CHIMERIC RESCUE OF *RB. CAPSULATUS* REACTION CENTER GENES WITH SEQUENCES FROM *RB. SPHAEROIDES*

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1. INTRODUCTION

Site-directed mutagenesis is becoming a very important technique in the study of photosynthetic reaction center structure-function relationships. However, there are certain disadvantages associated with this approach. First, it relies very heavily on the intuition of the investigator to select particular mutations that will give rise to interesting phenotypes; many times, mutagenesis can result in very little change in photosynthetic function, in complete loss of function, or in lack of assembly. Second, it is difficult to use specific mutagenesis to detect the concerted effect of several amino acids.

Here we describe methodology for generating, selecting, and rapidly screening large numbers of mutants in specific regions of the reaction center genes. One can generate mutants involving changes in both small and large numbers of amino acids and the mutant phenotypes always include stable reaction centers that are at least partially functional.

2. METHODS

2.1 Strains and Plasmids. *Rhodobacter capsulatus* strain U43 containing a deletion of the *puf* operon was generously provided by Dr. Douglas Youvan, as were plasmids pU29 and pU2922 (1, 2). pJW1 (3) was generously provided by Dr. George Feher. The M gene deletion used in this work is missing 48 bp between the *Bst*EII and *Sac*II restriction sites of M.

2.2 Chimeric Rescue. A detailed description of these procedures will be presented elsewhere.

2.3 Fluorescence Decay Measurements. The single photon counting instrument will be described in detail elsewhere. All measurements were made on whole cells using 870 nm excitation and 900 nm emission detection.

3. RESULTS

3.1 Mutagenesis via Chimera Rescue. We have developed a new form of mutagenesis to study the effects of interspecies differences on reaction center structure and function. In this approach, we select for recovery of photosynthetic activity in deletion mutants of the host gene (usually the *Rb. capsulatus* reaction center operon) by recombination with a donor gene from an organism with a related reaction center. For these experiments, we have constructed pCR, a derivative of pU2922 containing the *puf* operon of *Rb. capsulatus* as well as unique *Bam*HI and *Xho*I sites for insertion of donor genes into the plasmid.

We have used this system to repair a 48 bp deletion in the M gene of the *Rb. capsulatus* operon by recombination with sequences from the 4.5 kb *Pst*I fragment from the *sphaeroides puf* operon of pJW1 (Fig. 1). Even though most of the *sphaeroides* operon is present, the starting plasmid does not produce spectroscopically detectable amounts of reaction centers. The two operons were placed in the plasmid with opposite orientations to allow single, homologous

recombinational events between the two operons to occur without loss of sequence following inversion. When this plasmid was conjugated into U43 and put under photosynthetic selection, photosynthetic activity was recovered with a frequency of approximately 10^{-7}.

In initial studies, two photosynthetically competent chimeric reaction center genes were isolated and sequenced. In one case, repair of the deletion in the M gene of *capsulatus* resulted in the replacement of *capsulatus* gene sequence between position M669 and M780 with *sphaeroides* sequence. In the other case, positions M559 through M747 were replaced. Reaction centers isolated from these mutants had steady state absorbance spectra and P^+Q^- recombination kinetics comparable to wild type reaction centers. More recently, we have partially characterized 44 independently isolated mutations resulting in restoration of photosynthetic activity. Through restriction mapping, we have identified at least three classes of mutants: 1) single inversions in which some or all of the M gene and all sequence downstream of M in the *Rb. capsulatus* operon has been reciprocally exchanged with the analogous section of the *sphaeroides* operon 2) conversion events in which a section of the coding sequence of the *capsulatus* M gene has been replaced by *sphaeroides* sequences nonreciprocally (i.e., *sphaeroides* sequences do not appear to be altered) and 3) events which have resulted in photosynthetic rescue without repair of the lesion in the M gene of *Rb. capsulatus*. We suspect that the third class, which represents nearly half of the mutants analyzed to date, is due to conversion of the 5' sequences of the *sphaeroides* operon resulting in expression of the *sphaeroides* B875 and reaction center genes. In support of this, deletion of the *capsulatus* operon from the recombined plasmid and reintroduction of the plasmid back into U43 results in photosynthetic activity. This implies that the *sphaeroides* L and M genes can assemble with the *capsulatus* H gene and that it is possible to express the B875 antenna complex of *sphaeroides* in the *capsulatus* photosynthetic membrane at least in the presence of *sphaeroides* L and M reaction center subunits. Schematic representations of each class of mutation are shown in Fig. 1. Sequence analysis of these mutants is ongoing.

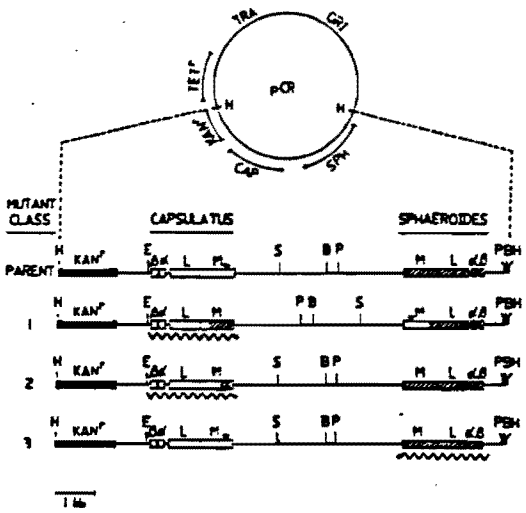

Fig. 1. Schematic diagram of pCR with the *sphaeroides* operon as a donor. Three of the classes of recombinants that arose from the chimera rescue experiments are also shown. The hatched boxes represent *sphaeroides* sequences; the open boxes represent *capsulatus* sequences. Only the *Pst*I sites that define the subclone of pJW1 (3) used in this experiment are shown. Restriction sites: B = *Bam*HI; E = *Eco*RI; H = *Hin*DIII; P = *Pst*I; S = *Sac*I.

3.2 Screening of Mutants Using Time-Resolved Fluorescence Spectroscopy. In order to screen many mutants quickly for potentially interesting phenotypes, we have employed time-resolved single photon counting techniques using whole cells directly from liquid culture. To avoid build-up of the long-lived P^+Q^- state, the quinone is chemically reduced with dithionite (approximately 20 mM). The fastest component of the decay is associated with the excitation trapping time and in simple antenna systems is thought to be proportional to the initial electron transfer rate in the reaction center (4). When the quinone is reduced, the initial radical pair state, P^+Bphe^-, lives for about 10 nanoseconds and back electron transfer from this state gives rise to nanosecond delayed fluorescence which is usually easy to distinguish from the much faster excitation trapping. The amplitude of the delayed fluorescence varies with the free energy difference between the states P^* and P^+Bphe^- (5). Thus, this technique allows one to quickly measure parameters that are sensitive to both the kinetics and thermodynamics of the initial

electron transfer in the reaction center and in this way identify mutants in which this reaction may be altered.

Representative fluorescence decays from whole cells in the presence of dithionite are shown in Fig. 2. The decay curve shown in the top panel is from cells harboring a plasmid that encodes wild type *capsulatus* reaction center and antenna proteins. The bulk of the decay occurs with a time constant of 40-45 ps, followed by a much lower amount of longer-lived delayed fluorescence with a multiexponential decay. The delayed fluorescence includes the 10 ns lifetime of P^+Bphe^-. The mutants examined thus far show one of two phenotypes with regard to their fluorescence decay curves. The decay kinetics from one group of mutants are similar to the parent strain (Fig. 2, middle panel). The other mutants tested all had emission decays that were very similar to each other but different from the decay of either the parent strain or the mutants described above. An example of one of these decays is shown in the bottom panel of Fig. 2. The biggest difference between this trace and those above it is the large increase in fluorescence on the 100 ps - 1 ns timescale.

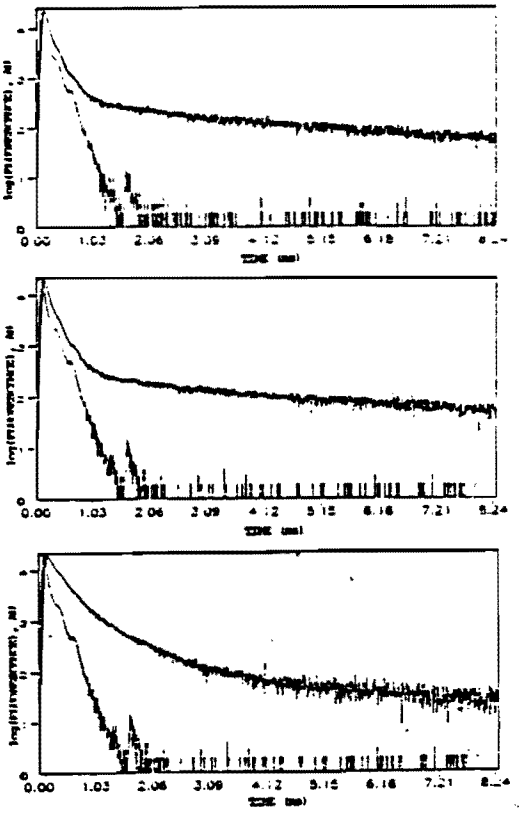

Fig. 2. Time-resolved fluorescence decays of whole cells of *Rb. capsulatus*. In each sample, the reaction center quinones were reduced with sodium dithionite. Excitation was at 870 nm, detection at 900 nm. For each decay, the instrument response function is also shown. Top decay: wild type reaction center chromophore. Middle decay: chimera #15 (very similar decays were observed for #4 and #32 as well). Bottom decay: chimera #5 (very similar decays were observed for #1 and #2 as well).

4. DISCUSSION

4.1 Chimeric Reaction Centers Can Be Used to Study Sequence Differences Between Homologous Reaction Center Genes. We have shown that it is possible to use chimeric rescue as a method for generating functional reaction center genes that utilize sequences from two different organisms. One can use this technique both to determine which homologous sequences between two reaction center genes perform similar roles and to identify the homologous regions that play different roles. Once regions with important differences are identified, specific mutagenesis techniques can be used to investigate the detailed sequence requirements further. We intend to expand this technique to compare more distantly related reaction centers (such as *capsulatus* and *viridis*) as well as to generate functional chimeric reaction center subunits with sequence from both the homologous L and M polypeptides.

4.2 A Subset of the Chimeras Show Altered Fluorescence Decays. The proper interpretation of the fluorescence increase we have observed in a number of the chimeras (Fig. 2, bottom panel) is not yet clear. Depending on how the data is fit to a series of exponentials, one either finds that the trapping time for the excitation has increased by about a factor of three, or one finds a smaller increase in the trapping time (50%) with a large increase in the amount of fluorescence decaying on a 100 ps to 1 ns timescale. It appears that there has been a significant change either in the rate that excitations reach the reaction center or in the kinetics or thermodynamics of the initial electron transfer reaction. The biochemical and spectroscopic properties of these mutants are being investigated in more detail.

It is interesting to note that those mutants that apparently express both the reaction center and antenna genes of *sphaeroides* also display the altered fluorescence decay kinetics shown in the bottom panel of Fig. 2. This suggests that the difference in the fluorescence decay is probably not due to an altered interaction between the chimeric reaction centers and the antenna. Possibly, the change is due to the interaction between the chimeric L and/or M subunits of the reaction center and the H subunit which in all cases is provided by the *capsulatus* genome.

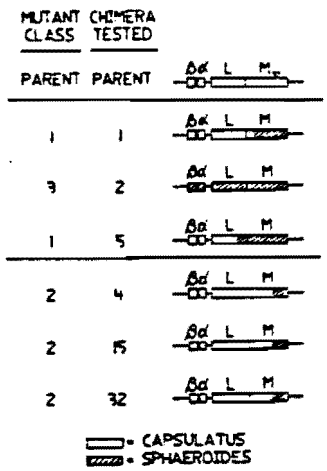

Fig. 3. Schematic diagrams of six *capsulatus/sphaeroides* chimeric *puf* operons generated by the chimera rescue technique described in the text. "Mutant class" refers to the three classes of mutants identified in the text. "Chimera tested" refers to the identification number of the specific mutant. The approximate boundaries between *capsulatus* and *sphaeroides* sequences was determined by restriction mapping.

4.3 The Fluorescence Decays are Correlated with Primary Structure. The effects of these mutations on excitation trapping and/or initial electron transfer events are correlated with their structure. Chimera mutants 4, 15 and 32 each show fluorescence decays very similar to the wild type (Fig. 2, top and middle panels). Mutants 1, 2 and 5 show the altered fluorescence decay (Fig. 2, bottom panel). This correlates quite well with the preliminary structures for these rearrangements determined by restriction mapping (Fig. 3). Mutants 4, 15 and 32 contain changes that are probably all confined to the 3' portion of M, while 1, 2 and 5 appear to extend through most or all of the 5' half of M as well. As we analyze a larger number of mutants in this way and obtain more detailed sequence data on the precise end-points of these rearrangements, it should be possible to locate the region of sequence responsible for the phenotypic variation. Directed mutagenesis techniques can then be used to determine exactly which amino acids are involved.

5. ACKNOWLEDGEMENTS

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