Membrane assembly of bacterio-opsin mutants expressed in halobacteria and incorporation of the proteins into phospholipid bilayers

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INTRODUCTION

Bacteriorhodopsin (BR) and many other integral membrane proteins rapidly and spontaneously insert into preformed unilamellar vesicles of dimyristoylphosphatidylcholine (DMPC) and other phospholipids without the use of detergents or sonication (1–4). BR incorporates in only one orientation, so that it pumps protons into the vesicles in response to light. We have investigated which parts of the BR structure are involved in its specific insertion into the membrane by removing portions of the protein either proteolytically or by deleting parts of the bacterio-opsin gene (bop) and expressing the mutant bop genes in *Halobacterium halobium.*

MATERIALS AND METHODS

DMPC was purchased from Avanti Polar Lipids, (Birmingham, AL); Lovastatin (also known as mevinolin) was a gift from Dr. A. W. Alberts (Merck, Sharp and Dohme, Rahway, NJ). Enzymes were from Sigma Chemical Co., (St. Louis, MO) or Boehringer Mannheim (Indianapolis, IN).

BR was purified from *H. halobium* strain E1 as described in reference 5, and the carboxyterminal hydrophilic peptide was removed by papain treatment (6). Bacterio-opsin (BO) and its chymotryptic fragment C-1 were prepared as described in reference 7, but were renatured using β-octyl glucoside instead of DMPC/cholate. Proteins were inserted into preformed vesicles of DMPC as described (1–3).

H. halobium strain SD16 (8), which has an ISH2 insertion in the coding region of the bop gene, was transformed using the plasmid pWL102 (9), which contains a gene conferring resistance to the HMGCoA reductase inhibitor lovastatin in *H. halobium.* Recombinant bop genes were inserted between the BamHI and XbaI sites of the vector. Spheroplasts were prepared using EDTA (which took < 10 min for this strain) and DNA was added, followed by polyethylene glycol 600, and then regeneration salt solution as described in (10). The cells were then centrifuged, resuspended in complex media (5) containing 15% sucrose, and incubated 18 h at 37°C. 100 μl samples were spread on plates containing complex media and 15% sucrose without drug and incubated for 5 d at 37°C. The bacteria were then harvested from the plates and spread on plates containing complex media and 16 μg/ml lovastatin. After ~10 d incubation at 37°C, colonies were picked, grown-up complex media, and their DNA and proteins were analyzed.

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RESULTS AND DISCUSSION

BR is a very stable integral membrane protein; even BO or its chymotryptic peptides can be renatured and functionally reconstituted after denaturation in formic acid or sodium dodecyl sulfate (7). When BR spontaneously inserts into phospholipid bilayers (1–4), it almost certainly remains in a fully folded state. We are interested in what drives this process and whether it has any relationship to the mechanism by which BR is assembled into the membrane in *H. halobium.*

BO in its native state inserted spontaneously into phospholipid bilayers in the same orientation as BR; the BO could then be reconstituted with retinal, and it pumped protons into the vesicles. BO is less stable than BR; at 25°C it slowly lost its ability to reconstitute with retinal. This denatured BO no longer inserted into phospholipid bilayers, indicating that for this process the protein had to be already folded in its native conformation rather than the presence of the bilayer causing the polypeptide to fold into the membrane. The chymotryptic fragment (C-1) of BO, which lacks the two aminoterminal helices of the seven membrane-spanning domains also spontaneously inserted into a phospholipid bilayer like BO. Apparently these sequences are not required to "lead" the polypeptide into the membrane. Proteolytic removal of the carboxyterminal hydrophilic domain of BR also had no effect on incorporation or the direction of insertion, indicating that this highly negatively charged portion of the protein was not acting as a barrier to incorrect insertion.

In studying what features of BR structure are important for membrane insertion in vivo, we first examined the role of the unusual 13-residue leader peptide which is encoded in the bop gene. It appears to be neither long enough or hydrophobic enough to act as a typical "signal sequence"; it appears to be proteolytically cleaved after membrane insertion because cleavage is not necessary

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for proper membrane assembly (11). We deleted the codons for these 13 residues by removing an NspI-AatII fragment from the bop gene and replacing it with a synthetic linker. We had previously created an XbaI site just downstream of the termination codon, allowing us to insert a BamHI-XbaI fragment containing the bop gene into the shuttle vector pWL102.

H. halobium strain SD16, which does not make BR, was transformed with recombinant plasmids containing either the wild-type bop gene or the leader sequence deletion mutant. Both resulted in lovastatin-resistant colonies that were purple due to production of BR at levels comparable to that of the wild-type strain E1. Efficient expression of the wild-type bop gene using a similar plasmid has been reported previously (12); these workers also included the brp gene, which is thought to regulate the bop gene, on their plasmid. We analyzed DNA from many of our transformants by digestion with SmaI or with BamHI and NotI, Southern blotting, and probing with a nick-translated bop gene fragment. Whereas pWL102 is stably maintained in *H. halobium*, the presence of a bop gene insert in each case caused the plasmid to integrate into the chromosome at the site of the endogenous bop gene. This means that if the crossover occurred downstream of the site of the mutation in the bop gene we inserted in the plasmid, integration would regenerate a functional wild-type copy of the gene and leave the mutation in the inactive copy with the ISH2 insertion. The Southern blot was probed with an oligonucleotide specific for the mutation to determine which copy of the gene it is in. The expected protein product is also the same, so we pulse-labeled spheroplasts of the transformed cells with [^{35}S]methionine because spheroplasts do not process the BO precursor (11), to determine whether the bop gene being expressed was in fact the leader sequence deletion mutant.

Transformation of SD16 cells with another mutant bop gene in which the membrane-spanning helix D had been deleted, resulted in purple colonies, which turned out to have regenerated a wild-type copy of the gene, as well as orange colonies, which were not making any BR-related protein by Western blot analysis. The mutant genes were being expressed, based on Northern blots of the mRNA, but the protein may not have folded properly and was degraded.

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