

# Genetic Engineering of Herbicide Resistance : Saturation Mutagenesis of Isoleucine 229 of the Reaction Center L Subunit

Edward J. Bylina and Douglas C. Youvan\*

Department of Applied Biological Sciences, Massachusetts Institute of Technology, P. O. Box 96, Cambridge, MA 02139, USA

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Oligonucleotide-mediated mutagenesis has been used to change isoleucine 229 of the reaction center L subunit of *Rhodospseudomonas capsulata* to seventeen other amino acids. A system of deletion strains and complementing plasmids has been used to assay the effects of changing this residue, which forms part of the secondary quinone  $Q_B$  binding site. The reaction center is assembled in all seventeen mutants. Ten of the seventeen mutations (Gly, His, Tyr, Trp, Asn, Asp, Gln, Glu, Arg, and Lys) result in the loss of photosynthetic growth. The remaining seven mutations yield varying levels of photosynthetic growth (Ile, Val, Ala, Leu, Met > Thr, Cys, Ser). Six of these mutations lead to varying atrazine resistance (Met, Leu > Ser, Ala, Thr, Cys > Val, Ile). None of the mutations lead to atrazine sensitivity. These results suggest that hydrophobic residues of moderate size function best at this position. This set of mutations at isoleucine L229 include the first examples of mutants with null quinone function and the first examples of crystallography-based genetic engineering in the design of herbicide resistant reaction centers.

## Introduction

The photosynthetic reaction center mediates the conversion of light energy into chemical energy in both photosynthetic bacteria and higher plants. The best characterized of these systems for studying the light reactions of photosynthesis are those of the photosynthetic bacteria [1, 2]. Information obtained from work with these bacteria may be applied to plant systems, since homologous and analogous structures are observed in the reaction centers of the purple non-sulfur bacteria and in the photosystems of higher plant chloroplasts [3, 4]. Recently, proteins D1 and D2 have been shown to form the core of the photosystem II reaction center, analogous to the subunits L and M in bacteria [5].

Herbicide resistant mutants have been generated and analyzed in both photosynthetic bacteria and chloroplasts, identifying amino acid residues important in herbicide binding. Spontaneous atrazine [2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine] resistant mutants isolated from *Rhodospseudomonas sphaeroides* contain an isoleucine to methionine change at position 229 of the L subunit [6, 7]. In the green alga *Chlamydomonas reinhardtii*, three different amino acid residues in D1- valine 219, phenylalanine 255 and serine 264- can be altered to obtain

varying resistance to *s*-triazine and urea-type herbicides [8]. The first two of these amino acids correspond to valine 194 and phenylalanine 216 in the *Rhodospseudomonas capsulata* L subunit when the two amino acid sequences are aligned [9].

Determination of the crystal structure of the reaction center of *Rhodospseudomonas viridis* at 3 Å resolution [10] provides spatial information which may be used to understand the molecular interactions involved in both quinone binding and herbicide resistance. Specific amino acid residues interacting with competitive inhibitors for the secondary quinone,  $Q_B$ , have been identified, but detailed interactions are not currently known for  $Q_B$  itself [11]. The *s*-triazine herbicide terbutryn [2-thio-methyl-4-ethylamino-6-*t*-butylamino-*s*-triazine] makes extensive contacts with both isoleucine L229 and phenylalanine L216, residues known to be involved in conveying herbicide resistance to reaction centers. Terbutryn also forms a hydrogen bond with serine L223, which may correspond to serine 264 in D1. Resistance at this position may be explained by a decrease in herbicide binding due to the loss of this hydrogen bond in the mutants analyzed.

Extensive sequence homology between *R. capsulata* and *R. viridis* [12] has allowed us to use the *R. viridis* structure in the design of mutagenesis experiments for *R. capsulata* reaction centers. In the vicinity of the iron and quinone binding sites, 11 of the 13 residues shown in Fig. 1 are identical between

Reprint requests to D. C. Youvan.

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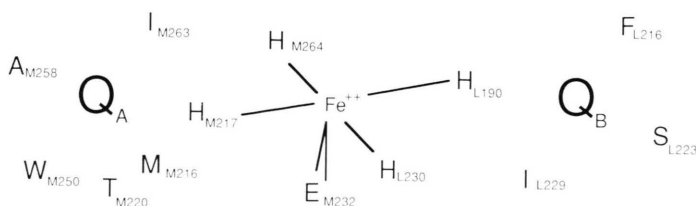


Fig. 1. Schematic diagram of the quinone and iron binding sites in the photosynthetic reaction center of *R. capsulata*, based on the crystal structure from *R. viridis*. The residues shown are identical between the two organisms, with the exception of an isoleucine to valine substitution at M263 and a methionine to alanine substitution at M216 in *R. viridis*. Results from this work show that an isoleucine to valine change in the  $Q_B$  binding site at L229 produces a pseudo wild-type phenotype. The large molar volume change in the substitution at M216 may compensate for the binding of the larger menaquinone in *R. viridis* at the primary quinone site.

the two species with the two changes being conservative ones. This is fortunate, since *R. capsulata* is eminently more suited for genetic analysis. Molecular genetic techniques for photosynthetic bacteria are most advanced in *R. capsulata*, which offers the additional advantage of possessing several alternative growth modes [2]. All mutants, whether photosynthetically wild-type, impaired or defective, may be propagated using one of these alternative modes. Mutants are never stressed under either photosynthetic or herbicide conditions in order to prevent spontaneous primary or secondary site mutations from being introduced into the reaction center (see Sinning and Michel this issue).

Sequence information from spontaneous mutants and structural information from the crystal structure complement each other in the design of mutagenesis experiments which have as their goal the generation of interesting phenotypes. The crystal structure indicates that isoleucine L229 forms part of the  $Q_B$  pocket and makes extensive contacts with terbutryn when this herbicide is bound to the reaction center. Mutant analysis indicates that this amino acid position is sensitive to subtle changes in structure, since a conservative mutation to methionine results in herbicide resistance. Saturation mutagenesis at isoleucine L229 should therefore generate a variety of interesting phenotypes which will help us to better understand the structural parameters at this position which affect quinone and herbicide binding.

In this communication we present the results of saturation mutagenesis at isoleucine L229. Seventeen of the nineteen possible amino acid changes at this position have been constructed and the phenotypes of these mutants ascertained by returning modified plasmids to deletion strains *via* conjugation.

These amino acid substitutions produce effects on both photosynthetic growth and herbicide resistance which define structural constraints for the  $Q_B$  binding site.

## Materials and Methods

### DNA methods

All procedures used were essentially as described by Maniatis *et al.* [13]. M13 replicative form (RF) and single-stranded DNA was isolated from phage grown in *E. coli* bacterial strain JM103. Plasmid DNA and M13 RF DNA were extracted and purified by the alkaline lysis technique. YIB11 was constructed by removing the polylinker *Bam*HI site from YIB4, a M13mp18 derivative containing the 1375 bp *Eco*RI-*Kpn*I fragment of plasmid pU29 [14]. The polylinker *Bam*HI site was removed from YIB4 as follows. YIB4 RF DNA was digested with *Bam*HI in the presence of dNTPs and Klenow fragment. Transformation into *E. coli* JM103 followed blunt end ligation. Plaques were screened for the loss of the *Bam*HI site by RF restriction analysis. For shuttling inserts, mutant RF DNA and vector DNA both digested with *Eco*RI and *Kpn*I were mixed and ligated using T4 DNA ligase in the recommended buffer for 8 h at 15 °C. The shuttling of the YIB12 insert into pU29, forming pU29(L228 *Bam*HI), was verified by the presence of an additional *Bam*HI site. Shuttling of L229 mutant inserts into pU29(L228 *Bam*HI) was verified by loss of the *Bam*HI site. Plasmid DNA was transformed into calcium chloride-treated HB101. Restriction endonucleases and other enzymes were purchased from Bethesda Research Laboratories, New England Biolabs, and Sigma.

### Site-specific mutagenesis

YIB12 was constructed by introducing a unique *Bam*HI site at the L229 coding sequence of YIB11 as previously described [14]. A C → G change in the third position of the glycine L228 codon in YIB11 generates this *Bam*HI site without changing the amino acid sequence of the L subunit (see Fig. 2). Introduction of site-specific mutations at isoleucine L229 followed this same protocol with the following modifications. Transformed JM103 was added to 10 ml YT media and 1 ml exponentially growing JM103 and incubated for 4 h at 37 °C instead of overlaying on YT plates. RF DNA was isolated and digested with *Bam*HI. Strain JM103 was retransformed with this digested DNA and overlayed on YT plates. Plaques were screened for the loss of the *Bam*HI site by RF restriction analysis. Mutations which lost the *Bam*HI site were analyzed by dideoxy sequencing [15]. Antisense oligonucleotides were synthesized by an Applied Biosystems 380B DNA synthesizer.

### Phenotypic assays

*R. capsulata* strain U43 (RC<sup>-</sup>LHI<sup>-</sup>LHII<sup>-</sup>) was used as the background [16] to determine phenotypes of mutations shuttled into pU29(L228*Bam*HI). Cultivation of *R. capsulata* and triparental conjugal matings were performed as previously described [17]. Plasmid pBR322 derivatives were returned from *E. coli* to *R. capsulata* by conjugation using a mobilizing plasmid [18]. *R. capsulata* cultures were grown semiaerobically in supplemented RCV medium [19]. Both photosynthetic growth assays and herbicide resistance assays were conducted by spotting mutants on MPYE plates and testing for growth within an anaerobic jar in the light. MPYE plates supplemented with 50–400 µM atrazine were used in the herbicide assays.

### Analysis of chromatophores

Chromatophore membranes were prepared by rupturing cells resuspended in buffer A (10 mM potassium phosphate, pH 7.4) in a French press (20,000 psi). Cell debris was pelleted (10K rpm; 10 min) and the supernatant saved. Crude chromatophores were pelleted from the supernatant by ultracentrifugation in a SW50.1 rotor at 45K rpm for 3 h. Reaction centers were purified from these chromatophores using a scaled down version of a

DEAE chromatography method [20]. Absorption spectroscopy was performed on resuspended crude chromatophores and purified reaction centers using a Lambda Diode Array Model 3840 spectrophotometer and Perkin-Elmer Model 7300 spectroscopic workstation.

### Results

A novel method of site-directed mutagenesis has been employed which facilitates our ability to introduce specific changes into G-C rich DNA. In this procedure a restriction site is introduced in the target region of the M13 template. In these experiments, a unique *Bam*HI site found at the L229 coding sequence of M13 derivative YIB12 was used. Site-specific mutations introduced at the isoleucine L229 codon of YIB12 simultaneously destroy the restriction site, making the M13 replicative form (RF) of these mutants resistant to *Bam*HI cleavage. Cleavage of RF DNA isolated after a mutagenesis experiment with *Bam*HI will selectively linearize unmutagenized YIB12 RF's. The uncut mutant RF's transform better than the linearized RF's, effectively enriching the population for mutants and eliminating the need for screening by plaque hybridization.

Isoleucine 229 of the reaction center L subunit, which forms part of the secondary quinone Q<sub>B</sub> binding site, was changed by this mutagenesis procedure to all other amino acid residues except proline and phenylalanine. Only frequent codons [9] were used; these changes are listed in Fig. 2. Mutations shuttled into pU29(L228*Bam*HI) were assayed in deletion strains [16] specifically engineered for this purpose.

Absorption spectra of chromatophore membranes from each of the substitutions at isoleucine L229 show that the reaction center assembles in every mutant generated at this position. The spectrum from the isoleucine to lysine mutant shown in Fig. 3A is typical of all of the mutants. In the LHII<sup>-</sup> background U43, RC absorption at 753 nm and 802 nm is clearly seen, along with LHI absorption at 878 nm. Reaction centers were purified from chromatophore membranes of each mutant. The reaction center spectrum of the isoleucine to aspartic acid mutant shown in Fig. 3B is typical of all the mutants and resembles the absorption spectrum obtained from wild-type reaction centers [20]. Physical characterization of these mutant reaction centers is in progress.

THR	LEU	GLY	ILE	HIS	ARG	LEU
5' ACG	CTC	GGC	ATC	CAC	CGC	CTC 3'
5' ACG	CTC	<u>GGG</u>	ATC	CAC	CGC	CTC 3'
<i>Bam</i> HI						
ANTI-SENSE OLIGONUCLEOTIDE	SEQUENCE		RESULT			
L228 <i>Bam</i> HI	5' GAG GCG GTG GAT CCC GAG CG 3'		INTRODUCES <i>Bam</i> HI SITE			
L229I → All	5'	(G/C)/NN	3'	Asp (GAC), Asn (AAC) Leu (CTC), Val (GTC) His (CAC), Lys (AAG) Ser (AGC), Thr (ACG)		
L229I → QE	5'	CT(G/C)	3'	Glu (GAG), Gln (CAG)		
L229I → GRW	5'	CC(A/G/C)	3'	Gly (GGG), Trp (TGG)		
L229I → M	5'	CAT	3'	Met (ATG)		
L229I → VA	5'	(G/C)(A/G/C)	3'	Ala (GCC)		
L229I → CY	5'	G(C/T)A	3'	Tyr (TAC)		
L229I → C	5'	GCA	3'	Cys (TGC)		
L229I → R	5'	CCG	3'	Arg (CGG)		

Fig. 2. Anti-sense oligonucleotides used in saturation mutagenesis at L229. L228 *Bam*HI introduces a restriction site into the target sequence shown without changing the amino acid sequence of the L subunit. The upper sequence is wild-type and the lower sequence carries the new restriction site (underlined). This *Bam*HI site is used as a selection against wild-type templates after subsequent mutagenesis. All of the oligonucleotides used at this position are identical, except for substitutions at the nucleotide triplet corresponding to the L229 codon (wild-type GAT) shown for each oligonucleotide. A mixture of all four nucleotides is represented by N; other mixtures are placed in parentheses with the mixed nucleotides separated by slashes (*i.e.* (G/C) represents a mixture of G and C). Mutations generated from each oligonucleotide are shown at the right. Only G and C were placed at the third position of codons based on the codon bias found in *R. capsulata*.

Mutations at isoleucine L229 result in varying levels of photosynthetic growth (see Fig. 4). Ten of the seventeen mutations (Gly, His, Tyr, Trp, Asn, Asp, Gln, Glu, Arg and Lys) result in the loss of photosynthetic growth, even under high light conditions ( $>10$  mW/cm<sup>2</sup>). The remaining seven mutations yield varying levels of photosynthetic growth (Ile, Val, Ala, Leu, Met > Thr, Cys, Ser) under low light conditions (1 mW/cm<sup>2</sup>) but are indistinguishable under high light.

Varying levels of atrazine resistance were generated in six of these mutants. With either methionine

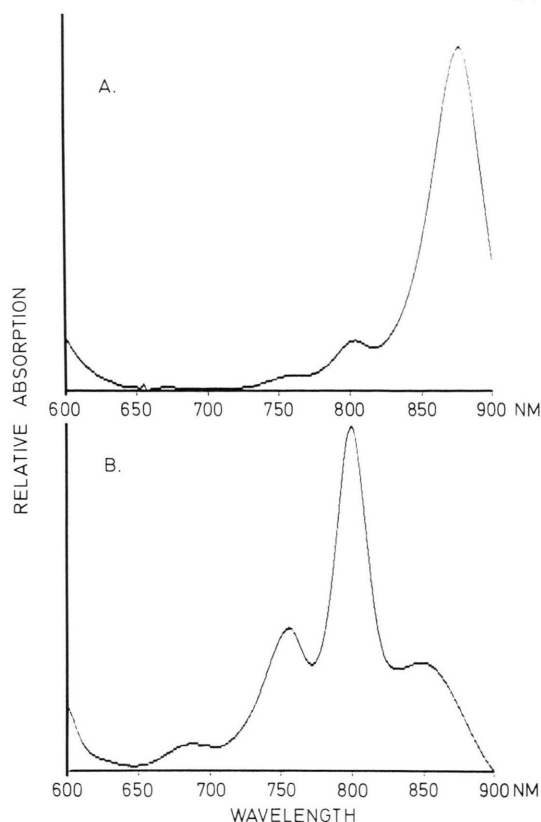


Fig. 3. The presence of reaction centers in each of the seventeen mutations at L229 is verified by absorption spectroscopy. Panel A: Chromatophore membranes from the isoleucine to lysine mutant. Peaks characteristic of reaction centers are seen at 753 nm and 802 nm. All seventeen mutations, when returned to the *puf* deletion strain (U43), are spectroscopically indistinguishable from the parental strain MW442 (LHII<sup>-</sup> mutant). Panel B: Purified reaction centers from the isoleucine to aspartic acid mutant. Reaction centers from all seventeen mutants are photochemically active as demonstrated by the reversible bleaching of the long wavelength absorption band by actinic light (spectra not shown).

or leucine at L229, photosynthetic growth was maintained in the presence of 400  $\mu$ M atrazine. Inhibition of photosynthetic growth in the presence of 200–400  $\mu$ M atrazine was observed for alanine, threonine, serine or cysteine at L229. Both valine and the wild-type isoleucine at L229 were inhibited in the presence of 50–100  $\mu$ M atrazine. None of the mutants analyzed at L229 showed greater atrazine sensitivity than the wild-type isoleucine. Assays for herbicide resistance were done under high light conditions to minimize any effect of differential photosynthetic growth rates among the mutants.



IL229		ATRAZINE RESISTANCE		
PHOTOSYNTHETIC GROWTH		-	±	+
	-	H,Y,W K,D,E,R G,N,Q		
	±		S,C,T	
	+	I,V	A	L,M

Fig. 4. Phenotypes for photosynthetic growth and atrazine resistance for amino acid substitutions at L229. Single letter amino acid symbols are used. Site saturation mutagenesis at this position generates a wide variety of phenotypes.

## Discussion

With the recent elucidation of the reaction center crystal structure [10, 11], the molecular basis of herbicide resistance at the  $Q_B$  site can now be addressed. Mutations which cause terbutryn resistance may disrupt hydrogen bond formation between protein (serine L223 or isoleucine L224) and herbicide by

(1) replacement of serine with an amino acid residue incapable of forming a hydrogen bond; (2) moving the ethylamino nitrogen of terbutryn away from the serine L223 side chain oxygen or N3 of the *s*-triazine ring system away from the peptide nitrogen of isoleucine L224 by affecting the position and orientation of the herbicide in the  $Q_B$  site; or (3) causing global changes in protein folding which move the hydrogen bonding residues away from the herbicide. The loss of this hydrogen bonding may decrease the affinity of terbutryn for the  $Q_B$  site. Alternatively, mutations may diminish herbicide binding by disturbing critical hydrophobic interactions, introducing steric hindrance or causing large-scale changes in protein structure which affect the  $Q_B$  site. Combinations of these factors may be at work in different resistant mutations. Chemical effects of the methionine sulfur atom are not required to explain resistance at L229 [11] since leucine and other residues which lack this moiety also convey herbicide resistance.

Hydrophobic residues of moderate size appear to function best at L229. An amino acid residue at this position must interact favorably with a hydrophobic quinone for proper reaction center function. Resi-

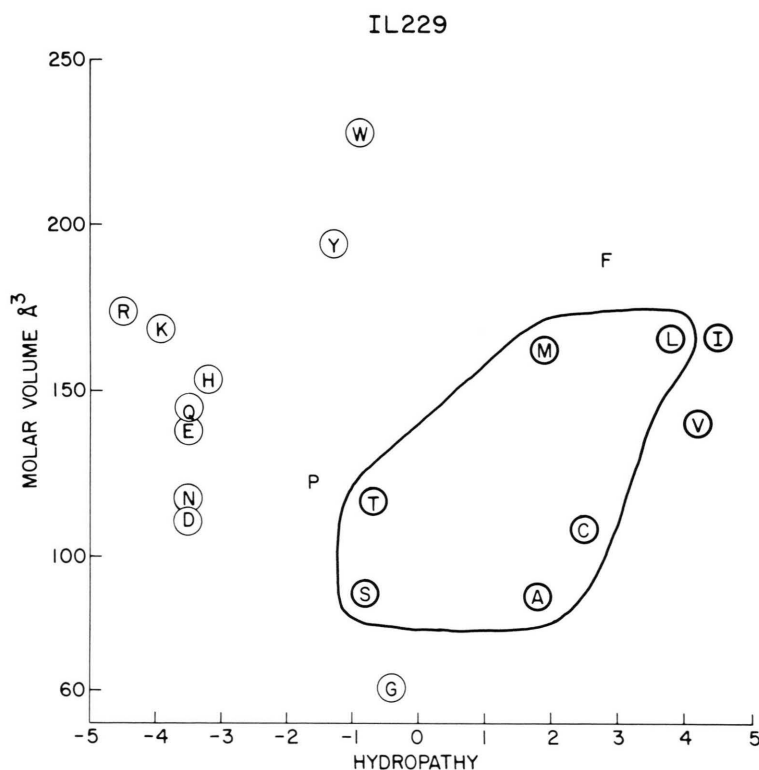


Fig. 5. Hydropathy-molar volume plot for L229. Residues which result in the loss of photosynthetic growth are enclosed in light circles; those residues which maintain some level of photosynthetic growth are enclosed in dark circles. Residues which were not tested are left unmarked. The heavy contour line encloses residues which convey some level of herbicide resistance. The hydropathy values are from Kyte and Doolittle [21]. The molar volumes of the amino acids are from Zamyatnin [22].

dues which convey similar phenotypes are clustered together in the hydropathy-molar volume plot found in Fig. 5. This diagram is a useful tool in understanding the results of saturation mutagenesis experiments by combining some of the physical properties of amino acid residues with the effect these properties have on a particular activity of the protein being studied. Consideration of experimental results in this way may help to better define the specific role of protein in altering the chemical properties of prosthetic groups.

Our results indicate that all mutations at L229 which maintain some level of photosynthetic growth also convey herbicide resistance, with the exception of valine. Analysis of spontaneous atrazine-resistant mutants in *R. sphaeroides* in two independent reports [6, 7] both find an isoleucine to methionine change at this position, yet four of the six herbicide-resistant mutants are accessible by single nucleotide mutations. Site-specific mutagenesis is therefore a necessary tool to investigate all potentially interesting residues at a particular position, whether or not the amino acid codon can be generated by a single point mutation.

Detailed physical analysis of these seventeen mutants at L229 is possible since the reaction center is always assembled. This suggests that a functional  $Q_B$  binding site and possibly the presence of the second

ary quinone itself are not required in the assembly pathway.  $Q_B$  may be unique in this respect since it is the only prosthetic group thought to leave the reaction center. Reaction center preparations from these mutants are similar in yield, suggesting that the photosynthetically impaired phenotype is not the result of a reduction in the number of reaction centers found in the membrane, but an effect on reaction center function itself.

Defects in reaction center function resulting from many of these mutations suggest important changes in the  $Q_B$  binding site. Reduction in quinone binding affinity may be caused by extensive perturbations in protein folding, increased steric hindrance, the introduction of unfavorable polar interactions, or the loss of important bonding interactions at the  $Q_B$  binding site. The equilibrium between  $Q_A^-Q_B$  and  $Q_AQ_B^-$  may be altered by changes in  $Q_B$  redox potential or  $Q_B$  orientation in the complex. Physical characterization of the mutations at L229 is currently underway in our laboratory to understand these changes. Future studies will provide a detailed understanding of the role of the protein in mediating quinone chemistry in the photosynthetic reaction center.

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