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Bacteriorhodopsin mutants containing single tyrosine to phenylalanine substitutions are all active in proton translocation*

(synthetic gene/site-specific mutagenesis/restriction fragment replacement/membrane protein reconstitution/liposomes)

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ABSTRACT **To study the possible role of the tyrosine residues in proton translocation by bacteriorhodopsin, we have replaced these residues individually by phenylalanine. The required codon changes were introduced in the bacterioopsin gene by replacement of appropriate restriction fragments by synthetic counterparts containing the desired nucleotide changes. The denatured opsin polypeptides obtained by expression of the mutant genes in *Escherichia coli* were purified and treated with a mixture of detergents, phospholipids, and retinal in a previously established renaturation procedure. All of the mutant proteins folded to regenerate bacteriorhodopsin-like chromophores. Three mutants with tyrosine to phenylalanine substitutions at positions 57, 83, and 185 regenerated the chromophore more slowly than the wild-type protein, and two of these mutants, Phe-57 and -83, showed slightly blue-shifted chromophores. When reconstituted into liposomes all of the mutant proteins with single Tyr → Phe substitutions pumped protons at rates and levels comparable to those of the wild-type bacteriorhodopsin. We conclude that single substitutions of tyrosine by phenylalanine do not affect folding, retinal binding, or light-dependent proton pumping in bacteriorhodopsin.**

Bacteriorhodopsin (bR), an integral membrane protein in *Halobacterium halobium*, carries out light-dependent proton translocation from the inside to the outside of the cell. The electrochemical gradient thus generated is used by the cell for ATP synthesis and other cellular processes. Elucidation of the mechanism of this light-driven proton pump is of fundamental interest from chemical and biological points of view. In our approach to the study of the mechanism, we are carrying out specific amino acid substitutions in the molecule by the techniques of recombinant DNA. The intended codon alterations are carried out in the bacterioopsin (bO) gene by replacement of short restriction fragments with synthetic counterparts that contain the required nucleotide changes (1, 2). To facilitate such mutagenesis in all parts of the bO gene, we have designed, synthesized, and cloned a bO gene that contains suitably spaced unique restriction sites (3). Systems for the expression of the mutant bO genes in *Escherichia coli* and isolation and purification of the expressed polypeptides have also been developed (4, 5). Finally, although the proteins thus obtained are in the denatured state, they renature in mixed detergent/phospholipid micelles to regenerate the bR-like chromophores. They can be reconstituted into liposomes for proton-pumping studies and, thus, the effects of the mutations on the functional properties of bR can be studied.

Using the above methods, we have prepared and studied a set of bO mutants that contain single amino acid substitutions in the putative helix F in the structure model shown in Fig. 1 (2). This region of the protein was chosen for mutagenesis to test a number of current proposals regarding bR structure and function. The mutants that we made showed interesting properties and, in addition, the mutant Pro-186 → Leu showed much reduced proton pumping (1).

As a next step in mutagenesis studies, we have studied mutants in which each tyrosine residue in bR has been replaced by a phenylalanine residue. The phenolic hydroxyl group in tyrosine is a good candidate for participation in a proton conductance pathway along with aspartic, glutamic, and other hydrogen bond-forming amino acids. Indeed, models have been proposed that involve specific tyrosine residues in hydrogen-bonded proton conductance pathways (6, 7). Further, protonation and deprotonation of at least two tyrosine residues during the photocycle have been suggested from Fourier-transform infrared spectroscopy (8, 9) and kinetic and low-temperature UV spectroscopy (10–12). Chemical modification studies also implicated Tyr-26 and Tyr-64 in proton-pumping mechanism (13, 14), although these conclusions were at variance with later work (15).

We have now individually replaced every one of the 11 tyrosine residues in bR, and the present paper reports the results obtained following these substitutions. All of the mutant proteins, when submitted to a standard renaturation procedure, folded and bound retinal to form a bR-like chromophore. A few mutants showed minor shifts in λ_{max}, but all of them pumped protons in proteoliposomes at rates that were similar to that of the native bR. We conclude that no single tyrosine in bR is obligatory for the proton translocation process.

MATERIALS AND METHODS

Materials. *Plasmids.* The plasmid pSBO2, which carries the synthetic bO gene, and the expression plasmid pPL1 have been described (3). The expression vector pPL2 was constructed by modification of pPL1 to stabilize the λP_L promoter. It contains a small *Pvu* I–*Hin*dIII segment of pPL1, which carries the λP_L promoter and a ribosome-binding site, and a large *Pvu* I–*Hin*dIII segment of pSP64TM (3), which carries a multicloning site, a transcription terminator, and a replication origin from a multicopy pUC plasmid. In the λP_L vector system, the mutant bO genes are under control of the λP_L promoter and a temperature-sensitive repressor, a product of *c*I857 gene. The T7 vector system (16) was modified to give the expression vector pT7-BO4, in which the bO gene is

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Abbreviations: bR, bacteriorhodopsin; bO, bacterioopsin; [Myr_2]PtdCho, L-α-dimyristoyl phosphatidylcholine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate. All of the tyrosine to phenylalanine substitutions are shown by Tyr-number → Phe.

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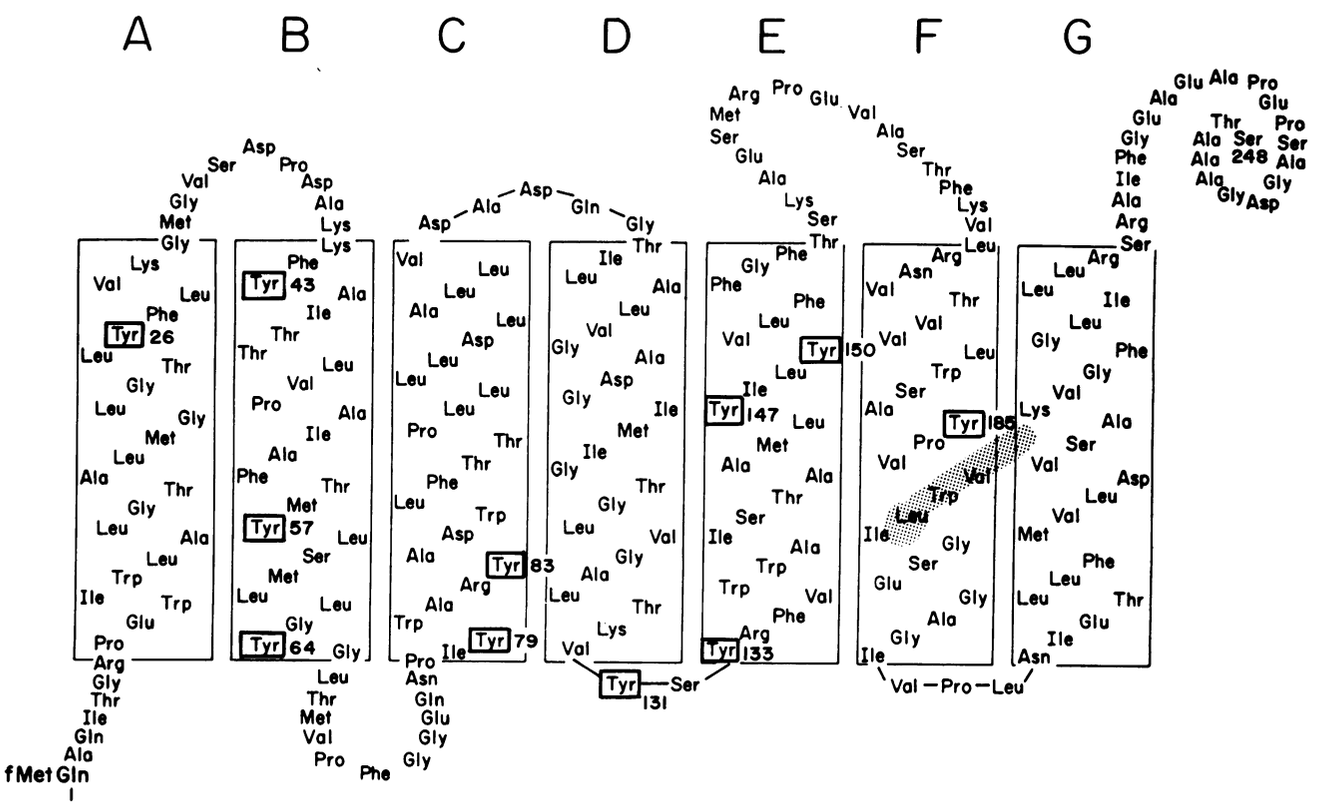

FIG. 1. Secondary structure model for bO. The amino acid sequence of bO encoded by the synthetic gene contains formylmethionine (−1) followed by glutamine (1) at the N terminus. The N-terminal amino acid residue in bO from purple membrane is pyroglutamic acid. Eleven tyrosine residues are boxed.

under the control of the T7 class III promoter and T7 RNA polymerase (S. Karnik and H.G.K., unpublished work).

Synthesis and characterization of single-stranded polynucleotides. All single-stranded polynucleotides required for preparing restriction fragments with altered codons (Fig. 2) and primers for sequencing purposes were synthesized by using an Applied Biosystems 380A DNA synthesizer.

Polynucleotides were purified by gel electrophoresis on denaturing polyacrylamide gels (10–18% acrylamide), labeled at their 5′ ends with [γ-^{32}P]ATP (10 Ci/mmol; 1 Ci = 37 GBq) and T4 polynucleotide kinase, and characterized by 5′-end nucleotide analysis (3).

Construction and cloning of the mutant bO genes containing specific Tyr → Phe codon alterations. For mutants containing single Tyr → Phe substitutions in the bO gene, the appropriate synthetic restriction fragments containing the mutations (Fig. 2) were introduced into the synthetic gene carried by the plasmid pSBO2. The synthetic single-stranded polynucleotides were annealed in appropriate combinations to form 11 double-stranded restriction fragments shown in Fig. 2. To reduce parental background in the transformation, three-fragment ligations were generally used as described (1). For example, a small *Pvu* I–*Bam*HI fragment [0.65 kilobase (kb)] and a large *Pvu* I–*Bsm* I fragment (2.4 kb), both of which are easily separated from the covalently closed circular DNA or the linear DNA (3.1 kb), were isolated separately and used for cloning of the much smaller *Bam*HI–*Bsm* I fragment carrying the codon change.

The synthetic duplex (0.2–1.0 pmol) and vector fragments (0.2 pmol each) were ligated by using T4 ligase at 4°C overnight. $CaCl_2$-treated *E. coli* DH1 cells were used for transformation and the appropriate regions of the plasmid DNA from ampicillin-resistant clones were sequenced.

Sequence Analysis. The sections of the mutant bO genes containing the inserted fragment replacements with the codon changes (Fig. 2) were sequenced. Direct plasmid sequencing was done by the dideoxy method (17, 18) using sequencing primers for the synthetic gene. Plasmid DNA was isolated by the alkaline extraction method (19), extracted with phenol, treated with RNase A, and purified by electrophoresis on a low-temperature-melting agarose (0.7%) (SeaPlaque, FMC, Rockland, ME). The mutants were sequenced as above, whereas the mutant Tyr-131→ Phe was sequenced by the dideoxy method after subcloning into M13mp11 and the mutants Tyr-147 → Phe and Tyr-150 → Phe were sequenced by the Maxam–Gilbert method (20).

Expression of Mutant Genes. The mutant genes were cloned into one of the expression plasmids, pPL1, pPL2, or pT7-BO4. The *Hin*dIII–*Eco*RI fragments that carried the entire gene and the terminator region (Fig. 2) were cloned into the corresponding sites of the P_L vector, and the ligation mixture was used to transform *E. coli* DH1/pcI857 (3). The transformed cells were induced for 20 min by shifting growth temperature from 30°C to 42°C. Similarly, the *Aat* II–*Sph* I gene fragment in pT7-BO4 was replaced by the corresponding fragment in pSBO2 that contained the altered codon. The resulting recombinant was introduced into *E. coli* W3110 *lacI*^{q} harboring a helper plasmid, pACT7. The expression of the bO mutant in the pT7-BO plasmid was induced at 37°C by addition of 1 mM isopropyl β-D-thiogalactoside at time 0, rifampicin (0.2 mg/ml) was added at 15 min, and expression was continued for 45 min.

Purification of bO Proteins. The bO mutant proteins produced in *E. coli* were purified by a two-step procedure as described (5).

Renaturation of the bO Mutants and Regeneration of the bR-Like Chromophore. The procedure for the regeneration of chromophore from bO using detergent/phospholipid mixed micelles has been described (5). The bR-like chromophores were regenerated using 0.4 mg of bO per ml with 2-fold excess of all-*trans*-retinal in 1% L-α-dimyristoyl phosphatidylcholine ([Myr_2]PtdCho)/1% 3-[(3-cholamidopropyl)dimethylam-

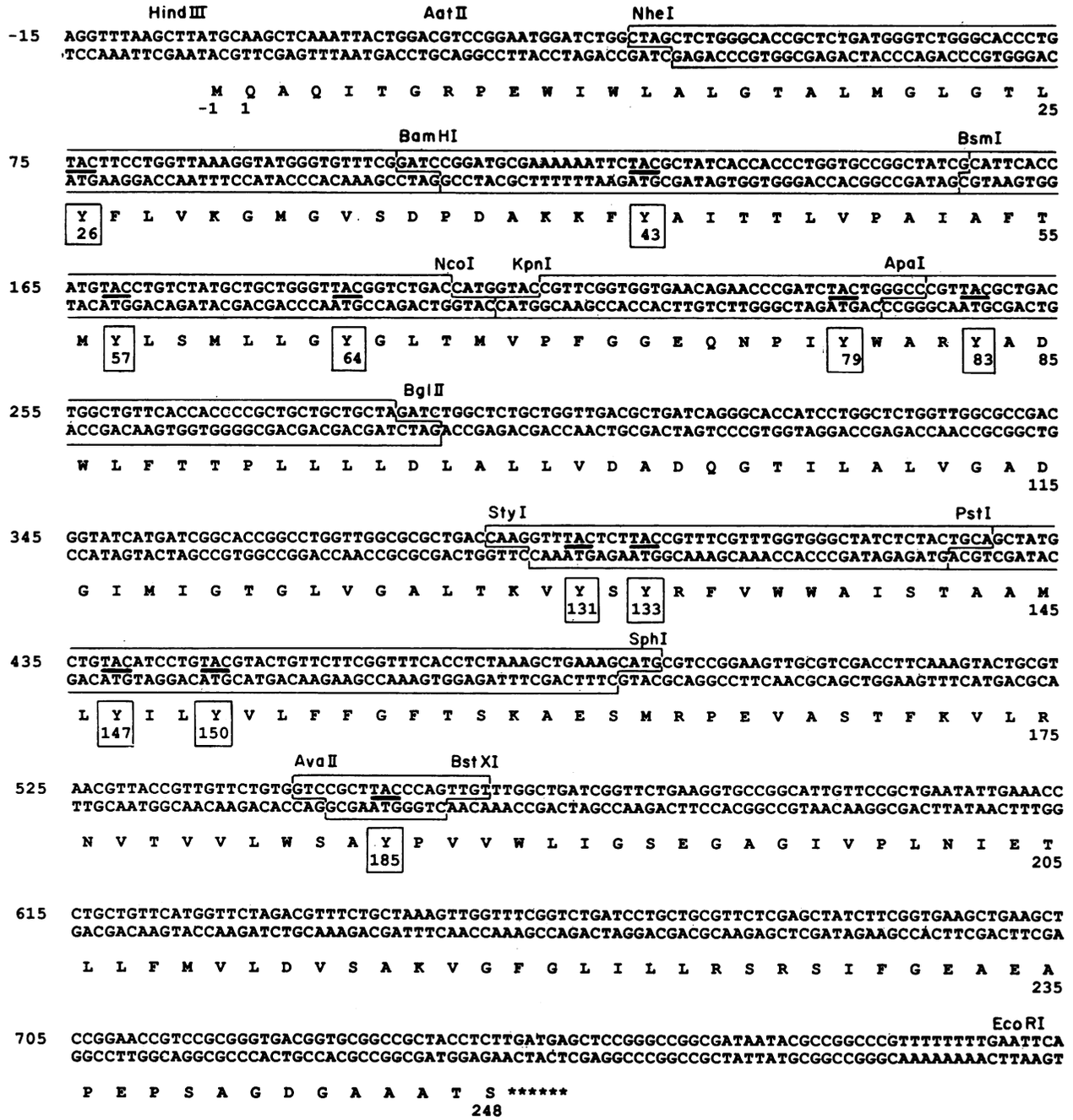

FIG. 2. Nucleotide sequence of the synthetic bO gene indicating the restriction sites and fragments used for Tyr → Phe substitutions. The numbering of nucleotides starts at the N-terminal glutamine codon of bO, whereas the numbering of amino acids starts at formylmethionine (−1). The locations of the restriction fragments that were replaced by synthetic counterparts containing single TAC → TTC codon changes are shown by boxes. Positions of tyrosine residues are numbered and boxed and the codons changed are underlined.

monio]-1-propanesulfonate (CHAPS)/0.2% $NaDodSO_4$/50 mM sodium phosphate, pH 6.0, in the dark at 20°C.

Proton-Pumping Assay. The mutant bR proteins with the regenerated chromophores were reconstituted into asolectin liposomes by the octylglucoside dilution method (21) with modifications as described (5). Typically, 10 μl of 10 μM bR solution was mixed with asolectin and octylglucoside at final concentrations of 1.36% (wt/vol) and 1.25% (wt/wt), respectively, and then diluted with 960 μl of 2 M NaCl/0.15 M KCl to form liposomes. Proton pumping was measured at 30°C under saturating illumination with a 430-nm long-pass filter. Light-dependent alkalinization of the medium was monitored by a pH electrode as described (5). Average values from at least six independently reconstituted vesicle preparations were recorded.

RESULTS

Synthesis, Cloning, and Characterization of the bO Genes Containing Single Tyr (TAC) → Phe (TTC) Substitutions. Single substitutions of the 11 tyrosine residues in bR by phenylalanine were carried out by replacements of small restriction fragments in the synthetic bO gene with synthetic counterparts containing the codon change TAC → TTC (Fig. 2; Table 1). The replacements were introduced by the three-component ligation procedure (*Materials and Methods*). The parental background was very low, usually <10%, as shown by sequencing of the regions corresponding to the inserted segments.

For expression of the mutant genes in *E. coli*, they were excised from pSBO2 and cloned into the expression vectors

Table 1. Characterization of restriction fragments in the bO gene containing phenylalanine in place of the tyrosine codon

Tyrosine replaced (no.)	Restriction fragment (nucleotide position)	Sequence confirmed
26	*Nhe* I–*Bam*HI (37–105)	18–197
43	*Bam*HI–*Bsm* I (106–157)	20–220
57	*Bsm* I–*Nco* I (158–200)	108–294
64	*Bsm* I–*Nco* I (158–200)	108–294
79	*Kpn* I–*Apa* I (209–243)	201–315
83	*Apa* I–*Bgl* II (244–285)	200–370
131	*Sty* I–*Pst* I (384–429)	375–444
133	*Sty* I–*Pst* I (384–429)	310–475
147	*Pst* I–*Sph* I (430–489)	381–493
150	*Pst* I–*Sph* I (430–489)	381–493
185	*Ava* II–*Bst*XI (546–563)	475–673

described in *Materials and Methods*. Mutant bO proteins were purified from *E. coli* crude membranes and were homogeneous, as judged by $NaDodSO_4$/polyacrylamide gel electrophoresis.

Characteristics of the Regenerated Chromophores in Detergent/Phospholipid Micelles. All of the bO mutants thus prepared regenerated bR-like chromophores in [Myr_2]PtdCho/CHAPS/$NaDodSO_4$ mixed micelles with all-*trans*-retinal. The $t_{1/2}$ for chromophore regeneration and the spectral characteristics observed are shown in Table 2. As is seen, substitutions at positions 57 (helix B) and 83 (helix C) (Fig. 1) caused small (about 15 nm) blue shifts of the absorption maximum as compared with the wild-type bO. Blue shifts in the spectra, large and small, have been observed with several mutants in the putative helix F (1). On light adaptation, all of the tyrosine mutants showed similar red shifts of about 10 nm, indicating that they may have similar photocycles. The substitution Tyr-57 → Phe dramatically decreased the rate of chromophore regeneration. Decreases in this rate were also observed for substitutions at positions 83 and 185 (Table 2). The slower rates of regeneration observed with the substitutions at positions 57, 83, and 185 did not appear to be due to changes in the affinity of the apoproteins for retinal. Thus, analysis by Michaelis–Menton plots showed that in all cases retinal had a similar binding constant (about 11 μM).

As reported, a decrease in the rate of Tyr-185 → Phe regeneration by a factor of 6 was observed with all-*trans*-retinal but not with 13-*cis*-retinal (1). However, in the present study, no significant difference was observed in the rates of chromophore regeneration for any of the other mutants using the two retinal isomers.

Table 2. Regeneration of chromophores and spectral characteristics of Tyr → Phe mutants of bR

Tyrosine replaced (no.)	Chromophore regeneration: $t_{1/2}$, min	Absorption maximum, nm	
		Dark-adapted	Light-adapted
bR	0.6	550	560
26	0.8	551	561
43	1.3	551	561
57	22.0	536	548
64	0.6	551	561
79	1.5	547	556
83	2.4	533	540
131	1.0	551	561
133	1.5	546	560
147	1.5	551	561
150	1.0	548	560
185	11.3	556	573

The $t_{1/2}$ of chromophore regeneration was calculated from the increase in absorbance at 536 nm for Tyr-57 → Phe, at 533 nm for Tyr-83 → Phe, and at 550 nm for all other mutants. Absorption maxima of the dark-adapted forms of the bR mutants were measured after storage overnight following chromophore regeneration. The spectra of the light-adapted forms were obtained following illumination for 10 min on ice using a 495-nm long-pass filter.

Table 3. Proton-pumping activity of Tyr → Phe mutants in bR

bR mutant (tyrosine no.)	Initial rate, H^+ per bR per sec	Steady state, H^+ per bR
bR	2.8	29
26	2.3	35
43	2.5	29
57	2.2	29
64	3.0	31
79	3.1	32
83	1.8	25
131	2.1	19
133	2.2	33
147	2.5	30
150	2.5	19
185	1.0	15

Proton-Pumping Activity of the bR Mutants. After chromophore regeneration, the substitution mutants in detergent/lipid mixed micelles were reconstituted into asolectin liposomes by the octylglucoside dilution method. Proteoliposomes prepared from all of the Tyr → Phe mutants uniformly showed proton pumping into the vesicles as in the case for wild-type bR. As shown in Table 3, all mutants showed initial rates and extents of proton pumping that were similar to those of the wild-type bR.

DISCUSSION

There are 11 tyrosine residues in bR, the majority of which are probably buried within the bilayer. To examine the possible role of the tyrosine hydroxyl groups in bR function, we have replaced individually every one of the 11 tyrosine residues by phenylalanine. The mutant Tyr-185 → Phe has been described previously (1). As before, all of the present substitutions were carried out by cassette mutagenesis in the synthetic bO gene (Fig. 2). The synthetic restriction fragments containing the altered codons all required the synthesis of only two single-stranded polynucleotides and the mutant genes were prepared by the three-fragment ligation method (1). The parental background among transformants was low, generally <10%. Confirmation of the desired mutations was achieved by sequencing the inserted synthetic fragments and the flanking regions. On the average, about 150-nucleotide-long regions were sequenced (Table 1).

All of the Tyr → Phe bO mutants were purified in a denatured state. They all regenerated bR-like chromophores under the standard conditions. The rates of regeneration were comparable to that of the wild-type bO except for the mutants Tyr-57 → Phe, Tyr-83 → Phe, and Tyr-185 → Phe, which regenerated more slowly. It is possible that the substitution of these amino acids in bR causes destabilization of the tertiary structure. In fact, the mutant Tyr-57 → Phe is unique in being quite unstable in octylglucoside.

Most important, all of the mutants showed proton pumping, comparable to that of the wild-type bR. This shows that none of the tyrosine hydroxyl groups in the tyrosine residues plays a critical role in proton translocation. Previously, models have been proposed in which proton translocation occurs along hydrogen-bonded pathways (6, 7), which include multiple tyrosine residues. Protonation–deprotonation of tyrosine residues, which has been identified by Fourier-transform infrared (8, 9) and by UV difference spectroscopy (11, 12), has been viewed as a support for the above proton translocation models.

It has also been proposed that a tyrosine or tyrosinate ion may stabilize the retinal protonated Schiff base (22) or may be required to deprotonate the Schiff base during the photointermediate M formation and decay (23, 24). Our present results show that if proton translocation occurs by proton conductance along amino acid side chains, then tyrosine groups are not obligatory participants. The following two qualifications may however be made. (*i*) Multiple pathways may exist for proton translocation and these may allow bypass of the mutated tyrosines. This possibility however does not seem to be very likely but it can be tested by multiple mutagenesis. (*ii*) Different steps in the process of proton translocation have different rates. The replacement of a tyrosine residue by a phenylalanine group may slow down a faster step, but this slowing down may not be detected because of a subsequent slow rate-limiting step. Therefore, it is important to include single proton release and uptake studies in our proton translocation assay.

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