EXPRESSION OF BACTERIOOPSIN GENES IN ESCHERICHIA COLI

EXPRESSION OF BACTERIOOPSIN GENES IN *ESCHERICHIA COLI*

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ABSTRACT: An inducible expression vector pUBO was constructed with native codons in order to express the gene of Bacteriorhodopsin (BOP) in *Escherichia coli* (*E. coli*). Vector pUBO contains lac-promoter followed by the partial structural gene of lacZ and the structural gene of BOP. The expression of this fusion protein was detected by ELISA with anti-BOP antiserum. The fusion protein obtained from *E. coli* trnsformed with pUBO formed approximately 0.1 % of the total protein of the *E. coli* membrane fraction.

Key words: Bacteriorhodopsin, Expression in *E. coli*

INTRODUCTION

Good crystals and typical samples of target substances are necessary for intensive research in materials physics, and similar requirements apply also in biophysical research. Recently, recombinant DNA techniques can be applied to prepare target substances for biophysical research and here we have applied these techniques for further investigations of bacteriorhodopsin (BR).

Bacteriorhodopsin is the sole protein constituent (a single polypeptide of 26 kD) of the purple membrane of *Halobacterium halobium*. It functions as a light-driven proton pump. A chromophoric retinal of BR is bound to lysine residue 216 via a protonated Schiff base linkage [1-4]. Light isomerizes chromophoric retinal from all-*trans* to 13-*cis* [5-8] and BR converts to bathochromic intermediate called K [9]. K finally reverts to the original BR

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Abbreviations: BR, bacteriorhodopsin; BOP, bacterioopsin; *E. coli Escherichia coli*; IPTG, Isoproryl-β-D-thiogalactopyranoside; ELISA, Enzyme linked immuno sorbent assay; HRPO, Horseradish peroxydase; OPD, orthophenylene diamine

via various intermediates with characteristic absorption spectra [10]. In the photocycle of BR, protons are released from BR to the outside of the cell. Vacant sites of BR are then filled with the protons from the protoplasm [11, 12]. Proton translocation to the outside of the cell produces an electrochemical gradient across the membrane, which the cell uses for ATP synthesis and transportation of ions and amino acids.

BR is a simple energy converter, so the molecular mechanism of the pump action has been studied intensively [13-17]. Site-directed mutagenesis is now a routine technique to analyze the roles of amino acid residues of proteins. To apply this technique, DNA encoding the modified protein has to be prepared and expressed in adequate systems. The Khorana group [18] constructed a DNA fragment encoding the apoprotein of BR (bacterioopsin; BOP) with codons which are suitable for use with *E. coli*, but different from the native ones, and expressed the protein. They made many mutants of BR expressed in *E. coli* and have revealed the roles of several amino acid residues in BR.

In this paper we report the expression of the BOP gene in *E. coli* with native codons and confirm that significant amounts of the expressed proteins are present in the bacterial membrane fraction.

MATERIALS AND METHODS

Plasmids The plasmid vector pHV-18.34-Δ-E3 [19] containing the native BOP gene was kindly provided by Dr. D. Oesterhelt. The plasmid pUC18 was from Toyobo Co. (Japan).

Construction of expression vector Restriction enzymes, T4-DNA ligase, bacterial alkaline phosphatase and T4 polynucleotide kinase were purchased from Toyobo Co. (Japan) and used as recommended. Restriction fragments were separated on low melting agarose gels and recovered by treatment with glass powder adhesion and separation. Synthetic oligonucleotides for use as linkers were synthesized by a DNA synthesizer (Applied Bio Systems 381A, USA) and purified by high performance liquid chromatography (Pharmacia LKB Biotechnology, Sweden). The synthesized oligonucleotides were desalted by Nap 10 columns (Pharmacia LKB Biotechnology, Sweden) and phosphorylated by T4 polynucleotide kinase. Ligation mixtures contained equimolar amounts of restriction fragments and synthesized oligonucleotides (0.1-1.0 pmol). Ligation was carried out at 14°C for 12 h. *E. coli* strain JM105 was transformed by the $CaCl_2$ procedure. Transformants were selected on LB plates containing 35 μg/ml ampicillin. The rapid boiling procedure was applied for small-scale plasmid preparation. Large-scale plasmid preparation involved extraction by alkaline SDS and centrifugation to equilibrium in CsCl gradients containing ethidium bromide.

The gene of native BOP contains two unique sites for restriction enzymes *Acc*III and *Xma*III. Digestion of the native genomic DNA of BOP with restriction enzymes *Acc*III and *Xma*III eliminates nucleotides corresponding to only seven amino acid residues. Deleted nucleotides encoding these seven amino acid residues were recovered by using synthesized oligonucleotides.

Construction of pUBO The plasmid pUCMini was constructed by insertion of a synthesized DNA linker with the *Acc*III-*Xma*III cloning site into the plasmid pUC18. A

DNA fragment of 710 base pairs encoding the main part of BOP, was obtained by digesting the plasmid pHV-18.34-Δ-E3 with restriction enzymes *Acc*III and *Xma*III. This fragment contains nucleotides of the BOP gene corresponding to amino acid residues 5 to 246 of mature BOP.

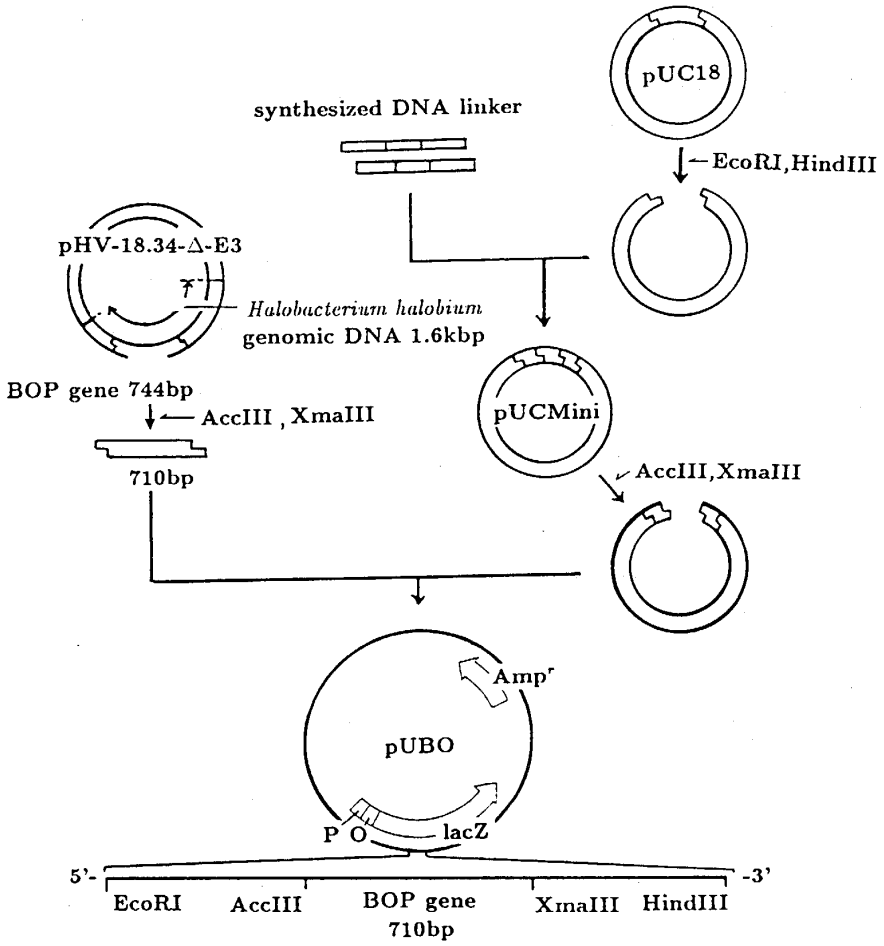

Fig. 1 The procedure of construction of pUBO. The pUCMini was constructed with the *EcoR*I-*Hind*III large fragment of pUC18 and synthesized DNA linker which has *Acc*III and *Xma*III cloning site. DNA fragment of 710 base pair (bp) encoding BOP peptide was cut out from pHV-18.34-Δ-E3 with restriction enzymes *Acc*III and *Xma*III. The pUBO was constructed by inserting the 710 bp fragment into the pUCMini.

The plasmid pUBO was constructed by inserting the DNA fragment into the plasmid pUCMini. *E. coli* strain JM105 was transformed by ligation mixture, and plasmid pUBO was identified in an ampicillin-resistant transformant. The sizes of the inserts were checked by agarose gel electrophoresis. The orientation of the inserts was confirmed by the nucleotide sequences.

Preparation of BOP peptide To express the BOP *in vivo*, cells were grown in LB broth containing 35 μg/ml ampicillin at a cell density of 0.1 optical density units (650 nm), IPTG was added to the culture at a concentration of 1 mM and the cells were cultured for more three hours. Cells were harvested, washed and suspended in MF buffer (50 mM of Tris-HCl, pH 7.0, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM 2-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride). DNase I (20 μg/ml) and RNase (20 μg/ml) were

added and the cells were sonified. Unbroken cells were removed by centrifugation at 10,000 $\times g$ for 10 min. A crude membrane fraction was collected by centrifugation at 100,000 $\times g$ for 1 hour. The membrane pellet was suspended in 50 mM Na-phosphate buffer (pH 7.0), and the protein concentration was determined by the Lowry method.

Detection of BOP peptide ELISA and western blotting were carried out as previously reported for bovine rhodopsin [20], using mouse anti-BOP antiserum. The amount of BOP in the membarane fraction was estimated by the same method. Horseradish peroxydase-conjugated anti-mouse goat IgG was used as the second antibody. Absorbance at 492 *nm* of the product of ortho-phenylene diamine (OPD) was monitored as the amount of BOP peptide. The membrane fraction was subjected to SDS-polyacrylamide gel electrophoresis and blotted to a nitrocellulose filter. The peptide bands on the filter were visualized by staining with amido black and the BOP band was also visualized by immunostaining with anti-BOP antiserum.

RESULTS

Confirmation of construction of pUBO by DNA sequencing DNA encoding of BOP (710 bp) was isolated by digestion of plasmid pHV-18.34-Δ-E3 with *Acc*III and *Xma*III. The isolated 710 bp fragment was inserted between *Acc*III and *Xma*III sites of pUCMini.

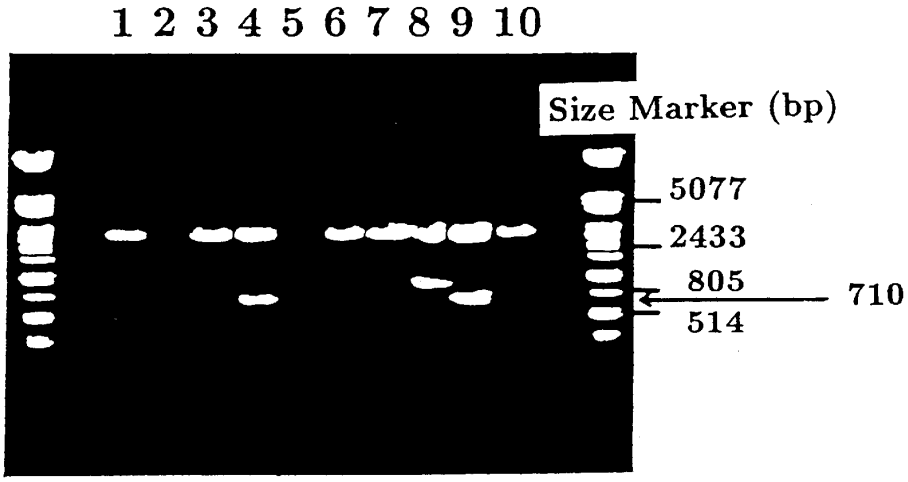

Fig. 2 The agarose gel electrophoresis of the digestive fragments of plasmids prepared from the transformants. Lanes 4 and 9 represents 710 bp fragment.

Among about a hundred of transformants we found two with the predicted DNA fragment size following electrophoresis on agarose gel (Fig. 2; Lanes 4 and 9). One of the two plasmids was sequenced by means of the chain terminator method and we confirmed the same sequence as that of BOP. The prepared plasmid was designated as pUBO.

The amount of BOP peptide in the membrane fraction of E. coli *E. coli* strain JM105 transformed by plasmid pUBO was cultured and the expression of fusion protein was induced by adding IPTG. Three hours after induction, cells were harvested and the membrane fraction of *E. coli* was prepared. The ELISA measurements indicated

the existence of BOP peptide in the membrane fraction, but not in the soluble fraction of *E. coli*. The amount of BOP peptide was estimated in the membrane fraction of cells transformed with expression vector pUBO (Fig. 3). Column a,b,c and d are the membrane

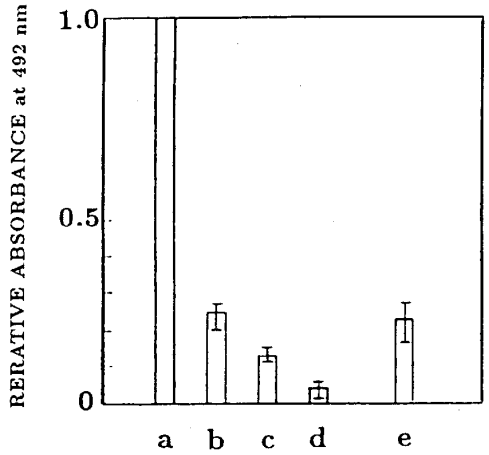

Fig. 3 The estimation of the amount of expression of BOP expressed in *E. coli*. Column (a,b,c) and (d) contained 1.28 %, 0.32 %, 0.08 %, and 0 % of authentic BOP in the membrane fraction of the cells transformed with pUC18, respectively. Column (e) was the membrane fraction of the cells transformed with pUBO. All the values were normalized by that of (a).

fractions of the cells transformed with pUC18 which contained various amount of authentic BR (1.28 %, 0.32 %, 0.08 % and 0 %, respectively). The amount of BOP peptide in cells transformed with pUBO was estimated to be about 0.32 % of the total protein of membrane fraction.

Immunoblotting with anti-BOP antiserum detected a band in the membrane fraction of cells transformed with pUBO but not with pUC18 (Fig. 4). The molecular weight of the stained band was close to that of BR. The detection limit of the anti-BOP antiserum was estimated to about 80 n*g* (data not shown). Taking account of the results shown in Fig. 4, where 80 μg of the membrane fraction was used for electrophoresis, the amount of BOP peptide existing in the membrane fraction agrees with that estimated by ELISA as shown in Fig. 3.

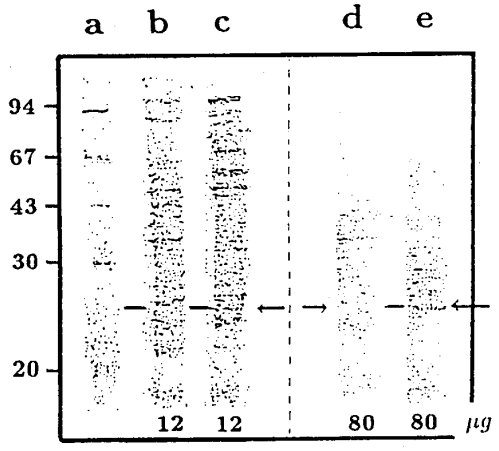

Fig. 4 SDS polyacrylamide gel electrophoresis of the membrane fraction of the cells. Lane (a) was the molecular weight marker. Lanes (b,d) were the membrane fraction of the cells transformed by pUC18. Lanes (c,e) were the membrane fraction of the cells transformed by pUBO. Lanes (b,c) were stained by amide black. Lanes (d,e) were immunostained.

DISCUSSION

From the above results, we conclude that it is possible to express BOP gene with native codons in *E. coli*. The amount of the expressed BOP peptide in the membrane fraction, at around 0.3 % of total membrane protein was small but was quantifiable by both ELISA and immunoblotting. In our experiments, the ratio of membrane proteins to the total protein of the cell was about 40 %, so that BOP peptide expressed is about 0.1 % of the total protein.

Dunn *et. al.* [18] reported that the amount of *E. coli* lipoprotein-BOP fusion protein, expressed using the lac promoter, was about 0.05 % of the total cell protein: only half of that produced in our system. Dunn *et. al.* [18] also reported that expression of the fusion protein, lipoprotein-BOP, caused inhibition of the growth of the host cells. We also observed this phenomenon in the course of our experiments (data not shown). Following the study of Dunn *et. al.* [18], Karnik *et. al.* [21] reported an improved expression system for the BR gene in *E. coli* by changing the codons of amino acid residues on the N-terminus into A=T rich ones. In both studies the promoter was lac promoter: the same one as in our present study. It is therefore clear that lac promoter limited the transcription level of the BOP gene to about 0.05-0.1 % of total cell protein, unless codons are artificially rearranged. Alteration of the codons might increase the level of expression of BOP. Nassal *et. al.* [22] reported that total synthesis of the BOP gene with *E. coli* favoring modifications progressed the further expression of BOP in *E. coli*. The amount of the expressed BOP protein was 0.5 % of the total proteins of the cell. The modification was certainly effective to improve the expression ratio of membrane protein such as BR which may inhibit the cell growth. However, judging from the fact that we used no *E. coli* favoring modifications in the construction of pUBO, it is clearly possible to express the BOP gene in *E. coli* without such modifications. This indicates that studies can now proceed using the unmodified native BOP gene.

The next step is to express partial peptides of BOP in *E. coli* which we shall report in the following paper.

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