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Reaction centers from triazine-resistant strains of *Rhodopseudomonas sphaeroides:* localization of the mutation site by protein hybridization experiments

M.Y. Okamura, E.C. Abresch and R.J. Debus

Department of Physics, University of California at San Diego, La Jolla, CA 92093 (U.S.A.)

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A procedure for dissociation and reconstitution of reaction centers has been used to hybridize reaction centers from three different herbicide-resistant mutant strains of *Rhodopseudomonas sphaeroides* with LM or H subunits derived from the native (susceptible) strains. All three mutant strains exhibited low rates of electron transfer. Hybridization of mutant reaction centers with native LM restored the high rates of electron transfer. Hybridization with native H did not. This procedure shows that the site of mutations in these mutant strains are on the LM unit.

The bacterial reaction center is the protein-bacteriochlorophyll complex responsible for the primary photochemistry in photosynthetic bacteria (for a review see Ref. 1). The electron acceptor complex in bacterial reaction centers contains two ubiquinone molecules, a primary acceptor (Q_A) and a secondary acceptor (Q_B) both coupled to Fe^{2+}. Electron transfer between Q_A and Q_B is inhibited by a number of compounds [2] including triazine herbicides [3–5] which are also potent inhibitors of electron transfer in PS II of green plants [6–8]. Understanding the mechanism of herbicide action in bacterial reaction centers may help to explain the mode of action of these herbicides in plants.

A promising approach for studying the mechanism of herbicide action involves the isolation and characterization of herbicide-resistant mutants of photosynthetic bacteria. Several groups have reported such studies [4,5,9]. The bacterial mutants not only display a decreased susceptibility to triazine herbicides, but also show alterations in electron-transfer characteristics within the quinone complex. In a previous study we showed that the herbicide resistance is associated with reaction centers isolated from the mutants [5]. Furthermore, these reaction centers displayed a remarkably low rate of electron transfer compared to normal reaction centers when cytochrome *c* was used as electron donor and UQ-0 as electron acceptor under conditions of continuous illumination. In this work we use the procedure of dissociation and reassociation of reaction center subunits [10] to prepare hybrid reaction centers containing a mixture of subunits from mutant and native strains of bacteria. These hybrid reaction centers were tested to see whether reconstitution with either isolated native LM or H could restore rapid electron transfer. This test was used to localize the subunit(s) that contained the mutant site.

The mutant strains were isolated by growing the native strain *Rps. sphaeroides* 2.4.1. in either atrazine (300 μm) or terbutryn (100 μm). (Herbi-

Abbreviations: Cyt, cytochrome; ATZ, atrazine; PS II, Photosystem II; L, M and H, reaction center protein subunits (light, medium and heavy).

cides were obtained from Chemical Services Inc., West Chester, PA). No mutagenic agents were used. The bacteria were grown photosynthetically at $T = 30°C$ in liquid culture using Huntners medium, at an average light intensity of $I \simeq 1$ mW/cm^2 (tungsten showcase lamps). The herbicide resistant cultures appeared after about 2 weeks. These cultures were then plated out on agar and grown aerobically. Single colonies of resistant strains were then picked and recultured in liquid medium. The growth rates of the resistant and native strains were similar (doubling times, approx. 15–20 h). The growth of the mutant strains was relatively unaffected (approx. 20% increase in doubling time) by addition of 300 μM atrazine or 100 μM terbutryn. The growth of the native strain was completely inhibited by these concentrations of herbicide. Reaction centers from the mutant strains were solubilized in LDAO by the procedures of Jolchine and Reiss Husson [11] and purified [12] using DEAE cellulose to a final purity $A_{280}/A_{800} = 1.2$. The reaction centers displayed characteristic absorption spectra in the infrared and visible regions. The long wavelength absorption band near 865 nm was completely reversible bleached by a laser flash. SDS polyacrylamide gel electrophoresis showed three bands corresponding to L, M and H subunits in mutant reaction centers which were indistinguishable from those of the native strains.

The hybridization procedure involved dissociation of mutant reaction centers in $LiClO_4$ followed by reassociation during dialysis in the presence of an excess of added native LM or H subunits which were obtained as previously described [10]. The amounts LM or H subunits were estimated using extinction coefficients of $\epsilon_{802}^{LM} = 288$ and $\epsilon_{280}^{H} = 46$ mM$^{-1} \cdot$ cm^{-1} [10]. Reaction centers from the mutants strains were dissociated by incubation in 0.75 M $LiClO_4$, 0.1% LDAO, 50 mM Tris-HCl (pH = 8) for 1 h at 4°C, at a concentration of 7 μM ($A_{800} = 2$). After this time 50 μl aliquots were mixed with 100 μl of purified native LM (14 μM) or H (6 μM) and dialyzed at 4°C for 24 h against 0.025% sodium cholate/0.1 mM EDTA/10 mM Tris-HCl (pH 7.7). Control samples (50 μl) of mutant reaction centers treated with $LiClO_4$ were diluted with 100 μl of buffer prior to dialysis. Control samples of reaction center, LM, and H (100 μl) were treated with $LiClO_4$, mixed with 50 μl of buffer, and dialyzed in parallel. Native LM (50 μl) and H (50 μl) were mixed together and dialyzed as a separate control. Following dialysis all samples were diluted 10-fold with dialysis buffer and optical absorption spectra were recorded. Aliquots of 50 μl of the diluted samples were mixed with 0.95 ml of assay buffer for electron-transfer activity measurements.

The rate of electron transfer was obtained by measuring the rate of cytochrome *c* photooxidation (monitored at 550 nm) using UQ-0 (2,3 dimethoxy-5-methyl benzoquinone, Biochemical Laboratories, Inc., Redondo Beach, CA) as the exogenous acceptor using a kinetic spectrometer [13]. The light source was a projector lamp (500 W tungsten lamp, $I = 1.0$ W/cm^2, CS 2-64 filter plus 3 cm water). The sample contained 100 μM UQ-0, 10 μM cytochrome *c* horse heart (Sigma type III), 10 mM Pipes buffer (pH 6.8) and 0.025% LDAO. The reaction center concentrations used in this assay were in the range of approx. 10 nmol.

The experimentally determined activities, expressed as a turnover number (Cyt/reaction center per s) are shown in Table I. Both the mutant reaction center and the isolated native LM subunit exhibited low activities. The activity in mutant reaction centers was relatively insensitive to atrazine compared to the reaction centers from R-26 or 2.4.1. (native strains). The alteration in electron transfer rate and sensitivity to herbicide mutants is believed to be due to a weaker binding of quinone and herbicide to the Q_B site [9].

The rates measured after reconstitution are shown in Table II. The rates of the hybridized samples (column c) are compared with the rates of the mutant reaction center and the isolated native LM or H from R26 (column a and b). The mutant samples hybridized with LM show large increases in rate (to approx. 50% of the activity of native reaction centers) over the sum of the individual components in the reaction mixture. Those samples hybridized with H do not show this increase. This enhancement in rate can be expressed quantitatively by the enhancement parameter *E*

$$E = \frac{R(\text{hybrid})}{R(\text{mutant}) + R(\text{native})}$$

TABLE I

TURNOVER NUMBER AND ATRAZINE (ATZ) SENSITIVITY OF REACTION CENTRES FROM NATIVE AND MUTANT (RESISTANT) STRAINS OF RPS. SPHAEROIDES

The turnover number is defined as the number of Cyt molecules/s oxidized per reaction center under the assay condition (see text). I_{50} is the concentration of ATZ required to reduce the rate by 50%. The small amount of activity in LM may be due to a slight (approx. 2%) contamination by reaction centers.

Strain	Turnover number (Cyt/s per reaction center)	ATZ inhibition I_{50} (μM)
R-26	176	5.2
24.1	215	6.5
2c(A)	13	>1000
5a(T)	2	>1000
11a(A)	9	>1000
R26-LM	6	5

where R(hybrid), R(mutant) and R(native) are the cytochrome photooxidation rates of the hybrid, mutant and native samples, respectively. The lack of enhancement upon addition of native H to the mutant reaction center is not due to degradation of H, since reconstitution of isolated native LM with H showed that they were both functionally active. The sensitivity of this method, due to the large change in activity upon hybridization to form native reaction centers, allows us to detect a change in rate due to the hybridization of 1% of mutant LM with native H for the case of mutants 11a and 2c, (assuming the hybridized protein was fully active and a limiting error of 20% in comparing the rates of the hybridized sample with the sum of the rates of its components). In practice the observed enhancement is limited by the extent of hybrid formed, e.g., 20% hybridization for the case of the LM and H control. This degree of reconstitution should give an enhancement well above the detection limit. The detection limit is even lower for mutant 5a due to its lower activity. The value of $E < 1$ observed in this case may be explained if some of the activity in the isolated native, H, due to residual LMH, is lost by hybridization with mutant LM.

The results of the hybridization experiment show that the addition of native LM to mutant

TABLE II

ACTIVITIES OF REACTION CENTERS MUTANTS HYBRIDIZED WITH R-26 LM OR H SUBUNITS

Cytochrome photooxidation rates were measured as described in the text. Reaction center concentrations were normalized to $A_{800} = 0.003$ (10 n molar). The enhancement is as defined in the text. The reconstituted samples column c) were prepared by hybridizing mutant reaction centers, with native LM or H. Equivalent amounts of mutant reaction centers (column a) and R-26 LM or H column b) were diluted with buffer and assayed for activity. The rate shown in column c) should be compared with the sum of the rates in columns a) and b). The small amount of the activity in the H sample is due to a contamination by reaction centers.

Experiment	Cytochrome Photooxidation Rate (μM/s)				Enhancement (E)
	Strain	a) Reaction center (mutant)	b) LM (R-26)	c) Reaction center (mut)+LM (R-26)	
LM Hybridization	11a	0.080	0.24	1.60	5.0±0.5
	2c	0.072	0.22	1.40	4.8±0.5
	5a	0.030	0.24	1.38	5.1±0.5
		Reaction center (mutant)	H (R-26)	Reaction center (mut)+H (R-26)	
H Hybridization	11a	0.080	0.02	0.10	1.0±0.2
	2c	0.072	0.02	0.10	1.1±0.2
	5a	0.030	0.02	0.03	0.7±0.2
		LM (R-26)	H (R-26)	LM (R-26)+H (R-26)	
Controls	R-26 LM	0.060	0.015	0.44	5.9±0.5
	R-26 RC	2.3	–	–	–

reaction centers restores high activity, but the addition of native H does not. Our interpretation of this result is that LM from the native strain hybridizes with H from the mutant to give the active species but the native H hybridized to mutant LM does not display high activity. Thus we conclude that the site of mutation is on the LM unit. Previous results had shown that the removal of the H subunit from the reaction center greatly decreased the rate of electron transfer from Q_A to Q_B [10], the susceptibility to inhibition by *o*-phen and triazine herbicide [10] and the binding of azido atrazine [5]. Thus, while the H subunit greatly modifies the Q_B binding site in bacterial reaction centers, this subunit is not the mutation site in the herbicide-resistant mutants assayed in this study.

These results are consistent with the finding that azidoatrazine predominantly labels the L subunit [14,9] and the observed sequence homology between the L subunit and the atrazine binding protein in green plants [15–17] which has been shown to be altered in herbicide resistant strains [18,19]. However, it is possible that the H subunit may be directly involved as part of the binding site or indirectly interacts with the site, e.g., by a conformational change. Thus it is possible that herbicide resistant mutants modified on the H subunit can be produced. The most precise localization of the mutant site is by sequence determination. However, this approach is very time consuming. The hybridization procedure presented here may be useful to screen large numbers of mutants for those altered on the H subunit.

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