Structure-Function Studies on Bacteriorhodopsin

V. EFFECTS OF AMINO ACID SUBSTITUTIONS IN THE PUTATIVE HELIX F

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To test structural and mechanistic proposals about bacteriorhodopsin, a series of analogues with single amino acid substitutions has been studied. Mutants in the proposed helix F of bacteriorhodopsin were chosen for investigation because of the probable interaction of this part of the protein with the retinal chromophore. Seven mutants of the bacteriorhodopsin gene were constructed by site-directed mutagenesis, and the gene products were expressed in Escherichia coli. The resulting mutant proteins were purified and assayed for their ability to interact with retinal in phospholipid/detergent micelles to form a bacteriorhodopsin-like chromophore. Four mutants, Ser-183 → Ala, Tyr-185 → Phe, Ser-183 → Ala, and Glu-194 → Gln, bound retinal to give chromophores that were blue-shifted relative to the wild type. Three mutant opsins bound retinal to give chromophores that were almost indistinguishable from the wild type, and the mutant Pro-186 → Leu gave a pigment with an absorption maximum of 470 nm. However, none of the amino acid substitutions eliminated the ability of the mutant bacteriorhodopsin to pump protons in response to illumination.

Substitution of specific amino acids in proteins provides an attractive and versatile approach to their structure-function studies. Such an approach is made possible by the techniques of recombinant DNA and is being currently used in studies of a number of proteins (1). Bacteriorhodopsin (bR), an integral membrane protein from Halobacterium halobium, carries out light-dependent proton translocation from the inside to the outside of the cell (2). Since it seems reasonable that proton translocation is mediated by functional groups present on the side chains of certain amino acids, we are investigating this possibility by producing specific amino acid substitutions in bacteriorhodopsin using the techniques of site-specific mutagenesis. With this aim, we have previously documented the isolation and characterization of the bacterio-opsin (bO) gene from H. halobium and have carried out an extensive study of the expression of this gene in Escherichia coli (3). Furthermore, we have developed rapid and convenient methods for the purification and characterization of the bO produced in Escherichia coli (4–6). To facilitate unrestricted site-specific mutagenesis of the bO gene, we have designed, synthesized, and cloned a bO gene that contains uniformly spaced unique restriction sites (7). This enables efficient mutagenesis in the gene by the method of cassette replacement.

In this paper, we describe our first results with a set of bO mutants that contain single and specific amino acid substitutions in the putative helix F (Fig. 1). We chose this region of the protein for our initial studies on the basis of the following considerations. In previous work on the location of retinal within the membrane-embedded part of bR, photolysis of a photosensitive analogue of retinal bound to Lys-216 resulted in cross-linking principally to Ser-193 and Glu-194 (8). This result indicated proximity of the β-ionone ring of retinal in bR to these two amino acids and presumably other amino acids in helix F. Therefore, judicious amino acid substitutions in helix F should yield information concerning interactions between retinal and this helix. Such studies would be relevant to the question of the cause of the large red shift in the spectrum of retinal upon binding to the opsin. The presence of an "external point charge" in the vicinity of the β-ionone ring of retinal in bR has been advanced as an explanation of the opsin red shift (9). Because of the uncertainties in the current structural models for bR, it is unclear which amino acid residue in the folded molecule may provide the charge, but the carboxylate of Glu-194 would appear to be a strong candidate in view of the cross-linking results.

Furthermore, there is an attractive postulate proposed independently by a number of groups (10–12) that protons may be conducted through proteins by continuous hydrogen-bonded systems probably involving the side chains of suitable amino acids. This idea could be tested by substitution of selected amino acids that can form hydrogen bonds by those that cannot. Thus, substitutions of amino acids such as phenylalanine for tyrosine, alamine for serine, or neutral amino acids for aspartic and glutamic acids can be used to test the validity of the above hypothesis. It has also been suggested that protons could move down a hydrogen-bonded system consisting of the polypeptide backbone (13, 14). Another possibility is a hydrophilic hole through the protein. Irrespective of the exact mechanism, it is likely that hydrophilic and/
or charged amino acids would be involved in the translocation process.

The ideas presented above have influenced our initial application of site-specific mutagenesis to bR. The amino acid replacements carried out in the present work are shown in Fig. 1. Thus, Trp-182 and Trp-189 were each replaced by phenylalanine; Ser-183 and Ser-193 were each replaced by alanine; Tyr-185 was replaced by phenylalanine; and Glu-194 was replaced by glutamine. Finally, Pro-186 was replaced by leucine to determine if the absence of the amide proton is necessary for pumping (15) or if the proline influenced the protein conformation or folding pathway significantly (16, 17). These mutations led to various phenotypes when studied by the procedures previously developed for the reconstitution of bO. Four mutations, comprising the two serine to alanine changes, Glu-194 + Ala, and Glu-194 + Lys, were each replaced by alanine, and to 0.1 pmol for Pro-186 + Leu. However, none of the mutations studied decreased the rate of lipid pumping when reconstituted into lipid vesicles, although the Pro-186 + Lys mutation decreased both the rate and extent of transmembrane proton pumping.

**MATERIALS AND METHODS**

**General—Recombinant DNA methods and the immunological detection of bO have been described (5). The procedures used for synthesis, purification, and characterization of oligonucleotides (7, 18), for purification of bO from E. coli cells expressing the bop gene, and for the regeneration of bO from bO in DMPC/CHAPS/SDS mixtures have been described previously (4, 6).**

**Mutagenesis of the bop Gene: Mutants in Helix F—Codon changes specifying the amino acid substitutions Trp-182 → Phe and Ser-183 → Ala were produced by cassette mutagenesis using the synthetic bO gene (7). Two restriction fragments (ScaI-BstXI) containing the desired mutations were synthesized. This required the synthesis of one oligonucleotide for each strand. The component oligonucleotides were quantitatively labeled at their 5' ends with [32P]phosphoryl groups and annealed appropriately. The fragments were ligated to equimolar amounts (0.1 pmol each) of the small KpnI-ScaI fragment and the large KpnI-BstXI fragment of pSB02. To prepare these restriction fragments, pSB02 was digested to completion with KpnI, and the products were run on a low melting point agarose gel. The linearized plasmid was recovered and purified on an Elutip-d (Schleicher & Schuell). It was further digested to completion with BstXI or ScaI, and the resulting fragments were purified on an agarose gel. The three-component ligation mixture described above was used to transform E. coli strain DH1, and four transformants corresponding to each mutant were chosen for further study. Plasmid DNA was prepared on a small scale and digested with EcoRI and HindIII. The small fragments which resulted were purified on an agarose gel and cloned between the HindIII and EcoRI sites of pPL1 (7). The ligation mixture was used to transform the E. coli strain C600 bearing the repressor plasmid pCL857 (19). Kanamycin- and ampicillin-resistant transformants bearing a plasmid of the expected size which produced bO (detected immunologically) upon temperature shift-up were retained as frozen stocks.

**Expression of Mutants in the Native bop Gene—Preparation of the five mutants, Trp-185 → Phe, Pro-186 → Leu, Trp-189 → Phe, Ser-193 → Ala, and Glu-194 → Gln, has been described previously (18).** These were prepared by replacement of an AvaI-XhoI restriction fragment by synthetic counterparts containing the desired nucleotide changes. The five mutants were subcloned from pIN-B02 to pPL-BO6 (3) by the following method. A small EcoRI-SphI fragment which specifies a Shine-Dalgarno sequence and the first 183 amino acids of bO were purified from pPL-BO6. SphI-BamHI fragments were prepared from the derivatives of pIN-B02 specifying the replacements in the bop gene. The Pvu I-promoter was provided by pPL2853 (20) following digestion with EcoRI and BamHI. The three restriction fragments were purified on agarose gels. They were ligated, and the ligation mixture was used to transform C600 bearing the temperature-sensitive repressor plasmid pCL857. Kanamycin- and ampicillin-resistant transformants which harbored plasmids of the expected size and which produced bO upon induction were retained as frozen stocks.

**Growth of Cells for Bacterioopsin Production**—All cultures were grown at 30°C in the presence of 50 µg/ml ampicillin and 100 µg/ml kanamycin. Cells were streaked from the frozen stocks. A single colony was picked and cultured overnignt in 100 ml of a medium consisting of 80 mM potassium phosphate, pH 6.5, 15 mM ammonium sulfate, 4 mM sodium citrate, 2 mM magnesium chloride, 0.2 mM calcium chloride, 29 µM ferric sulfate, 2 µM each ammonium molybdate, boric acid, zinc sulfate, cobalt chloride, copper sulfate, and manganese chloride, 2% (w/v) glycerol, and 0.1% each (w/v) casamino acids and yeast extract. This was subcultured into 1 liter of the same medium with 10% glucose and grown at 37°C for 24 h and used to inoculate 2 liters of the same medium in a Biosafety BL15P fermentor. Ammonium hydroxide (5 M) was added to maintain the pH at 6.9. The agitation rate was periodically increased to maintain a dissolved oxygen concentration of greater than 50% saturation. During the culture, 1 liter of nutrient was added, consisting of 25% (w/v) glucose, 5% (w/v) each yeast extract and casamino acids, at a rate proportional to the agitation speed. When the cells reached a density of 4 g, dry weight/liter, the temperature of the culture was rapidly raised to 42°C to induce bO production. Cells were harvested after 15 min and frozen as quickly as possible.

**Sequencing of Mutant Genes—Cells from the fermentor batch were used for plasmid DNA preparation (5). The bop gene was excised and subcloned into M13mp11 (21). The sequence of the region carrying the mutation was determined by the chain terminator method (22) and was confirmed to be correct. Because of the efficiency of mutagenesis by the cassette procedure, sequencing of mutations before this stage was considered unnecessary; and, in fact, the first candidate grown for each mutant had the correct sequence for the expected mutation. For the mutant Trp-189 → Phe, the DNA sequence of the whole bop gene was determined to ensure that the observed phenotype resulted from only the single mutation desired.

**Expression of Bacterioopsin—** The cells were disrupted by passage at 10,000 p.s.i. through a French press, and a membrane fraction was collected by centrifugation at 40,000 × g for 60 min. The membranes were lyophilized, and bO was extracted by the chloroform/methanol/triethylamine extraction procedure (6). bO was purified to homogeneity by gel filtration and ion-exchange chromatography in chloroform/methanol and transferred to 17% SDS, 0.025% sodium azide (6). The concentration was determined using an εnm of 66,800 cm−1 M−1 for bO in either SDS or DMPC/CHAPS (23).

**Folding of bO and Regeneration of Bacteriorhodopsin-like Spectrum—** bO, 1% SDS was brought to 0.2% SDS, 1% DMPC, 1% CHAPS, 0.025% sodium azide, 30 mM NaP, pH 6.0, by the addition of water and concentrated buffer and lipid/detergent solutions. Retinal was added from a concentrated stock solution in ethanol (final retinal concentration, 0.25%). The rate of increase of absorbance at 550 nm was recorded at 20°C. To determine the rate and efficiency of retinal binding, an estimated 1.5-fold excess of retinal was used. Absorption spectra were scanned after overnight incubation in the dark by subtraction of a blank containing no protein or retinal. To determine the absorption maxima, 0.2-0.5 eq of all-trans or 13-cis-retinal was added, and the spectrum was scanned at intervals during regeneration of the chromophore.

**Proton Pumping by Lipid Vesicles Containing Bacteriorhodopsin—** The detergent dilution procedure of Racker et al. (24) was modified as follows. bR was regenerated as described above except the buffer concentration was lowered to 1 mM. An aliquot (10 µl containing 30–100 pmol of bR) of the regeneration mixture was added to 27.2 µl of 2% soybean lipids and 2.8 µl of 0.5 M octyl glucoside, and the mixture was incubated on ice under argon for 1 h. This sample was injected into 1 ml of argon-saturated 2 M NaCl in a jacketed reaction vessel with ports for a pH probe and an argon line. The pH was adjusted to 7.0 with aliquots of 10 mM HCl or NaOH, and the base line was allowed to flatten (about 10 min). The sample was then irradiated with a 250-watt projector lamp through a heat-absorbing filter and a 455-nm-long pass filter and focused on the cell. This light intensity was saturating for all the mutants. Three light-dependent proton uptakes were recorded with recovery intervals of more than 3 min. The system was calibrated by 0.5–1 µl injections of 1 mM HCl. The initial rates and extents of the light-induced pH change were linear with bR concentration in the range studied.
RESULTS

Preparation of Wild Type and Mutant Bacterio-opsins

Mutagenesis of the Synthetic bop Gene—In total, we have studied seven mutants in the putative helix F of bacteriorhodopsin (Fig. 1). In addition to the five mutations described previously (18), Trp-182 was mutated to phenylalanine and Ser-183 to alanine. These two mutations were created by cassette mutagenesis of the synthetic gene. The codons for these two amino acids, TGG and TCC, overlap with the closest restriction site (AvaII) (Fig. 2). Therefore, the next available flanking sites were used, a Scal site corresponding to position 173 of the protein and a BstXI site corresponding to position 186 (Fig. 2). Two synthetic restriction fragments were prepared consisting of only one oligonucleotide for each strand, each containing a single codon change (Duplexes I and II, Fig. 2).

To clone such synthetic duplexes, it is usually sufficient to cut the vector with the flanking restriction enzymes, purify the cut vector fragment, and ligate it with the newly synthesized duplex. In the present case, this approach was not possible due to the presence of an additional Scal site in the β-lactamase gene of the vector. Therefore, a three-component ligation strategy was employed (Fig. 3). The procedure involved reconstructing the mutant bop genes from two restriction fragments (fragments A and B, Fig. 3) and the synthetic duplexes (Fig. 2). Fragment A is the long BstXI-KpnI fragment comprising the vector and the 5’ and 3’ ends of the bop gene that encode amino acids 1–69 and 187–248. Fragment B is the short KpnI-Scal fragment; it encodes amino acids 70–173. The mutagenic duplexes specifying amino acids 174–186 complete the bop gene.

This approach allowed cloning of Duplexes I and II with high efficiency. To estimate the frequency of mutagenesis, a control ligation and transformation was performed using fragments A and B (Fig. 3), but in the absence of the synthetic duplex. The number of transformants obtained relative to the complete ligation and transformation suggested a mutagenic frequency in the range of 90%. This was borne out by analysis of the DNA of eight random transformants, four for each mutant. All of these indeed lacked the AvaII site, as expected from the desired codon changes introduced. For each mutant, the DNA of one clone was sequenced through the synthetic fragment and flanking restriction sites. Only the nucleotide changes introduced by synthesis were found.

Expression of Mutant Bacterio-opsins—The expected amino acid sequences of the mutant bacterio-opsins are shown in Fig. 4. All of the mutants differ slightly from the native bacterio-opsin from Halobacterium halodotum (Fig. 4a) not only in helix F, but also at the N and C termini. The two mutants prepared from the synthetic gene (W182F and S183A) were cloned into vector pPL1 to give analogues of pPL-SD02 (7). Their predicted amino acid sequences are shown in Fig. 4b. The five mutants previously described in the native bop gene (Y185F, P186L, W189F, S193A, and E194Q) were cloned into pPL06 (3). The resulting plasmids encode the N- and C-terminal amino acid sequences shown in Fig. 4c.

Fermentation of Cells Containing bO Expression Plasmids—To produce large amounts of bO, cells harboring the expression plasmids were grown in a high efficiency fermentor. Using the conditions described under “Materials and Methods,” yields of about 50 mg of bO (estimated immunologically) were obtained from 10-liter cultures. We have tested several strains for bO production and find C600 (19) to be the best. Optimal production of bO was obtained when the culture was induced by temperature shift-up in the midexponential phase of growth. Glucose was the best carbon source for growth, but high concentrations caused nonscheduled induction of bO. Therefore, it was added slowly during the culture. To ensure that the repressor and expression plasmids were stable during fermentation, cells were sampled immediately before induc-

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**Fig. 1. Structural model of bacteriorhodopsin showing the helix F mutants.** The two boxes enclose the amino acids proposed to form helices F and G of the protein. The residues circled were each mutated to the residue shown in the corresponding rectangle.

<table>
<thead>
<tr>
<th>Protein Sequence</th>
<th>Synthesis of Synthetic Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys Val Leu Arg Asn Val Thr Val Val Leu Trp Ser Ala Tyr Pro Val Val Trp Leu...</td>
<td></td>
</tr>
<tr>
<td>...AAA GTA CTG CTT GAT ACC GTT GTG CTT CTG TCC GCT TAC CCA CCT GTG...</td>
<td></td>
</tr>
<tr>
<td>Scal</td>
<td>AvaII</td>
</tr>
<tr>
<td>Phe</td>
<td>BstXI</td>
</tr>
</tbody>
</table>

**Fig. 2. Synthetic duplexes for the mutagenesis of Trp-182 and Ser-183 of the bop gene.** The amino acid sequence of a part of the proposed helix F (Fig. 1) of bO is shown with the corresponding DNA sequence of the synthetic gene (7). The recognition sites of three restriction enzymes are shown in boxes, BstXI and Scal cut at the positions indicated by the arrows. To mutagenize Trp-182 to phenylalanine, Duplex I was synthesized as one oligonucleotide for each strand. Similarly, Duplex II was synthesized to mutagenize Ser-183 to alanine.
that the plasmids were stable throughout the subculture and plated with or without antibiotic selection. The purified. This was further digested with either efficiency of plating was the same on both media, suggesting ble.

were prepared from the cells, and bO was extracted with basic fermentation steps. After 20 min of induction, the amount of bop was digested to completion with

with the synthetic Duplex I or

was digested to completion with

b.

The vector pSBO2 contains the whole synthetic bap gene (black arc) followed by a transcription terminator. pSBO2 was digested to completion with KpnI and, the linearized plasmid was purified. This was further digested with either BsrXI or ScaI to yield the fragments shown. Fragments A and B were purified and ligated with the synthetic Duplex I or II (Fig. 2) to reconstruct the synthetic gene.

and plated with or without antibiotic selection. The efficiency of plating was the same on both media, suggesting that the plasmids were stable throughout the subculture and fermentation steps. After 20 min of induction, the amount of bO in the culture began to decline. To minimize loss of the protein, the cells were harvested in two batches at 10 and 35 min after induction and frozen as pellets as quickly as possible.

Purification of Bacterio-opsins—Crude membrane fractions were prepared from the cells, and bO was extracted with basic chloroform/methanol/water (6). The extract was subjected to gel filtration to remove lipids, and bO was purified by ion-exchange chromatography and transferred to SDS (6). All seven mutants, including E194Q which has a charge difference from the wild type, could be purified by the standard procedure. The samples all appeared homogeneous by SDS-polyacrylamide gel electrophoresis.

Properties of Mutant Bacterio-opsins

Chromophore Regenerations and Spectral Ratios—The standard chromophore regeneration assay involves the addition of retinal to bO in DMPC/CHAPS/SDS mixed micelles. The formation of bR chromophore is rapid and quantitative (4). The wild type bO and all seven mutant bacterio-opsins purified from E. coli bound retinal to give a bR-like chromophore (Fig. 5). The absorption maxima of the resultant chromophores were dependent on the mutants, varying from 559 nm for the wild type to 476 nm for the mutant P186L.

To estimate the extent of chromophore regeneration for each mutant, a spectral ratio was calculated (Table I). This value is the ratio of the protein absorbance at 280 nm to the absorbance maximum of the peak in the visible region. The values were recorded after overnight regeneration with a small excess of retinal. For a completely regenerated sample of bO from H. halobium, this ratio would be 1.3. The wild type bO purified from E. coli and the mutant Y185F both gave a ratio of 1.6, probably indicative of incomplete chromophore regeneration (6). Other mutants gave higher spectral ratios, and this was most apparent for two mutants, W182F and P186L, with the lowest absorption maxima (Table I). This probably reflects both incomplete chromophore regeneration and a decrease in the visible extinction coefficient.

Precise determination of the $\lambda_{max}$ of the bR-like chromophores was not possible in the presence of excess retinal since the absorption spectra of the reconstituted proteins and free retinal show some overlap. In addition, for several mutants, it was not clear if the chromophore observed after overnight incubation contained a 1:1 mixture of the all-trans- and 13-cis-isomers of retinal, as is characteristic of the native dark-adapted bR. Therefore, each mutant was regenerated with a limiting concentration of either all-trans- or 13-cis-retinal, and the absorption spectrum was recorded immediately after

Amino Acid Substitutions in Bacteriorhodopsin

FIG. 3. Three-piece ligation approach to cloning synthetic Duplexes I and II. The vector pSBO2 contains the whole synthetic bap gene (black arc) followed by a transcription terminator. pSBO2 was digested to completion with KpnI and, the linearized plasmid was purified. This was further digested with either BsrXI or ScaI to yield the fragments shown. Fragments A and B were purified and ligated with the synthetic Duplex I or II (Fig. 2) to reconstruct the synthetic gene.

FIG. 4. Predicted protein sequence of mutant bacterio-opsins produced in E. coli. The predicted protein sequences in the expression plasmids are shown. The sequences at the N and C termini and in helix F are shown. a, the amino acid sequence of the bacterio-opsin from H. halobium which has a pyroglutamate (<Glu) residue at position 1 and lacks the C-terminal aspartate residue specified by the gene. b, the amino acid sequence encoded by expression plasmids used to produce mutants of the synthetic gene. These two mutants encode, in addition to the intended mutations in helix F, an N-terminal fMet residue and glutamate in place of pyroglutamate. c, the predicted amino acid sequence of the expression plasmids containing mutations in the natural gene. These encode an N-terminal fMet residue in place of pyroglutamate and an additional C-terminal aspartate residue.
regeneration (Fig. 5, insets). In all cases, the affinity for retinal was sufficiently high so that no free retinal was seen in the spectra recorded after regeneration. The $\lambda_{\text{max}}$ of the resulting chromophores for the different bO mutants and the calculated peak widths are shown in Table I. Dark- and light-adapted $\lambda_{\text{max}}$ for each mutant fell within the bounds defined by the all-trans and 13-cis $\lambda_{\text{max}}$ (data not shown).

Neutralization of the Carboxyl Group of Glu-194: the Mutant E194Q—When regenerated with 13-cis-retinal, the mutant E194Q gave a chromophore of the same $\lambda_{\text{max}}$ and peak width as the wild type bO (Table I). However, with all-trans-retinal, the regenerated chromophore had a $\lambda_{\text{max}}$ of 545 nm, slightly blue-shifted relative to the $\lambda_{\text{max}}$ of 559 nm observed for the wild type protein.

Serine to Alanine Substitution—Neither of the two mutants in which serine was replaced by alanine (positions 183 and 193) showed marked changes in the properties of the regenerated chromophores. The mutant S183A displayed the same spectrum as the wild type after regeneration with both all-trans- and 13-cis-retinal. For S193A, the phenotype was similar to that seen for E194Q. Thus, when 13-cis-retinal was used, the $\lambda_{\text{max}}$ was the same as for the wild type; however, the spectrum observed with all-trans-retinal was slightly blue-shifted. The chromophore peak obtained in the latter case but not with 13-cis-retinal was notably broadened, suggesting the presence of more than one species.

Tyrosine 185 → Phenylalanine—Substitution of Tyr-185 by phenylalanine did not affect the chromophore when regen-

![Diagram](image-url)
Bacteriorhodopsin proteins were regenerated as described for Table I. At time zero, 1.5 equiv of all-trans- or 13-cis-retinal was added from a concentrated stock solution, and the increase in absorbance at 500 nm (W182F and P186L) or 550 nm (all others) was measured. Semilog plots such as shown in Fig. 6 were constructed from A_{max} and the initial slope. A half-time was calculated which reflects the early phase of chromophore regeneration.

Table II

<table>
<thead>
<tr>
<th>Mutant</th>
<th>All-trans-retinal t&lt;sub&gt;1/2&lt;/sub&gt;</th>
<th>13-cis-Retinal t&lt;sub&gt;1/2&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>91</td>
<td>74</td>
</tr>
<tr>
<td>W182F</td>
<td>320</td>
<td>230</td>
</tr>
<tr>
<td>S183A</td>
<td>102</td>
<td>102</td>
</tr>
<tr>
<td>V185F</td>
<td>220</td>
<td>60</td>
</tr>
<tr>
<td>P186L</td>
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<td>39</td>
</tr>
<tr>
<td>W189F</td>
<td>106</td>
<td>72</td>
</tr>
<tr>
<td>S193A</td>
<td>166</td>
<td>149</td>
</tr>
<tr>
<td>E194Q</td>
<td>125</td>
<td>48</td>
</tr>
</tbody>
</table>

Fig. 7. Light-dependent transmembrane proton pumping by bacteriorhodopsin mutants. The mutant proteins were regenerated as described under "Materials and Methods," and the Br concentration was adjusted to 3.3 μM with the regeneration buffer (using ε<sub>max</sub> = 52,000). Vesicles were made from 10 μl of these solutions by the dilution method as described under "Materials and Methods." Proton pumping assays were performed using a pH electrode after the pH had equilibrated at 6.9-7.0. Illumination was indicated as ON and terminated where marked OFF.

Calculation of initial rate for each mutant was performed to characterize the differences. In all the chromophore regenerations, an excess of retinal was used since under these conditions, the rate is independent of the retinal concentration (23). The kinetic plots obtained with different mutants are shown in Fig. 6. The regeneration of at least some of the mutants and the wild type involved at least two exponential processes. This was evident particularly for the regeneration of P186L with 13-cis-retinal. In view of this, we have calculated from the initial rate for each mutant a half-time of folding which reflects the kinetics of the first phase of regeneration. These data are recorded in Table II. For several mutants, the rate of regeneration with 13-cis-retinal was substantially higher than the rate with all-trans-retinal. This difference was largest for two mutants at adjacent residues, P186L and Y185F.

Proline 186 → Leucine—Replacement of Pro-186 by leucine led to a striking shift in the absorption maximum. With both all-trans- and 13-cis-retinal, the reconstituted mutant BO showed a chromophore with λ<sub>max</sub> at 470 nm. The absorption peak was somewhat broadened with both isomers (Fig. 5).

Tryptophan to Phenylalanine Substitution—Striking spectral shifts also resulted when Trp-182 and Trp-189 were substituted by phenylalanine. In the case of W182F, an extremely broad peak centered at 480 nm resulted with all-trans-retinal, whereas reconstitution with 13-cis-retinal gave a chromophore with a λ<sub>max</sub> of 500 nm. This difference in the spectra between the two isomers was of the opposite sense than seen for the wild type. This was also true for the W189F mutant, which gave a λ<sub>max</sub> of 524 nm with all-trans-retinal and a λ<sub>max</sub> of 531 nm with 13-cis-retinal. This mutant also gave broadened peaks, although this was not as pronounced as for W182F.

Rates of Chromophore Regeneration—The rate of regeneration varied with different mutants, and it also depended on the isomer of retinal being used. A series of measurements was performed to characterize the differences. In all the chromophore regenerations, an excess of retinal was used since under these conditions, the rate is independent of the retinal concentration (23). The kinetic plots obtained with different mutants are shown in Fig. 6. The regeneration of at least some of the mutants and the wild type involved at least two exponential processes. This was evident particularly for the regeneration of P186L with 13-cis-retinal. In view of this, we have calculated from the initial rate for each mutant a half-time of folding which reflects the kinetics of the first phase of regeneration. These data are recorded in Table II. For several mutants, the rate of regeneration with 13-cis-retinal was substantially higher than the rate with all-trans-retinal. This difference was largest for two mutants at adjacent residues, P186L and Y185F.

Proton Pumping by Mutants—The ability of the mutant proteins to pump protons across a bilayer in response to light was also investigated. Mutant bacteriorhodopsins were reconstituted into lipid vesicles by a dilution procedure (see "Materials and Methods"). Pumping was monitored using a pH electrode to give traces such as those shown in Fig. 7. The 90% response time of the system was severalfold faster than the rise time for proton uptake by the vesicles. At saturating light intensities, all but one of the mutants pumped protons to essentially the same extent and at the same initial rate as the wild type (Table III). The exception was P186L, which pumped at one-quarter the rate and to a steady-state value that was half that of the wild type (Table III and Fig. 7).

**DISCUSSION**

It has been shown earlier that, following denaturation, BO refolds correctly and binds retinal to regenerate the BR chromophore. Therefore, mutants of BR prepared by amino acid substitutions can be studied for their ability to fold to the correct three-dimensional structure and regenerate the chromophore. All mutants described in the present work regenerated a BR-like structure. Other mutants that do not regenerate a chromophore have also been obtained (25).

The preparation of large number of mutants relies heavily on the efficiency of the techniques for site-specific mutagenesis. Previously, we investigated, among other methods, oligonucleotide-directed mutagenesis in M13 subclones. This...
was unsuccessful with the *bop* gene (18). Subsequently, we used the restriction fragment or cassette replacement method (19) and found this to be by far the most efficient method. To facilitate this approach, we designed and carried out the total synthesis of a *bop* gene that contained uniformly spaced unique restriction fragments (7). The mutagenesis approach thus involves replacing a restriction fragment of the gene by a synthetic duplex containing altered codons. In the example described in the present work, two *ScaI*-BstXI fragments were synthesized and cloned.

To clone a mutagenic restriction fragment, it is generally sufficient to cut the vector with the appropriate restriction enzymes, purify the vector fragment, and ligate to it the newly synthesized fragment to reconstitute the gene. It is important that the vector fragment is uncontaminated by singly digested vector which would readily circularize to reform the wild type gene. It is difficult to separate the singly digested vector from the correct product if, as in the present work, they differ by only about 40 base pairs in a total of 3100 base pairs. Therefore, it is essential that the digestion of the vector with both enzymes goes to completion. This can be ensured by prior titration of the restriction enzymes. However, although a number of mutations in both the synthetic and native bacterio-opsin genes were made in this way, the efficiency of mutagenesis was low in some cases. This can be caused by contamination of commercial restriction enzyme preparations by exonucleases so that complete digestion of a vector does not result in the correct ends for ligation to the synthetic DNA. In addition, some restriction sites, while unique in the synthetic gene, may occur in the vector. It was for such situations that the three-component ligation strategy described for Fig. 3 was developed. This allowed partial digestion by the flanking restriction enzymes but ensured the complete separation of the desired restriction fragments from the partial digestion products. Thereby, the effect of inadvertent exonuclease digestion of the fragments is minimized. This approach was used in the cloning of the two additional mutants of the *bop* gene, Ser-183 → Ala and Trp-182 → Phe. The efficiency was extremely high, demonstrating the utility of this approach and reinforcing the value of cassette mutagenesis in the context of a synthetic gene.

Focusing on site-specific mutagenesis in putative helix F, we have herein described seven amino acid substitutions. In particular, the results bear on the nature of the interaction between the retinal and the opsin and on the mechanism of proton pumping. Binding of retinal as a protonated Schiff's base to the opsin results in a red shift of about 150 nm. The mutants S183A, Y185F, S193A, and E194Q, after regeneration in lipid/detergent micelles, all display a similar spectrum as the native bO. On the other hand, the substitution of the Trp-182 and Trp-189 by phenylalanine causes, in both cases, large spectral shifts. These results could indicate that the interaction of Trp-182 and Trp-189 with retinal is important in the opsin shift. A direct interaction of residues 189 and 182 with the β-ionone ring is possible, as indicated by their proximity to the site of cross-linking previously established using m-diazirinophenylretinal (8). We have also noticed that visual rhodopsins (26, 27) have a tryptophan residue in approximately the same position, relative to Lys-216, in their proposed secondary structure models.

The substitution of Pro-186 by leucine also caused a striking blue shift from the wild type spectrum. This appears to be caused by a perturbation of the tertiary structure, a conclusion also suggested by the diminished rate of proton pumping which was seen only for this mutant. In view of the known destabilization of α-helices in proteins by prolines (16), a structural change resulting from proline removal is quite likely. It was also striking that the removal of the proline residue reduced the rate of retinal binding specifically with the all-trans-isomer. The data may suggest a role of the proline residue in folding. The need for isomerization of the peptide bond at proline residues for the folding process has been established for a number of water-soluble proteins. We plan to carry out additional substitutions of Pro-186 (e.g., alanine and glycine). Studies of these mutations should allow some insights into the kinetics of folding of the protein.

The hypothesis has been advanced (9) that the opsin shift is caused in part by the proximity of a negative charge to the β-ionone ring of retinal. As described above, the carboxyl group of Glu-194 appeared to be a likely candidate for the above interaction. However, replacement of this glutamic acid residue by glutamine had no significant effect on the spectrum of the protein. Systematic individual substitutions of glutamic and aspartic acid residues are needed to obtain definitive data on the above postulate.

All the mutants studied retained the ability to pump protons in response to illumination when incorporated into phospholipid vesicles. Whereas there was scatter in the measurements, only one mutant, P186L, reproducibly gave a lower rate (~25% of wild type) and extent of proton pumping. Since mutants with wide spectral shifts were capable of translocating protons, it appears that bacteriorhodopsins can be activated by light over at least the wavelength range of 470–570 nm (Table I) covered by our mutants. This supports and complements data on bacteriorhodopsin reconstituted with retinal analogues, which showed that a normal chromophore is not necessary for proton pumping (28, 29).

Some proposals concerning proton translocation across the membrane become unlikely in view of our observations. It has been proposed that the amide nitrogen of a proline residue becomes transiently protonated and deprotonated during proton pumping (14). We can state that Pro-186, 1 of just 3 intramembrane prolines in the present model, does not play such a role in a unique proton pathway, although alternative pathways may exist. It has also been proposed that contiguous hydrogen-bonding amino acids provide a conduction pathway through the membrane. Such a "proton wire" passing down one face of helix F or, indeed, any unique proton wire involving the side chains of Tyr-185, Ser-183, Ser-193, or Glu-194 can be excluded. Spectroscopic evidence and protein modification data (30, 31) have strongly implicated tyrosinate protonation-deprotonation as playing a role in proton pumping by bR. The present data show that such an ionization either does not involve Tyr-185 or, if it does, that the ionization is not necessary for proton pumping.

The present approach can be used to test other mechanistic proposals concerning bacteriorhodopsin. It seems important to test comprehensively the possible role of 1 or more tyrosine residues. Therefore, all the tyrosine groups are being individually substituted by phenylalanine. Similarly, aspartic and glutamic acid residues are also being replaced by asparagine and glutamine residues, respectively. Aspartic and glutamic acids are the only residues proven to be critical in the F_{o} component of proton-translocating ATPases and in the proton/lactose symport carried out by lactose permease of *E. coli* (32, 33). Experimental evidence has also been provided that 3 or more aspartic acid residues undergo protonation-deprotonation during the bR photocycle (34).

Finally, it should be emphasized that the large number of mutants being generated should provide a variety of opportunities for biochemical and biophysical studies of bacteriorhodopsin: Fourier transform IR, resonance Raman, low tem-
temperature ultraviolet difference, and flash kinetic spectroscopy studies are underway.

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