Generation and analysis of bacteriorhodopsin mutants with the potential for biotechnological applications

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Keywords: absorption spectra, bacteriorhodopsin, mutants, proton pumping, site-directed mutagenesis

The properties of bacteriorhodopsin (BR) can be manipulated by genetic engineering. Therefore, by the methods of gene engineering, Asp85 was replaced individually by two other amino acids (D85V, D85S). The resulting recombinant proteins were assembled into soybean vesicles retinylated to form functional BR-like nano-particles. Proton translocation was almost completely abrogated by the mutant D85S, while the D85V mutant was partially active in pumping protons. Compared with wild type, maximum absorption of the mutants, D85V and D85S, were 563 and 609 nm, which illustrated 5 nm reductions (blue shift) and 41 nm increases (red shift), respectively. Since proton transport activity and spectroscopic properties of the mutants are different, a wide variety of membrane bioreactors (MBR) have been developed. Modified proteins can be utilized to produce unique photo/Electro-chromic materials and tools.

Introduction

Bacteriorhodopsin is a model system in nanobiotechnology. To turn this molecule into an evolutionarily-optimized biomaterial for the information technology sector requires the controlled modification of its molecular properties, accomplished by a combination of genetic and biotechnological methods. The majority of these approaches rely on the absorption properties of the retinal chromophore, mutant conformation and subsequent changes in protein activity. Mutant proteins with the Asp85 substitution represent Schiff base deprotonation which affects the color of the chromophore and shows interesting changes comparable with the wild type. The mutants, based on their spectral properties, show either essentially the same absorption spectra as the native BR, or relatively spectral shifts from its spectrum. Replacements of Asp85 were performed with Valine and Serine. The mutant proteins were folded accurately by the binding of all-trans retinal and BR-like chromophores regenerated, proton-pumping activities were assayed and the spectroscopic characterization of bacteriorhodopsin mutants were determined.

Results

Two pairs of primers (F1, xR1 and xF2, R2) were employed to produce two separate gene fragments (300 bp and 500 bp) using the cloned bo gene as the template with three (splice by overlap extension) SOE PCRs. The resulting gene fragments were purified on a 1% agarose gel and served as templates for a third PCR reaction using the first and last primers (F1, R2). PCRIII produced the full-length mutant genes (800 bp) (Fig. S1). Both constructs with the desired mutations (D85V and D85S) were verified by DNA sequencing (Fig. S2) and subsequently aligned by using the MEGA4 software package (Fig. S3).

The positively transformed samples were cultured and induced and all three bacterio-opsin (bO) mutants were expressed in E. coli (DE3). The mutants were overexpressed under various induction protocols (1–100 mM IPTG). A faint 29.6 kDa band (including the short part of the β-galactosidase gene + 6His tag) was detected in the SDS-PAGE gel (Fig. 1). Relatively sharp bands were seen in the western blots treated with both anti-BR and anti-His tag-conjugated antibodies (Fig. S4).

His-tagged mutants of bo proteins were purified by His-Bind columns, with the affinity of histidine to nickel. As a quick method for protein purification. Using pre-packed His columns was effective, easy to control, and permitted the parallel preparation of a number of samples. As Figure 2 shows, the 29.6 kDa mutant proteins were eluted. However, the presence of impurities was unavoidable.

After chromatopore regeneration, both Asp85Val and Asp85Ser mutants were reconstituted in SBP vesicles. Proteoliposomes prepared from Asp85Val mutant showed proton pumping into the vesicles inverse of the case for wild-type BR.
Overall, this approach was more efficient than the previously existing protocols. Even after considering the E. coli codon usages and changing a number of codons, protein expression remained poorly detectable. The weak expression of trace amounts of the proteins under certain conditions indicated that some of the BR variants might be misfolded, proteolysed and/or aggregated. Impurities might include histidine-rich E. coli proteins that compete for binding with the His-tagged mutant proteins. The extra protein band of around 50 kDa might include proteins such as cytochrome oxidases derived from the host E. coli cyton. The 116 kDa band could be aggregated bOs. However, these contaminations and His-tag sequences do not interfere with the photocycle measurements or the efficiency of the photosomeration step, and as such can be ignored.

Both mutant proteins were assayed in phospholipid vesicles as a function of pH. Variant D85V was partially active in proton translocation whereas, D85S was almost completely inactive with severely diminished proton pumping activity (Fig. 3). The absorption characteristics of BR mutants were analyzed using only LA (light Adopted) forms. Variants D85V and D85S exhibited peaks at 563 and 609 nm, respectively as mentioned in Table 1. The absorption spectra of the regenerated mutants are shown in Figure 4.

Discussion

Site-directed mutagenesis of the kO gene was employed in functional studies of BR. Prior approaches, which have employed techniques of mismatched oligonucleotides or restriction fragment replacement, have been mostly ineffective. In the current study, previous techniques were improved via a different approach, SOE PCR, with two pairs of primers, and the kO synthetic gene was used as a template. Mutations were introduced into the gene with an expression vector to create specific amino acid replacements. Overall, this approach was more efficient than the previously existing protocols.

Under standard conditions, protein expression in E. coli was not detectable. Even after considering the E. coli codon usages and changing a number of codons, protein expression remained poorly detectable. The weak expression of trace amounts of the proteins under certain conditions indicated that some of the BR variants might be misfolded, proteolysed and/or aggregated. Impurities might include histidine-rich E. coli proteins that compete for binding with the His-tagged mutant proteins. The extra protein band of around 50 kDa might include proteins such as cytochrome oxidases derived from the host E. coli cyton. The 116 kDa band could be aggregated bOs. However, these contaminations and His-tag sequences do not interfere with the photocycle measurements or the efficiency of the photosomeration step, and as such can be ignored.

The mutant proteins expressed in E. coli could be built-in as monomers in lipid vesicles derived from endogenous sources (e.g., polar lipids isolated from H. halobium) or exogenous sources (e.g., mixed lipid/detergent micelles, sulfolipids or SBPs). Upon illumination, protons are transmitted from the environment to the inside of reconstituted vesicles of the BR mutants in SBPs. Hence, the BR mutants have an inside-out orientation, which differs from what is found in vivo with native BR. The observed low proton-translocation activity may be the result of ineffective vesicle formation or random orientation of BR. However, it may also indicate the probability that the vesicles were not spherical, or that the vesicle consisted of equal amounts of inside-out and right-side-out orientations of protein particles. Substitution of Asp85, the proton acceptor, in consequence inhibition of the pumping activity in some BR mutants is due to electrostatic interactions. Probably the reverse polarity of the photoelectric response of the D85X mutants rather than WT BR is not for structural or fundamental changes but due to a reverse orientation of recombinant proteins in micelles. The functional differences in the exchanged amino acids could be due to changes in the kinetics of the photo cycle and the proton pumping activity in the mutants. The small difference in reported absorptions is most likely due to the use of a different reconstitution system. The D85V mutant has blue shifted chromophore with only a slight shift in maximum absorption at 563 nm. The D85S mutant is very different, which in turn produces the observed red shift in λmax 690 nm. This result is in contrast with the results of Facciotti MT, et al. which suggests a blue shift for D85S; moreover, D85V shows a slight activity about 20% wild type, while the proton pumping of D85S is almost inactive (less than 7% WT), as a rule; the loss of a proton-accepting group at this position entirely defeats proton pumping. Different maximum absorptions of recombinant proteins result in specific properties and consequently diverse certain applications. For instance, they can be the basis of Electro-chromic materials of different colors. Replacement of Asp85 by serine causes considerable alterations of the functional and spectroscopic properties of the D85S mutant. In wild-type BR, the carbonyl group of Asp85 is negatively charged, whereas the serine in D85S mutant, is neutral, which results in a red shifted absorption spectrum and a deep-blue color. The D85S mutant’s excited-state lifetime is considerably longer compared the wild-type BR. This aspect makes
the D85S mutant suitable to study some processes in the protein matrix in response to chromophore excitation.6,7

In spite of extensive applications of these photosynthetic proteins in a variety of different technologies, efforts continue to utilize them as light sensitive elements in image detectors in applications such as: artificial retinas, spatial light modulators and holographic memories and to make BR and associated mutants a protein of interest. Moreover, proteins with different absorption-wavelength characteristics can be used as the foundation of a color sensitive sensor. This study provides further evidence that these mutant proteins are powerful technological systems with the potential to change solar energy into electrical or chemical energy. Either natural or engineered proteins can serve as a basis for the evolution of biosensors and biosips, photovoltaic and photovoltaic devices and perhaps alternative computers.15-18

The structure of mutants with red-shifted absorption has probably been expanded, such that retinal is more exposed and can be excited with longer waves and less energy. In contrast, the structure of mutants that display a blue-shift may be compacted and retinal is not being well exposed; therefore, these mutants need more energy and shorter waves for excitation.

Altogether the properties of BR can be regulated by the development of gene engineering which made it possible to obtain BR analogs with the individual amino acid replaced by others. Thus, a wide variety of types of membrane bioreactors (MBr) have been developed to carry out many industrially important processes.

Materials and Methods

Solutions. PBS Buffer: 1.9 mM NaH2PO4·2H2O, 8 mM Na2HPO4, and 0.15 M NaCl; Lysis Buffer: 0.5 M NaCl, 20 mM TRIS-HCl (pH = 8.0), 10 mM imidazole and 0.005% PMSF; Buffer A: 0.01 M TRIS-HCl, 0.15 M NaCl, 0.25% (w/v) deoxycholic acid and 0.025% (w/v) NaN3 titrated to pH 8.0 with NaOH; Buffer B: 0.01 M Na2HPO4, 1% (w/v) SDS and 0.025% (w/v) Na2S (pH 8.0); Buffer C: 0.15 M KCl, 2% (w/v) sodium cholate and 0.025% (w/v) Na2S (pH 8.0); Buffer D: 0.01 M Na2HPO4, 0.15 M NaCl and 0.025% (w/v) Na2S (pH 8.0); and Buffer E: 0.15 M NaCl and 0.025% (w/v) Na2S (pH 8.0).

Mutagenesis of the synthetic bacterio-opsin (dBO) gene. Site-directed mutagenesis was performed with the SOE PCR method: The synthetic bacterio-opsin (dBO) gene (Cinagene) cloned in pET21a was used as a template, and specific primers were applied. The vector, including the dBO gene and considering the E. coli codon usages, was fused with part of β-galactosidase (β-gal) gene (13 aa) at the N-terminus. The primer sequences were designed to substitute Asp85 with two other amino acids; namely valine (13 aa) at the N-terminus. The primer sequences were designed with consideration of the desired replacement in these sequences; VR1: 5′-GAA CAG CCA A TGC ATA GCG-3′ (XhoI site is shown in bold). The first reverse (XR1) and the second forward (XF2) primers (mutagenic primers) were designed to add the XhoI site at the end of the multiple cloning sites. The final reverse primer was designed with the consideration of restriction sites; VR2: 5′-GAG CCA CAG CTC TTT TTT TGG CAG-3′ (XhoI site is shown in bold). The final reverse primer was designed with the consideration of restriction sites; VR2: 5′-GAG CCA CAG CTC TTT TTT TGG CAG-3′ (XhoI site is shown in bold).

A total of 35 amplification cycles were performed in accordance with the protocols of the Roche kit. The PCR amplification consisted of the following: 4 μM of each primer, 100 μM of mixed dNTP (Fermentas), 2 μL of template DNA, and 1 unit of Tag DNA polymerase (Fermentas) to a total volume of 25 μL. A total of 35 amplification cycles were performed as follows: 1 min at 94°C (denaturation step); 1 min at 57, 66 and 65°C (for 300 bp, 500 bp and 800 bp fragments, respectively) (primer annealing step); and 1 min at 72°C (polymerization step). The calibrated PCR procedure was repeated with Pfu DNA polymerase (Fermentas) for enzyme fidelity. PCR products were confirmed by agarose gel electrophoresis and purified using a high-performance spin column (Fermentas). The PCR product was cloned into pGEM-T Easy vector and transformed into E. coli DH5α (Fermentas) for in vitro screening. The correct clones were sequenced using the BigDye® Terminator Cycle Sequencing Ready Reaction Kit (Fermentas) according to the manufacturer’s instructions.
were extracted with the High Pure PCR Product Purification Kit (Bioneer, South Korea) according to the manufacturer's manual. 

**E. coli** TOP10F’ (Stratagene) was employed as a cloning host. 

**Figure 4.** Absorption spectra of the regenerated mutants, D85V (Asp85Val) and DB5V (Asp85Ser).

**Protein expression and purification.** The proteins were initially transformed into TOP10F’ and the expressed plasmids were again transformed into BL21 (DE3). Transformed cells were grown at 37°C in 5 mL of sterile LB Broth (Scharlau) in medium supplemented with 5 μg/mL ampicillin. Upon reaching an OD of 0.6 to 0.7 at 600 nm, 10 mM isopropylthio-β-D-galactoside (IPTG, Fermentas) was added to induce protein synthesis. After an induction period of 3–4 h, the cells were harvested to determine the expression level by using SDS-PAGE with Coomassie Blue. Expression analyses were complemented by western blots. 

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**Cell cultures of interest were grown in the presence of ampicillin (100 μg/mL) at 37°C.** Expression was induced by adding IPTG (10 mM) at an OD 600 of 0.8. The cells were harvested (centrifuged at 6,000 g for 6 min at 4°C) 3 h after induction. The yield was ~2 g (wet weight) of cells per liter of culture. Pellets were washed and resuspended in the lysing buffer. Finally, cells were disrupted by ultrasonication (for 40 cycles of 10 sec). Membranes were collected via centrifugation (1 h, 20,000 g, 4°C). The cell lysates were filtered through a 0.45 μm (Millipore) filter and passed through pre-packed His-Bind columns (Novagen), in accordance with the manufacturer’s instructions.

The concentrations of the purified proteins were determined spectrophotometrically by Bioral assay. The proteins were concentrated to 0.5 mg/mL in a CF-25 Centri cone. The eluted protein was dialyzed consecutively against PBS (2 × 1 L) at 4°C for 1 d, against buffer A (2 × 1 L) at 25°C for 1 d (sodium deoxycholate was used to remove any residual cell debris particles); and against buffer B (4 × 1 L) for 2–3 d. Dialysis was performed in a dialysis tube (MW cut-off 3500, Spectrum Laboratories, USA). The concentration of the obtained solution (BO/SDS) was the same as before (0.5 mg/mL). Each sample was lyophilized to dryness and stored at 4°C.

**Proton pumping assay.** The BR mutants were regenerated with all-trans retinal (Sigma) and reconstituted into exogenous dried soy phospholipids (SBP) (Fluka) vesicles. A 1 mg sample of all-trans retinal was dissolved in 1 mL of ethanol. This solution was newly prepared and was kept at -20°C in the dark for no more than 2 weeks. The concentration of the solution was determined by measuring the absorption at 380 nm. A 0.2 mL aliquot of each apoprotein was mixed in 2.5 μL of all-trans retinol solution (BO/Retinal). The mixture of 20 mg of SBP with 1 mL of buffer C was sonicated until it was clear and was then rapidly mixed with 100 μL of BO/Retinal. After 30 min, the chromophore regeneration was completed. The solution was incubated at 25°C for 2 h, was dialyzed against buffer D (4 × 1 L) for 2 d, and eventually dialyzed against buffer E (2 × 1 L) for 1 d to eliminate any free retinol in the environment.

**Proton pumping assay.** The BR mutants were regenerated with chromophore and reconstituted into the SBP liposome with modifications as described. Algolos (50 μL) of vesicles containing bacteriochlorophyll were assayed for proton-pumping activity in a freshly prepared solution of 3 M KCl and 80 mM MgCl2 at pH 7.1, irradiated at 30°C with a 200 W tungsten lamp. A calibrated glass electrode was used to monitor the pH of the external medium over a period of 60 sec. **Absorbance measurements.** After an overnight dark adaptation, reconstituted BR mutants (100 mg/mL) were irradiated with light for 5 min by using a 200 W projector lamp to obtain light-adapted variants. Spectrum analysis was performed from 250 to 700 nm with a UV-Visible (Unicam UV-300) spectroscope. 

**Disclosures of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Supplemental Material**

Supplemental materials may be found here:

http://www.landesbioscience.com/journals/bioe/article/21048/
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