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Biochemistry

Specific amino acid substitutions in bacterioopsin: Replacement of a restriction fragment in the structural gene by synthetic DNA fragments containing altered codons

(purple membrane/proton pump/oligonucleotide purification/site-specific mutagenesis/T4 DNA ligase)

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ABSTRACT **To study the mechanism of light-dependent proton translocation by bacteriorhodopsin, we have introduced single-codon changes in the gene so as to produce the following specific amino acid substitutions in the protein: Tyr-185 to Phe, Pro-186 to Leu, Trp-189 to Phe, Ser-193 to Ala, and Glu-194 to Gln. The strategy involved replacement of a 62-base-pair restriction fragment by synthetic DNA duplexes containing the modified nucleotide sequences. This required a unique restriction site (*Xho* I) at Ile-203 which was created by oligonucleotide-directed point mutagenesis. The six DNA duplexes corresponding to the modified native and mutant restriction fragments were all prepared by DNA ligase-catalyzed joining of chemically synthesized deoxyribooligonucleotides. The bacterioopsin expression plasmids reconstructed by using the synthetic DNA fragments were characterized by restriction analysis and DNA sequence determination. An extremely rapid, efficient, and general method for purification of the synthetic oligonucleotides and of DNA fragments was developed.**

The purple membrane in *Halobacterium halobium* carries out light-dependent proton translocation (1). Bacteriorhodopsin (BR) is the only protein present in this membrane, and it contains one molecule of retinal as chromophore linked to Lys-216 as a Schiff base (2). The amino acid sequence of the protein has been determined by both protein (3, 4) and gene analysis (5), and a secondary structure model has been proposed (6) that attempts to correlate the sequence with electron (7) and neutron diffraction (8), proteolysis (9), and crosslinking data (10).

Proton translocation is important in a variety of biological processes, and BR offers a well-defined model for its study. It seems plausible that proton translocation by this protein is mediated by the functional groups present in certain amino acids. Therefore, one general approach to the study of the mechanism is that of specific amino acid replacements. This is now feasible by the techniques of recombinant DNA. With this aim, this laboratory recently has identified the bacterioopsin (BO) gene in *H. halobium* (5) and cloned it into a set of expression vectors (11), investigating different methods for specific codon alterations in the BO gene (11, 12). In this paper we report a number of codon changes in the BO gene effected by replacement of a restriction fragment with synthetic DNA. The changes cause amino acid substitutions in the presumed helix 6, which was chosen because of its probable interaction with the chromophore (Fig. 1).

We synthesized six DNA duplexes encoding amino acids 182–203. One duplex corresponds to the native sequence, while five duplexes contain the single-amino-acid changes indicated in Fig. 1. The synthetic DNA duplexes were inserted into the gene, replacing a 62-base-pair (bp) restriction fragment, and the cloned mutant BO genes were characterized.

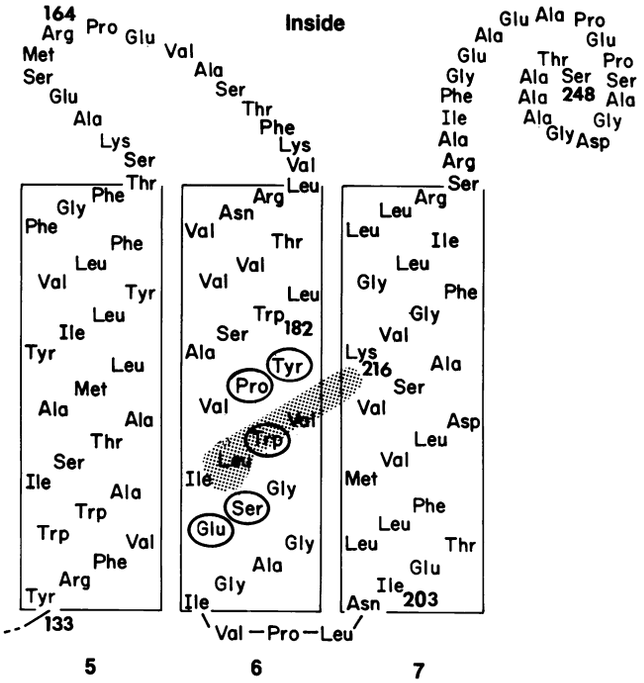

FIG. 1. A secondary structure model for amino acids 133–248 of BR. The vertical boxes show the α-helical segments (5, 6, and 7) that are embedded in the bilayer. The shaded area represents the approximate orientation of retinal linked to the ε-amino group of Lys-216. The five amino acids mutated in the present work are circled.

Finally, we describe an extremely rapid and general method for the purification of synthetic oligonucleotides and DNA duplexes.

MATERIALS AND METHODS

Materials. Polynucleotide ligase was purified (13) from *Escherichia coli* R594 lysogenized with the phage NM989 λT4 lig kindly provided by N. E. Murray (14). Other enzymes and chemicals were from commercial sources. Sep-Pak C_{18} cartridges (Sep-Pak) were purchased from Waters Associates. Elutip-d columns were from Schleicher & Schuell. Triethylammonium bicarbonate ($NHEt_3HCO_3$) (1 M) was prepared as described (15).

Oligonucleotide Synthesis. The protected deoxyribonucleosides were prepared as described (16, 17). All oligonucleotides were synthesized by the solid-phase "phosphite tries-

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Abbreviations: BO, bacterioopsin; BR, bacteriorhodopsin; bp, base pair(s); $NHEt_3HCO_3$, triethylammonium bicarbonate; $(MeO)_2Tr$, dimethoxytrityl.

ter" method (18) using 10 molar equivalents of fully protected 2'-deoxyribonucleoside-3'-*N*,*N*-dimethylamino or *N*-morpholinophosphoramidites (19, 20) for each condensation. After completion of synthesis, the methoxy and the *N*-protecting groups were removed (18), ammonia was evaporated in the presence of 1 M $NHEt_3HCO_3$ (0.5 ml), and the crude dimethoxytrityl [$(MeO)_2Tr$]-oligonucleotide(s) was finally dissolved in 10 ml of 25 mM $NHEt_3HCO_3$.

Purification of 5'-*O*-$(MeO)_2$Tr-Oligonucleotides from Failed Sequences on Sep-Pak. The Sep-Pak was connected to a 10-ml syringe and before use was washed successively with 10 ml of acetonitrile, 5 ml of 30% acetonitrile in 100 mM $NHEt_3HCO_3$, and finally, 10 ml of 25 mM $NHEt_3HCO_3$. The $NHEt_3HCO_3$ solution of crude $(MeO)_2$Tr-oligonucleotide(s) was passed through the Sep-Pak (in about 20 sec). (The total capacity of a Sep-Pak is about 100 A_{260} units; if the solution contains >50 A_{260} units, a repeat passage may be necessary.) The failed sequences containing a terminal 5'-hydroxyl group were eluted with 10–15 ml of 10% acetonitrile in 25 mM $NHEt_3HCO_3$, and the $(MeO)_2$Tr-oligonucleotide(s) was eluted with 5 ml of 30% acetonitrile in 100 mM $NHEt_3HCO_3$. Aliquots of the 10% acetonitrile eluate (containing about 1 A_{260} unit) and of the 30% acetonitrile eluate (about 0.1 A_{260} unit) were tested for the $(MeO)_2$Tr group (16). While the latter solution gave a positive color test, the 10% acetonitrile eluate was negative.

Further Purification of Synthetic Oligonucleotides by Gel Electrophoresis. After treatment with 80% acetic acid (18), the fully deprotected product was further purified by electrophoresis on a preparative 20% polyacrylamide gel run under denaturing conditions. Gel containing the desired band was excised, crushed, and extracted with 1 M $NHEt_3HCO_3$. The gel extract was diluted 1:5 with water, filtered, and passed through Sep-Pak. The urea and salts were removed by washing with 10 ml of 25 mM $NHEt_3HCO_3$. The oligonucleotide was eluted with 3 ml of 30% acetonitrile in 100 mM $NHEt_3HCO_3$, and the solution was lyophilized.

5'-^{32}P-Phosphorylation of Oligonucleotide, Purification, and Characterization. 5'-Phosphorylation of the oligonucleotides with T4 polynucleotide kinase was carried out as described (21). The reaction mixture was passed through Sep-Pak, the P_i and ATP were eluted with 10 ml of 5% acetonitrile in 25 mM $NHEt_3HCO_3$, and the 5'-phosphorylated oligonucleotide was then eluted with 2 ml of 30% acetonitrile in 100 mM $NHEt_3HCO_3$. The purity of each ^{32}P-labeled oligonucleotide was checked by gel electrophoresis and by sequence analysis [two-dimensional homochromatography (15, 22)].

DNA Ligase-Catalyzed Joining of Oligonucleotides to Form DNA Duplexes. All oligonucleotides, except the two that contain the external 5' termini of the duplex, were preparatively phosphorylated (21) at the 5' ends to a specific activity of 4 Ci/mmol (1 Ci = 37 GBq), and the products were purified by using Sep-Pak. T4 DNA ligase-catalyzed joining of oligonucleotides was performed as described (21). The products were purified on a 12% polyacrylamide gel run under denaturing conditions, recovered from gel extract, and purified by Sep-Pak.

Characterization of Synthetic DNA Duplexes. Each synthetic duplex was characterized by its electrophoretic mobility on gels and by 5'-end-group determination after complete digestion with P1 nuclease (15). The accuracy of joining reactions was confirmed by nearest-neighbor analysis (21).

Oligonucleotide-Directed Point Mutagenesis. This was performed in the BO expression plasmid pLBB (Fig. 2*A*), the preparation of which has been described (5). A published mutagenesis procedure (23) was modified as follows. Plasmid DNA (30 μg) was nicked by 150 units of *Sph* I endonuclease in the presence of 20 μg of ethidium bromide in a 0.37-ml vol for 90 min at room temperature. The DNA was recovered and digested with 25 units of exonuclease III in 40 μl for 15 min at room temperature. Gapped DNA was mixed with a 15-fold molar excess of the mutagenic oligonucleotide (Fig. 2*B*), which had been quantitatively 5'-phosphorylated by using T4 polynucleotide kinase. The mixture (40 μl) was incubated at 14°C for 16 hr in the presence of 4 units of the large fragment of DNA polymerase I, 60 units of T4 DNA ligase, and 0.5 mM each of the four dNTPs and ATP. The progress of these *in vitro* reactions was monitored by running aliquots on 1% agarose gels containing ethidium bromide (0.6 μg/ml). The DNA produced was used to transform *E. coli* strain HB101 by a high-efficiency procedure (24). Amplified colony replicas were prepared (25) and probed with the same oligonucleotide used for mutagenesis (26). Autoradiography of the filters after washing at sequentially higher temperatures allowed colonies containing the mutant plasmids to be identified.

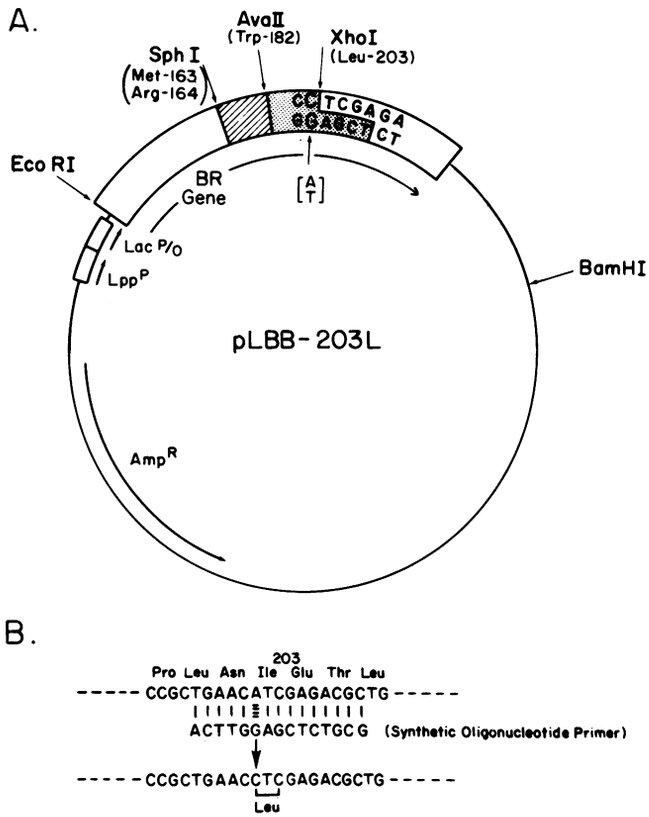

FIG. 2. (*A*) The BO expression plasmid pLBB-203L and the strategy for codon alterations by chemical synthesis. A unique *Xho* I site was introduced at Ile-203 by oligonucleotide-directed mutagenesis as shown in *B*. The *Sph* I–*Ava* II fragment was isolated as described. *Ava* II–*Xho* I fragment was replaced by synthetic counterparts (see Fig. 3) containing modified nucleotide sequences. The plasmid was reconstructed from the large *Sph* I–*Xho* I vector fragment, *Sph* I–*Ava* II fragment, and the synthetic duplex. (*B*) Oligonucleotide-directed mutagenesis to create a unique *Xho* I site. The top DNA sequence is the sense strand of the parental plasmid pLBB, the middle sequence is the mutagenic primer with one base mismatch, and the bottom sequence is the sense strand of the mutant plasmid pLBB-203L. The *Xho* I recognition site is C-T-C-G-A-G.

Restriction Fragments from the BO Expression Plasmid. *The* Sph *I*–Ava *II fragment.* This fragment is shown in Fig. 2*A*. The plasmid pLBB (1 mg) was double-digested with *Sph* I (300 units) and *Bam*HI (500 units) in 1 ml for 5 hr. Separation on low-melting-point agarose gel yielded 83 μg of the *Sph* I–*Bam*HI fragment. This fragment (20 μg) was further cut with *Ava* II (10 units) in 150 μl for 4 hr. A portion of the *Ava* II digest was 5'-^{32}P-phosphorylated with T4 polynucleotide kinase. The labeled material was mixed with the remain-

der and run on an 8% polyacrylamide gel under native conditions. The desired band was extracted with 1 M $NHEt_3HCO_3$, and the DNA was precipitated with ethanol twice.

The large Sph *I–*Xho *I vector fragment.* This fragment (shown in Fig. 2*A*) was obtained by digestion of pLBB-203L (400 μg) with *Sph* I/*Xho* I (200 units each) in 500 μl for 5 hr. Isolation was by electrophoresis on low-melting-point agarose gel.

Reconstruction of the Plasmid and Transformation of *E. coli*. The large *Sph* I–*Xho* I vector fragment (0.06 pmol), the *Sph* I–*Ava* II fragment (0.3 pmol), and the synthetic duplex (0.6 pmol) with 5′-hydroxyl termini were incubated with 2 units of T4 DNA ligase in 10 μl of standard ligation buffer at 15°C overnight. After inactivation of the ligase, the DNA was digested with *Xho* I (1 unit) at 37°C for 1 hr. Aliquots of the incubation mixture were used to transform HB101 cells (27). Colonies were picked, and DNA was prepared by the alkaline lysis method (28). The DNA was further purified by Elutip-d columns before restriction analysis. Colonies that yielded DNA that was cut by *Sph* I but not by *Xho* I were selected.

Subcloning of the *Eco*RI–*Bam*HI Fragment into M13mp8. The *Eco*RI–*Bam*HI fragment (shown in Fig. 2*A*) containing the BO gene from the selected colonies was subcloned into M13mp8. JM103 cells were made competent with calcium chloride (27) before transfection. White plaques were picked from minimal plates, and single-stranded DNA was prepared. The correct insertion of the synthetic duplex was confirmed by sequence determination through the *Sph* I and *Xho* I (see below) sites.

DNA Sequence Determination. The sequence of phage DNA was determined by the dideoxy method (29) using as a primer the synthetic oligonucleotide complementary to amino acids 209–215 of the BO gene.

RESULTS

Synthetic Strategy. To limit the amount of DNA synthesis, a new *Xho* I site was planned at Ile-203. This, used in conjunction with the unique *Sph* I site at Arg-164, would give a fragment with a single *Ava* II site at *Trp*-182 (Fig. 2*A*). Thus, the total strategy (Fig. 2) involved: (*i*) oligonucleotide-directed mutagenesis to introduce the required A-to-C base change to create a unique *Xho* I site; (*ii*) preparation of the large *Sph* I–*Xho* I vector fragment from the mutated plasmid; (*iii*) preparation of the short *Sph* I–*Ava* II fragment; (*iv*) synthesis of native and mutant DNA duplexes corresponding to the *Ava* II–*Xho* I restriction fragment (Fig. 3); and (*v*) ligation of the three fragments to reform the BO gene.

The plan for the synthesis of one DNA duplex (Duplex I) containing the native amino acid sequence and five mutant DNA duplexes (Duplex II–VI) is shown in Fig. 3. The oligonucleotides synthesized had mostly overlaps of four nucleotides at each cohesive end (21). Four silent changes to A/T were introduced in the DNA duplexes to decrease the G/C content of the oligonucleotides to be synthesized. Further, in the synthetic duplexes, the last base pair before the A-G-C-T cohesive end is A·T, as in the native sequence. This end is designated *Xho* I′ because it will ligate with an *Xho* I cohesive end but will not regenerate the *Xho* I site. Upon reconstruction with the synthetic duplex and the *Sph* I–*Xho* I vector fragment, the Ile-to-Leu change at position 203 created by point mutagenesis thus would be reversed.

Oligonucleotide-Directed Point Mutagenesis to Produce an *Xho* I Site. The BO expression plasmid, pLBB, was nicked with *Sph* I endonuclease at a unique site 120 nucleotides upstream from the site to be mutated. Subsequent digestion with exonuclease III formed a gap (about 150 nucleotides), which was filled in the presence of the mutagenic oligonucleotide. The plasmid was used to transform *E. coli*, and colonies were screened with the same oligonucleotide as a probe. On washing the hybridized filters at 52°C, 2 positives were identified among 300 colonies.

Synthesis, Purification, and Characterization of Oligonucleotides. Deoxyribonucleoside *N*-morpholinophosphoramidites were superior to the corresponding *N,N*-dimethylamino analogues with regard to yield, purity (^{31}P NMR), and stability. Further, the yields during the coupling steps (>95%) and of the isolated products were consistently higher with the morpholino derivatives. The overall yield for a purified dodecamer with the morpholino derivatives was 11–14%, compared with 3–5% obtained with the *N,N*-dimethylamino analogues.

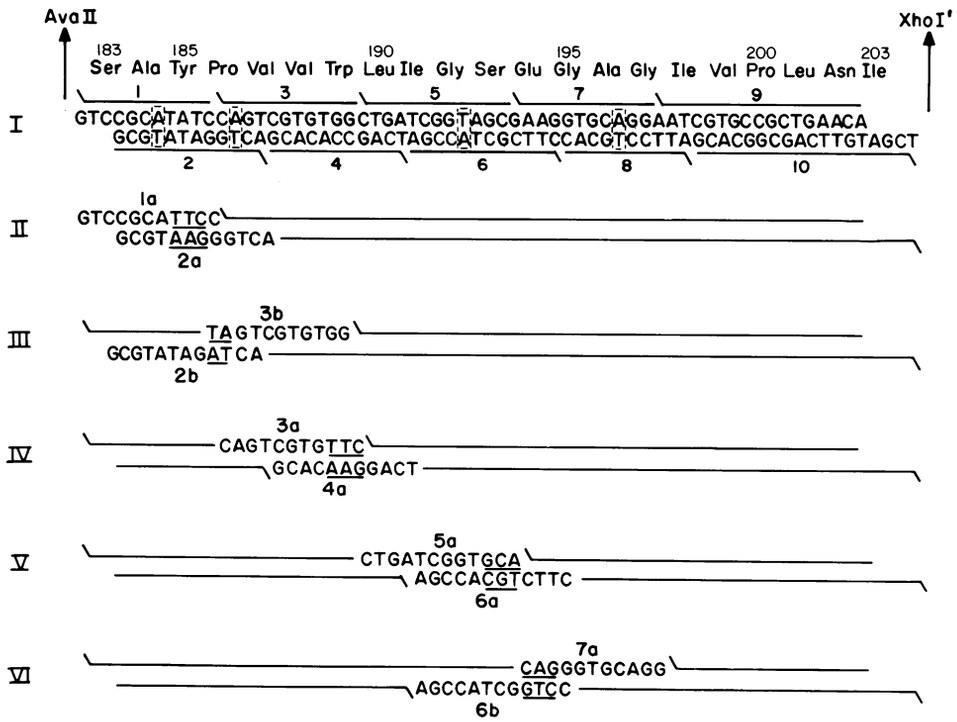

FIG. 3. Synthetic plan for the modified native and mutated *Ava* II–*Xho* I′ fragments. Each DNA duplex consisted of 10 oligonucleotides joined by T4 DNA ligase. The mutant duplexes were produced by replacing two oligonucleotides of the native sequence by two that contain the desired codon changes underlined. Four silent G/C-to-A/T changes were introduced where shown by the dotted boxes. Duplex I encodes the native amino acid sequence, while the others have the following substitutions: II, Tyr-185 to Phe; III, Pro-186 to Leu; IV, Trp-189 to Phe; V, Ser-193 to Ala; and VI, Glu-194 to Gln.

The use of the Sep-Pak allowed rapid separation on a preparative scale of the failed sequences from the required $(MeO)_2$Tr-containing product (Fig. 4*A*). Similarly, after 5′-^{32}P-phosphorylation of the oligonucleotides, the products were separated from excess [γ-^{32}P]ATP and $^{32}P_i$ (Fig. 4*B*).

All oligonucleotides were purified by this procedure and were >95% pure by electrophoretic and sequence analysis. The Sep-Pak method also was used for desalting of eluates of oligonucleotides and DNA duplexes from gel slices. Recoveries were >90%, and the lyophilized materials were used directly for enzymatic reactions.

Joining of Synthetic Oligonucleotides to Form DNA Duplexes (*Ava* II–*Xho* I′ Fragments). The T4 DNA ligase-catalyzed joining of oligonucleotides was performed in two steps (Fig. 5). The product was isolated by gel electrophoresis in an overall yield of 35–55% and characterized as described.

Reconstruction of Modified Native and Mutant BO Genes Containing the Synthetic *Ava* II–*Xho* I′ Duplexes. The gene was reconstructed in a three-component ligation involving the large *Sph* I–*Xho* I vector fragment, the *Sph* I–*Ava* II fragment, and the synthetic duplex. The *Sph* I–*Ava* II fragment was isolated through the *Sph* I–*Bam*HI fragment (Fig. 2*A*) because an *Ava* II cut of the small *Sph* I–*Xho* I fragment gives two fragments of almost identical size. Before transformation, the ligation mixture was digested with *Xho* I to reduce the background due to the parental plasmid. Of the total transformants, 30% were linearized by *Sph* I but not by *Xho* I. The remainder contained a deletion through both of these sites.

Sequence Characterization of the Mutant BO Genes. The BO genes of plasmids identified by restriction analysis to be positives were subcloned into M13mp8. The sequences of all clones were determined through the synthetic section to the *Sph* I site to ensure that the correct constructions had been obtained. Out of a total of 11 clones, 9 had the expected sequence (Fig. 6). The data demonstrate the correct insertion of the synthetic duplexes into the gene and further confirm the accuracy of the sequences within the synthetic oligonucleotides.

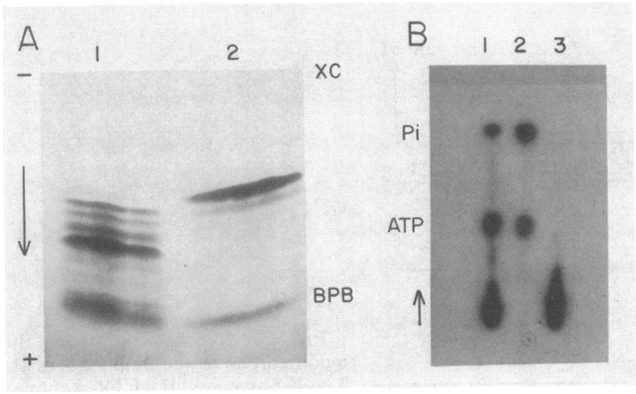

FIG. 4. (*A*) Analysis by polyacrylamide gel electrophoresis of Sep-Pak separation of synthetic $(MeO)_2$Tr-oligonucleotide(s) from the failed sequences. Electrophoresis was on a preparative gel (0.2 × 20 × 40cm^3) run under denaturing conditions at 1000 V. Lanes: 1, 50 A_{260} units of 10% acetonitrile eluate; 2, 25 A_{260} units of 30% acetonitrile eluate after detritylation. BPB:bromophenol blue; XC: xylene cyanol. The gel was placed on top of a fluorescent TLC plate and visualized under UV. (*B*) Sep-Pak purification of the reaction products obtained on 5′-end phosphorylation of an oligonucleotide with polynucleotide kinase and [γ-^{32}P]ATP. The separation was monitored on a polyethyleneimine plate run in 0.75 M sodium phosphate buffer (pH 3.5). Lanes: 1, the total reaction mixture; 2, 5% acetonitrile wash, which removed all the P_i and ATP; 3, the 30% acetonitrile eluate, which contained >90% of the pure oligonucleotide in a 2-ml volume.

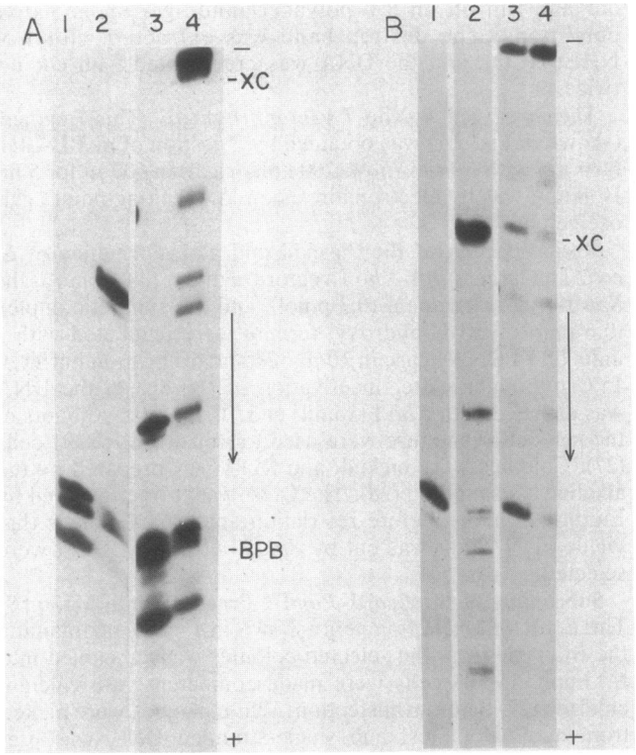

FIG. 5. T4 DNA ligase-catalyzed joining of synthetic oligonucleotides to form DNA duplexes. The ligation mixture was analyzed on a 12% polyacrylamide gel run under denaturing conditions at 1500 V. The oligonucleotides 1–4 and 5–10 were first ligated separately at 40 μM concentration of each oligonucleotide by using 1000 units of T4 DNA ligase per ml. (*A*) Lanes: 1 and 3, oligonucleotides 1–4 and 5–10, respectively, at zero time; 2 and 4, the two ligation mixtures after 1 hr at 7°C. (*B*) Lanes: 1 and 2, the ligation mixtures 1–4 and 5–10, respectively, after 3 hr at 7°C. The two mixtures were then mixed, and fresh ATP, dithiothreitol, and ligase (500 units/ml) were added. The extent of ligation was analyzed after 1 hr (lane 3) and 3 hr (lane 4) at room temperature.

DISCUSSION

Classically, protein structure–function studies have been carried out by using chemical modification techniques. However, the reactions often lack selectivity, and the modifying groups may cause nonspecific perturbations. We took the alternative approach of introducing mutations in the gene in an expression vector to bring about specific amino acid replacements.

The amino acids we mutated were chosen so that some of the ideas previously proposed for proton translocation and structural interactions within BR can be examined. Thus, the removal of the hydroxyl groups from Tyr-185 and Ser-193 allows us to determine if they are required for the formation of a proton conductance wire (30). The substitution of Pro-186 by Leu should test whether this proline creates a "proton hole" that could be involved in proton translocation (31). The replacement of Trp-189 by Phe should provide a spectroscopic variant of BR if this amino acid interacts with the chromophore. Finally, the change of Glu-194 to Gln may bear on the external point charge model (32) since this negatively charged amino acid is adjacent to the β-ionone ring of retinal (10).

The genes specifying the above amino acid substitutions were produced by replacing a restriction fragment of a BO expression plasmid by synthetic DNA. To limit the amount of chemical synthesis, a unique *Xho* I restriction site was created by an A-to-C change at Ile-203. This point mutation was first attempted by using a single-stranded M13mp7 clone

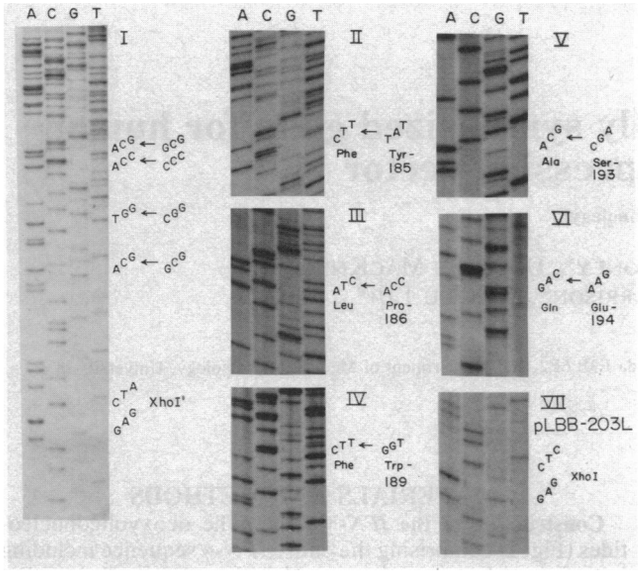

FIG. 6. Sequences of the modified regions of the BO gene. Sequence I shows the four G/C-to-A/T base changes and the *Xho* I' site; sequences II–VI show the amino acid codon substitutions; sequence VII shows the *Xho* I site created by point mutagenesis.

of the BO gene as template. Although the formation of covalently closed, circular DNA after correct extension of an oligonucleotide primer with a mismatch was demonstrated, no mutants were recovered (unpublished results). We attribute this to the preferential mismatch repair of the strand synthesized *in vitro* because of its unmethylated state. Subsequently, the required mutant was successfully produced directly in the expression vector by point mutagenesis with a gapped double-stranded template.

The Sep-Pak method described is effective in rapid separation of failed sequences from the required $(MeO)_2Tr$-containing products, for recovery of oligonucleotides after preparative polyacrylamide gel electrophoresis, and for separation of 5'-end-phosphorylated oligonucleotides after polynucleotide kinase reactions. It is equally applicable to the purification of double-stranded DNA (we have tested restriction fragments of up to 1600 bp), with almost quantitative recoveries. The Sep-Pak procedure, together with rapid methods available for the synthesis of oligonucleotides, further expedites the preparation of synthetic DNA.

The synthetic method for site-specific mutagenesis offers important advantages over alternative procedures. We extended the scope of the synthetic method by introducing a new restriction site by the oligonucleotide-directed mutagenesis technique because selective restriction sites are not available naturally. Once the oligonucleotides required for the synthesis of a given DNA segment have been prepared, all possible mutants within that region can be efficiently produced. New synthesis is limited to the immediate region where a mutation is required. Thus, we are now in a position to introduce many mutations into helix 6 of BO.

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