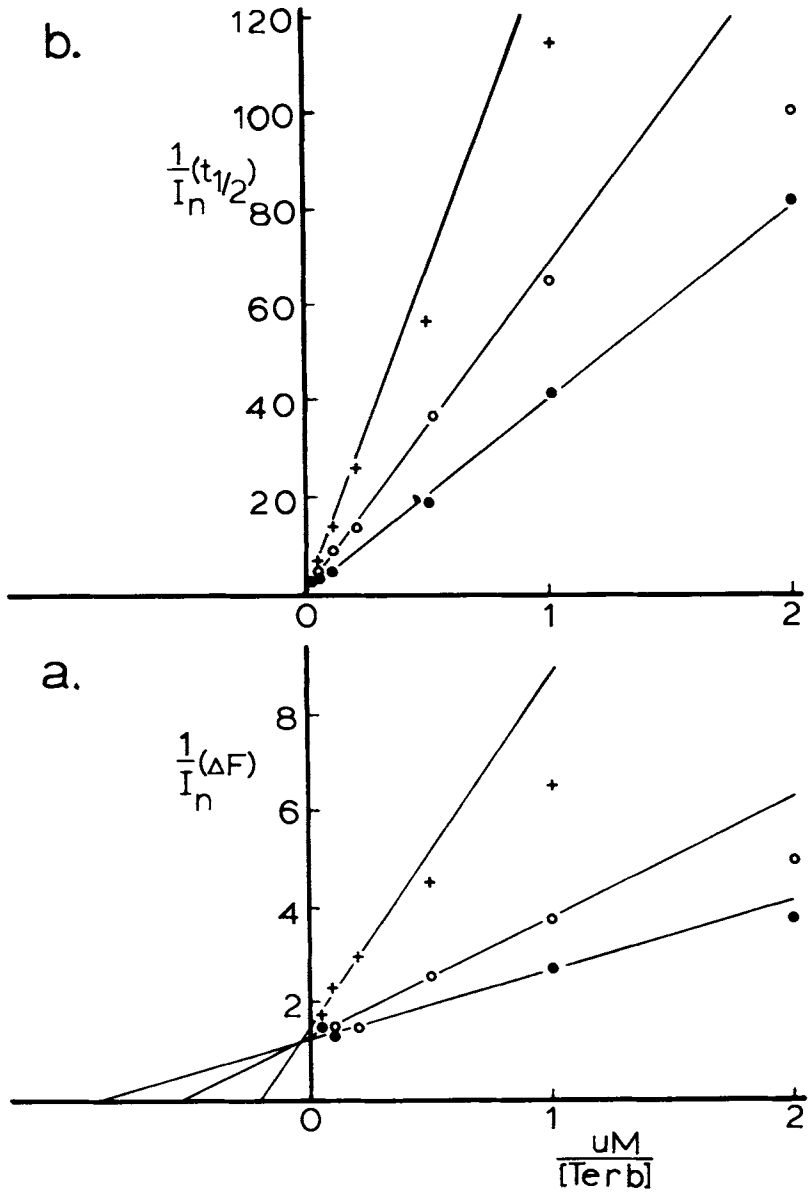


Fig. 1. Effect of terbutryn on the back-reaction kinetics of isolated RCs. Double reciprocal plots of normalized inhibition calculated from $I_n = [I(i) - I(i = 0)]/[I(i = \infty) - I(i = 0)]$ as described in the text. All samples contained 0.5 μM RCs in 10 mM Tris, pH 8.0, 0.14% LDAO. a) Normalized inhibition measured by the fraction of the fast phase of the back reaction: I = ΔF, the relative amplitude (fraction) of the fast component of P^+ decay; $I(i = \infty) = 1.0$. b) Normalized inhibition measured by the rate of the slow phase of the back-reaction: $I = 1/t_{1/2}$(slow), the reciprocal halftime of the slow component of P^+ decay; $I(i = \infty) = 0.065$ sec. ● = 1 μM UQ; ○ = 3 μM UQ; + = 30 μM UQ.

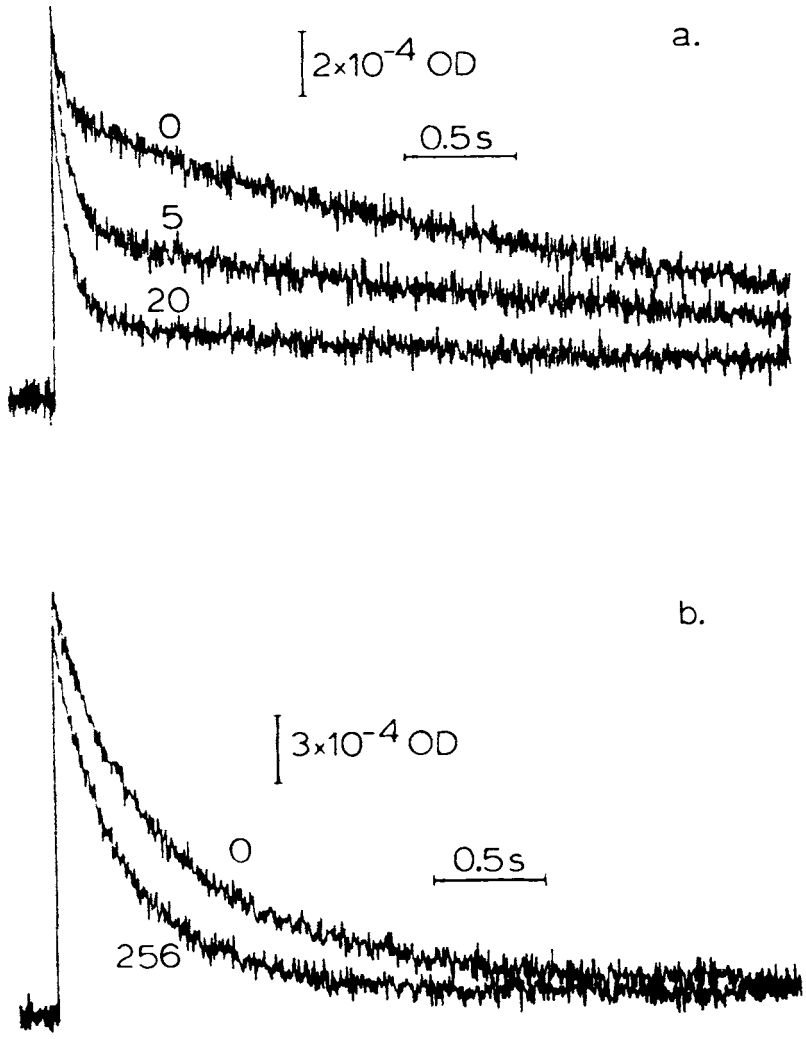


Fig. 2. Back-reaction in Ga and mutant ngC chromatophores. Chromatophores (~0.1 μM RC) in 10 mM Tris, 100 mM KCl (pH 8.0), 2 μg/ml valinomycin, 2 μg/ml antimycin A, E_h = ~445 mV (adjusted with ferricyanide). Terbutryn concentrations (μM) are indicated on the figure. a) Ga chromatophores. b) Mutant ngC chromatophores.

behavior is clearly competitive in both cases. The quinone concentration dependence is close to expectation for the two lower concentrations. For the fraction fast phase, the slope is equal to $K_i^D(1 + (q)/K_q^D)$, giving $K_i^D = 0.8$ μM, $K_q^D = 2$ μM. The latter value is in good agreement with direct titrations of Q_B-activity with Q-10 ($K_q^D = 2.8$ μM) (not shown). For inhibition determined from the half-time of the slow phase (Fig. 1b), the slope is equal to $K_i^L[1 + (1 + K_2)(q)/K_q^L]$. Comparison of any two curves should allow the estimation of K_i^L and $K_q^L/(1 + K_2)$, but the value of (q) is uncertain because, as have previously shown [13], quinone transfer between detergent micelles is slow and the effective concentration is not equal to the total concentration,

nor is it the same as (q) in the dark equilibrium. An effective value of $K_q^L/(1 + K_2)$, related to the nominal concentration of quinone, can be obtained ($\simeq$ 2.2 μM), but it is clearly of little significance. However, if the effective concentration of quinone is linearly related to the total amount of quinone present, the terms in $(1 + K_2)(q)/K_q^L$ can be eliminated and estimation of K_i^L is not subject to the uncertainty in (q). Utilizing this assumption, the data for the two lower quinone concentrations yield K_i^L = 20 μM. Alternatively, solving for an effective quinone concentration that allows K_i to remain unchanged, ie $K_i^L = K_i^D$, one finds that the apparent quinone affinity increases significantly ($K_q^L < K_q^D$).

In both parts of Figure 1, but especially for the half-time measurement, the data for the highest level of quinone indicate an effective concentration considerably lower than the nominal 30 μM, and there is invariably a problem with solubilizing high concentrations of Q-10 even in detergent suspensions. The hexane:water partition coefficient for Q-10 has been estimated to be about 10^{20} [Dutton PL: personal communication]! This extreme hydrophobicity impinges on all measurements of secondary acceptor activity with Q-10. The accessibility and effective quinone concentration are dependent on detergent concentration and type, and on temperature [13; and Wraight CA, Stein RR: in preparation].

Triazine-Resistant Mutants

Herbicide resistant mutants can be readily derived from Rp sphaeroides using the Ga strain. All of the triazine-resistant mutants obtained and tested so far are resistant at the reaction center. We have not detected any significant contribution from metabolic sources such as detoxification. Thus, triazine-containing medium is still bacteriostatic for non-resistant strains after supporting several generations of growth by a variety of resistant mutants. (The used medium was sterilized by filtering and was resupplemented with growth factors and carbon source).

Five triazine-resistant mutants obtained by nitrosoguanidine treatment were investigated using chromatophores. At high redox potentials (E_h > 440 mV) the back-reaction can be studied as all donors to P^+ are chemically oxidized. In resistant and susceptible strains, alike, the rate of recombination of the flash-induced charge separation was accelerated by terbutryn (Figs. 2,3). In nonresistant Ga chromatophores, the slow phase of the back-reaction occurs with a characteristic half-time of about 2 sec, much slower than for RCs in detergent but similar to RCs in phospholipid vesicles [4,5]. Terbutryn caused a marked and progressive increase in the fraction of the fast-decaying component and also accelerated the slow-decaying component. The I_{50} values for these effects differ by a factor of about 8 (7 μM for the fraction of fast phase vs 55 μM for the half-time of the slow phase). In the mutants, the uninhibited back-reaction was usually substantially faster ($t_{1/2}$ = 0.3–0.4 sec) but was quite variable in different preparations. In contrast to the susceptible strains, the effect of terbutryn in the mutants was only to accelerate the monotonic decay: biphasic kinetics were not observed. The I_{50} for all five mutants was about 0.3 mM, using the half-time as the measure (Table II). The fast phase, observable in the presence of an effective inhibitor like o-phenanthroline, was unaltered in the mutants, with $t_{1/2}(Q_A)$ = 45 msec. This value for chromatophores is significantly faster than for isolated RCs, $t_{1/2}(Q_A)$ = 65 msec, even when incorporated into phospholipid vesicles.

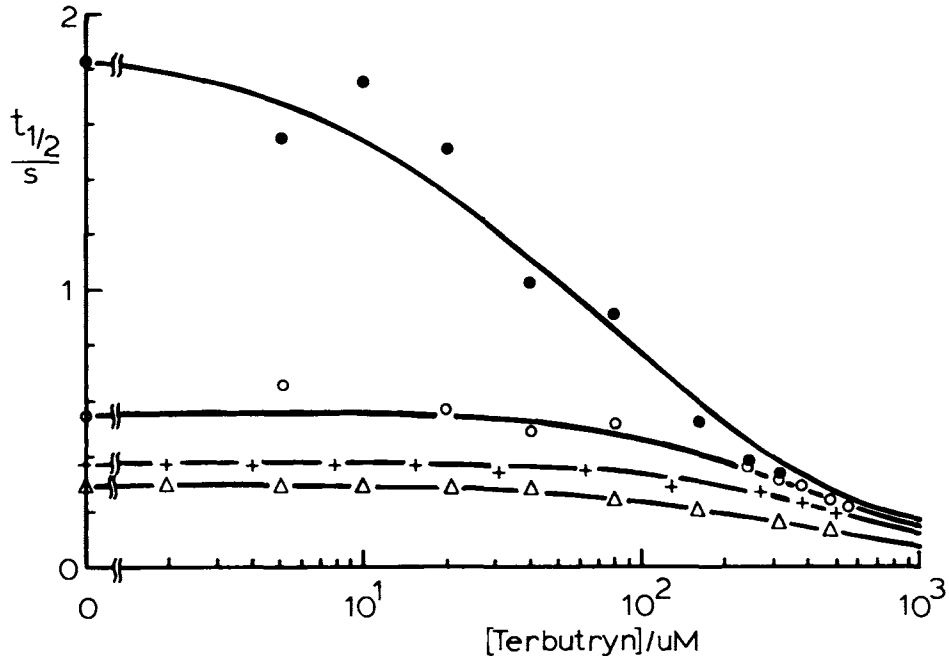


Fig. 3. Effect of terbutryn on the half-time of the back-reaction in Ga and herbicide-resistant mutant chromatophores. Conditions were as described in Figures 2. ●, Ga (slow phase); ○, mutant ngA; +, mutant ngB; △ , mutant ngC.

TABLE II. Characteristics of Herbicide-Resistant Mutants*

	Whole cells, I_{50} (μM)					Chromatophores		
Mutant	Atra	Ioxy	Brom	Terb	DCMU	Terb, I_{50} (μM)		$t_{1/2}$ (msec)
Ga	47	55	110	8	208	50	(8)	1,850
ngA	500	38	75	300	150	280		313
ngB	330	58	150	510	525	350		423
ngC	600	55	66	650	260	350		403
ngD	550	36	30	260	150	270		311
ngE	325	25	90	80	175	240		314

*Whole cell measurements were made as described in Materials and Methods. Chromatophore measurements were made as in Figure 2. Chromatophore I_{50}'s were determined where the rate of the slow back-reaction was doubled, except for the value in parentheses for Ga, which was taken from the fraction of fast phase. Abbreviations used: Atra, Atrazine; Ioxy, Ioxynil; Brom, Bromoxynil; Terb, Terbutryn.

DISCUSSION

The reaction center from Rp sphaeroides is well characterized [see 20,21 for review]. It consists of three polypeptides, termed L, M, and H (22, 24 and 28 kilodaltons [kD]), respectively, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE] and contains four bacteriochlorophylls, two bacteriopheophytins, two ubiquinones (Q-10), and an iron atom. The LM structure, which retains

primary photochemical activity, contains all the chromophores, the iron atom, and the primary quinone, Q_A. Photoaffinity labeling has implicated the M subunit in the binding site for Q_A [22], and antibody studies have suggested a role for M in the Q_B-binding also [23]. The H subunit is of uncertain function at this time, but removal of the H subunit almost completely inhibits Q_A^- to Q_B electron transfer activity [24]. This is consistent with a role for H in the Q_B binding site. The function of the iron atom is also unknown and, although both semiquinones (Q_A^- and Q_B^-) interact with it magnetically [25], it is probably not involved directly in the electron transfer. It may be important in determining the redox and binding properties for the quinones [4,26].

The homology between bacterial and PS II RCs is striking at the functional level, especially in the acceptor quinone complexes, and even for earlier intermediates [1,4,21]. Both utilize a two-electron gate consisting of two quinones and an iron atom. Similarity at the structural level, however, is less obvious. As isolated, PS II "core" particles have been considered to contain three polypeptides: M_r = 9, 27, and 43 kD [21]. The smallest of these has been assigned to cytochrome b_{559}, which has an enigmatic role in PS II activity. Recent studies, however, have shown the polypeptide composition to be more complex [27]. The 43-kD band on normal SDS-PAGE is actually composed of two polypeptides—43 and 47 kD. Both components have been previously associated with RC function [28]. The 27-kD band, formerly thought to be the light-harvesting pigment protein, also consists of two polypeptides—32 and 34 kD. The 32-kD protein was identified as the well-known herbicide-binding protein. It is evidently present in substoichiometric amounts due to loss during isolation. The two 40–50-kD proteins and the 32-kD protein seem to provide a functional unit equivalent to that of the bacterial RC. PS II particles, which generally lack much of the 32-kD protein, have a diminished number of triazine binding sites but also have impaired Q_B activity [7,27], suggesting a functional homology between the H subunit and the 32-kD protein. We are currently trying to establish this by the use of photoaffinity labels—note that azidoatrazine is a potent inhibitor (Table I).

In view of the differences between bacterial and PS II RCs, it is not surprising that the affinities for triazines are also rather different (Table I). The additional polypeptides of the intact PS II RC appear to contribute to the affinity and broad sensitivity to herbicides seen in chloroplasts, and various treatments, including isolation or trypsin digestion, diminish the herbicide sensitivity of PS II. In fact, however, I_{50} values are a poor criterion for comparison if herbicide activity is competitive with quinone, because of differences in the membrane quinone level. The photosynthetic bacteria are known to have very high quinone contents (30–50 Q/RC [3,31]), consistent with their lower sensitivity to herbicides. Nevertheless, it is likely that the binding constants themselves would also reflect a significantly stronger binding in chloroplasts. A more detailed comparison is not profitable at this time because even the minimal competitive binding scheme described here (Scheme 2) has the complexity of binding equilibria before and after flash activation and there is no a priori reason to suppose that K_i^D and K_q^D are equal to K_i^L and K_q^L. Indeed, earlier studies on the effect of o-phenanthroline and ubiquinol on the redox properties of Q_A in bacterial RCs showed them to bind less strongly in the presence of Q_A^- [9,15].

The competition between terbutryn and ubiquinone, implied in Scheme 2, is demonstrated in Figure 1. The occurrence of biphasic kinetics, as observed in this work, indicates that binding equilibrium is not reestablished rapidly after the flash,

ie, compared to the *fast* back-reaction—rapid binding equilibrium would lead to a monophasic decay of varying half-time [5,13]. Thus, the fraction fast phase reflects the distribution of inhibitor and quinone at the time of the flash and Figure 1a shows the competitive binding equilibrium of terbutryn and ubiquinone set up before the flash, ie, involving $K_i^D = 0.8\ \mu M$ and $K_q^D = 2\ \mu M$. K_q^D is strongly dependent on the detergent concentration above the critical micelle concentration [McComb JC, Wraight CA: unpublished observations], which causes the I_{50} for terbutryn to be detergent-concentration dependent also. Preliminary studies show K_i^D to be moderately detergent dependent. There is definite curvature in the double reciprocal plots at low inhibitor concentrations. This is also seen in some situation in direct binding studies with chloroplasts and has led to suggestions of noncompetitive inhibition [29]. However, this has recently been reinterpreted [30] as arising from unmonitored alterations in the free concentration of one modifier, ie, Q-10 in our case, as the concentration of the other (terbutryn) is raised. We hope to address this problem shortly by direct binding studies.

The half-time of the slow phase reflects competition between inhibitor and quinone binding to the B-site after the flash, with Q_A reduced. The apparent affinity for quinone is magnified by the electron transfer equilibrium because Q_B^- is very tightly bound: $K_q^{app} = K_q^L/(1 + K_2)$. In isolated RCs this is complicated by the slow rate of transfer of quinone between micelles, which gives rise to biphasic kinetics even in the presence of excess quinone [13]. The remaining slow phase, however, largely reflects the expected competition between inhibitor and quinone. Much experimental evidence now indicates that in phospholipid membranes even the larger prenyl-substituted quinones diffuse quite rapidly ($D = 10^{-9}\ cm^2\ sec^{-1}$ [2]). Thus, for RCs in chromatophores or incorporated into vesicles, quinone should be rapidly and homogeneously available, limited only by its concentration in the membrane. Addition of terbutryn, however, induced a fast-decaying fraction, even at the high quinone levels found in chromatophores (Fig. 2). This is understandable if the inhibitor unbinds slowly. Following the flash, inhibitor-bound RCs will undergo a fast back-reaction before the inhibitor can unbind to allow postflash equilibration with the quinone. In chromatophores, the fraction fast phase becomes constant at 60–70% at high terbutryn, suggesting an unbinding half-time of about 80 msec competing with the fast back-reaction time of 45 msec. This interpretation is supported by measurements of the rate at which electrons on Q_A^- leak through the inhibitor block to Q_B: the extent of cyt c oxidation by a second flash increases progressively as the flash spacing is increased [5,18]. In isolated RCs the half-time of the leak process is about 240 msec for terbutryn and is not significantly dependent on either terbutryn or quinone concentrations (not shown). For the back-reaction, the fraction fast phase becomes constant at high terbutryn at about 80% (Fig. 1a), consistent with a slower unbinding of inhibitor (240 msec) and the slightly longer half-time of the back-reaction ($t_{1/2}[Q_A] = 65$ msec) seen in isolated RCs. From the unbinding rate constant ($k_{off} = 0.69/t_{1/2} = 3s^{-1}$), and K_i^L, one can calculate k_{on}. However, the value of K_i^L is uncertain, as discussed above: for $K_i^L = 20\ \mu M$, $k_{on} = 2 \cdot 10^5\ M^{-1}\ sec^{-1}$; whereas for $K_i^L = K_i^D = 0.8\ \mu M$, $k_{on} = 4 \cdot 10^6\ M^{-1}\ sec^{-1}$.

In Ga chromatophores, the amplitude of the fast phase titrates in with $I_{50} = 7\ \mu M$ terbutryn (Table II). In view of the high quinone levels in chromatophores, K_i^D must be somewhat smaller than this. Acceleration of the slow phase of the back-

reaction, however, requires substantially higher concentrations of terbutryn (Table II and Fig. 3). One might expect to attribute this to the enhancement of quinone binding by the effect of the electron transfer equilibrium (K_2). However, the I_{50}, defined at the midpoint of the titration from the uninhibited to the fully inhibited state, is independent of K_2 (it cancels since the uninhibited rate is also dependent on K_2). Thus, the I_{50} values for the fast fraction and the slow half-time provide a direct comparison of $K_i^D(1 + (q)/K_q^D$ and $K_i^L(1 + (q)/K_q^L)$, indicating that $K_i^L > K_i^D$ and/or $(1 + (q)/K_q^L) > (1 + (q)/K_q^D)$. The first inequality is consistent with our previous finding that reduction of Q_A suppresses the binding of o-phenanthroline, albeit only above the pK of Q_A^- [5]. The quinone content of chromatophore membranes is high (20–50 per RC; ref. 31), and is far in excess of the number of specific binding sites, including RCs, cytochrome b-c_1, succinate and NADH dehydrogenases, etc. Thus, the free quinone level in the membrane is likely to be rather constant and the second inequality would imply that $K_q^L < K_q^D$. The general conclusion, that reduction of Q_A affects the affinity for quinone and/or terbutryn, is the same as that arrived at above, for isolated RCs. As we have pointed out before [3,4], the redox properties of Q_B show that quinol is bound more weakly than quinone to the B-site, a situation consistent with the functional turnover of the acceptor complex. The possibility, suggested here, that reduction of Q_A enhances the binding of quinone would, together with our previous observation that Q_A^- suppresses the binding of quinol [5], provide a further, positive contribution to the turnover of the B-site. Quantitatively, we view the isolated RC data with some skepticism because Q-10 is slowly transferred between micelles and is not freely accessible on the time scale of the back-reaction [13]. Qualitatively, however, this behavior is also seen with chromatophores and with RCs in vesicles, where the quinone is rapidly available in the membrane.

The triazine-resistant mutants exhibit the expected low sensitivity to terbutryn, but their behavior is also qualitatively different from the nonresistant strain. The major response to terbutryn in the susceptible Ga strain is an increase in the fraction of fast phase, which, we suggest, arises from a slow unbinding rate for the inhibitor. In the mutants, however, the only significant effect is the acceleration of the slow phase, suggesting that the lower binding affinity is accompanied by an enhanced off-rate.

As described above, I_{50}, defined when the slow rate is doubled, is independent of K_2: $I_{50} = K_i^L(1 + (q)/K_q^L)$. However, the concentration necessary to accelerate the slow phase so that it is half as fast as the fast phase is $I'_{50} = K_i^L[1 + (1 + K_2)(q)/K_q^L]$. The latter value is little different for the resistant and nonresistant strains. This can be explained if both K_i^L and K_q^L are altered, in the mutants, to a similar extent. If $(q)/K_q^L$ is not much larger than 1, the effect of K_i^L alone would be seen in I_{50}. When $(q)/K_q^L$ is multiplied by $1 + K_2$ (at least 45 from a comparison of fast and slow rates in uninhibited Ga chromatophores) it becomes much larger than 1 and similar changes in K_i^L and K_q^L would cancel in I'_{50}. A similar cancellation could be effected by changes in K_2, which can reflect alterations in the binding of the semiquinone, Q_B^-. As well as being generally faster in the mutants, the slow back-reaction is also more variable. This is consistent with a diminished quinone-binding affinity at the B-site in the mutants. The quinone dependence of the electron transfer equilibrium would then be less saturated in the mutants and more responsive to variations in the membrane quinone level. We are currently testing this in isolated RCs and by direct assay of

quinone levels in Ga and mutant strains grown under a variety of conditions.

Inspection of Table II reveals that, for Ga, the I_{50} for growth is very similar to that for the fraction fast phase in chromatophores. In the mutants, the correlation is with the acceleration of the slow phase. This suggests that the resistance strategy is to avoid the slow unbinding of the inhibitor, even at the expense of stabilization of the electron on Q_B—which is determined by K_q^L and K_2. However, *in vivo* P^+ is rereduced by cyt c_2 in 200 μsec, much faster than the fast back-reaction at 45 msec. The electron on Q_A^- will, therefore, be trapped (stabilized) anyway, and the activity of the RC will be relatively insensitive to the value of K_2. If K_2^{app} is normally 45, the electron will be on Q_B—and the RC will, therefore, be "open"—98% of the time. If K_2^{app} is decreased tenfold, the RC will still be open 80% of the time. The inhibitory effect in susceptible strains seems to be related to the introduction of a new and slower rate-limiting step for electron flow. In the absence of inhibition the rate-limiting step is associated with turnover of the cyt b/c_1 complex and is about 2 msec [2].

So far we have not detected any differences in the RC polypeptides of the mutants using SDS or LDS-PAGE. Thus, there do not appear to be gross structural alterations, such as deletions. Nevertheless, two of the mutants (ngA and ngC) had significantly altered redox midpoint potentials (E_m) for P/P^+ (not shown), indicating that modifications of the acceptor complex can have far-reaching effects. This result also shows that the five mutants described here represent at least two distinct classes.

The back-reaction kinetics of the nonresistant Ga strain invariably show a significant but variable amount (15–35%) of fast decay even in the absence of inhibitor. We have also observed this in R-26 and in the related species, Rp capsulata. It is not apparent in the triazine-resistant mutants. At the present time we have no explanation of this behavior in the nonresistant strains.

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