Folding and Assembly in Rhodopsin

EFFECT OF MUTATIONS IN THE SIXTH TRANSMEMBRANE HELIX ON THE CONFORMATION OF THE THIRD CYTOPLASMIC LOOP

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Previous studies on bovine opsin folding and assembly have identified an amino-terminal fragment, EF(1–232), which folds and inserts into a membrane only after coexpression with its complementary carboxyl-terminal fragment, EF(233–348). To further characterize this interaction, EF(1–232) production was examined upon coexpression with carboxyl-terminal fragments of varying length and/or amino acid composition. These included fragments with incremental deletions of the third cytoplasmic loop (TH(241–348) and EF(249–348)), a fragment composed of the third cytoplasmic loop and sixth transmembrane helix (HF(233–280)), a fragment composed of the sixth and seventh transmembrane helices (FG(249–312)), and EF(233–348) and TH(241–348) fragments with Pro-267 or Trp-265 mutations. Although EF(1–232) production was independent of the third cytoplasmic loop and carboxyl-terminal tail, both the sixth and seventh transmembrane helices were essential. The effects of mutations in the sixth transmembrane helix on EF(1–232) expression were dependent on the length of the third cytoplasmic loop. Although Pro-267 mutations in EF(233–348) failed to stabilize EF(1–232) expression, their introduction into TH(241–348) was without discernible effects. However, Trp-265 substitutions in the EF(233–348) and TH(241–348) fragments conferred significant EF(1–232) production. Therefore, key residues in the transmembrane helices may exert their effects on opsin folding, assembly, and/or function by influencing the conformation of the connecting loops.

Rhodopsin is the photoreceptor that mediates vision in dim light. Bovine rhodopsin is composed of the apoprotein opsin, a single polypeptide chain of 348 amino acids, and an 11-cis-retinal chromophore (1–4). The apoprotein folds into a structure of seven transmembrane (TM) helices connected by solvent exposed polypeptide segments on the intradiscal and cytoplasmic surfaces. These seven membrane-spanning helices form a binding pocket for the retinal chromophore (5). Light-induced conformational changes in rhodopsin mediated by retinal isomerization expose cytoplasmic binding sites for the heterotrimeric guanine nucleotide-binding protein (G-protein), the interface between the receptor and effector molecules in visual transduction (6, 7).

An understanding of how rhodopsin adopts its tertiary structure is important not only to clarify details of the folding and assembly process but also to gain insight into the severe visual impairments occurring as an immediate consequence of natural mutations affecting opsin structure and function. One approach that has been used successfully to study the mechanism of protein folding and assembly is to use fragments of a polypeptide. In earlier studies (8, 9), we have examined whether expressed complementary bovine opsin fragments separated in the intradiscal, membrane-embedded, and cytoplasmic regions contain sufficient information to independently fold, insert into a membrane, and assemble into a functional pigment. Virtually all of the singly expressed fragments fold to a conformation that allows for membrane insertion and, in some cases, form the rhodopsin chromophore with 11-cis-retinal when coexpressed with their complementary partners. Thus, these results suggest that the functional assembly of bovine rhodopsin is mediated by the association of multiple folding domains and demonstrate the utility of defined polypeptide fragments for studying the mechanism of bovine opsin folding and assembly.

We have now focused on the nature and specificity of the fragment interaction(s) by identifying determinants that lead to proper folding, membrane insertion, and assembly. For this purpose, we utilized an amino-terminal five-helix opsin fragment, EF(1–232), which is stably produced only upon coexpression with its corresponding carboxyl-terminal partner, EF(233–348) (9). This finding suggests that regions within the 1–232 opsin polypeptide do not fold independently of interactions with other domains present in the complementary portion of the polypeptide chain (amino acids 233–348). To study this in greater detail, eight additional carboxyl-terminal bovine opsin fragments were constructed and expressed (Fig. 1). These include a fragment composed of the third cytoplasmic loop and sixth TM helix (HF(233–280)), a fragment that lacks the third cytoplasmic loop and carboxyl-terminal tail (FG(249–312)), and EF(233–348) as well as TH(241–348) fragments with Pro-267 → Gly/Leu or Trp-265 → Phe mutations. Two previously reported (8, 9) bovine opsin fragments with incremental deletions of the third cytoplasmic loop (TH(241–348) and EF(249–348)), were also utilized. The opsin gene fragments were expressed in COS-1 cells singly or in combination with EF(1–232), and the polyepptide fragments were examined for stable production and their ability to form the rhodopsin chromophore with 11-cis-retinal. Coupled with our earlier findings, the present results suggest that specific amino acid residues in the TM helices can exert their effects on opsin folding, membrane insertion, and/or assembly by influencing the conformation of the solvent exposed connecting loop regions.

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1 The abbreviations used are: TM, transmembrane; DM, n-dodecylβ-D-maltoside; PAGE, polyacrylamide gel electrophoresis.
**Folding and Assembly of Opsin Fragments**

**EXPERIMENTAL PROCEDURES**

**Materials**

Restriction endonucleases were from New England Biolabs or Roche Molecular Biochemicals, and horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG was from Promega. The enhanced chemiluminescence detection system was from Amersham Pharmacia Biotech, and the 3', 5', 5', 5'-tetramethylbenzidine Peroxidase Substrate was from Kirkegaard and Perry Laboratories. The B6–30N, rho 4D2, K42–41L, and rho 1D4 monoclonal antibodies, which are specific for amino acid sequences 3–14, 2–39, 276–286, and 341–348 of bovine opsin, respectively, have been described (10–12). An anti-rhodopsin polyclonal antibody, opi, was a gift from B. Knox (SUNY Medical School, Syracuse, NY). The sources of other materials used in this investigation have been reported (8, 9).

**Methods**

**Construction of Opsin Gene Fragments**—All opsin gene fragments were constructed by restriction fragment replacement of the bovine opsin gene in the pMT-3 expression vector (13, 14). The HF(233–280), EF(233–348/P267G), EF(233–348/P267L), EF(233–348/W265F), TH(241–348/P267G), TH(241–348/P267L), TH(241–348/W265F), and FO(249–312) gene fragments (see Table I) were constructed by replacing the appropriate restriction fragment with a synthetic oligonucleotide duplex containing a CCACC consensus sequence (15) and a Met codon (ATG) to provide a translation initiation site. Construction of the EF(1–232), EF(233–348), TH(241–348), and EF(249–348) gene fragments has been described (8, 9). The sequences of the opsin gene fragments were confirmed by the dideoxynucleotide chain termination method of DNA sequencing (16).

**Expression and Purification of Opsin Polypeptide Fragments**—Procedures for the transient transfection of COS-1 cells with the opsin genes and gene fragments have been described (8, 17). The transfected cells were harvested 55–72 h after addition of DNA and washed with phosphate-buffered saline (10 mM NaH2PO4, pH 7.0/150 mM NaCl). The cells were either solubilized with 1% (w/v) DM in phosphate-buffered saline/1 mM phenylmethylsulfonyl fluoride or incubated with 5 μM 11-cis-retinal at 3 h at 4 °C in the dark. The retinal reconstituted proteins were solubilized with 1% DM in phosphate-buffered saline/0.1 mM phenylmethylsulfonyl fluoride and purified on immobilized rho 1D4 antibody or concanavalin A as described (8, 17).

**SDS-PAGE Analysis of Opsin Polypeptide Fragments**—Protein samples were analyzed by reducing SDS/Tris-glycine PAGE (18) with a 5% stacking and a 15 or 16% resolving gel and electroblotted onto poly(vinylidenefluoride) membranes (19). In some cases, the proteins were analyzed by nonreducing SDS/Tris-tricine PAGE (20) with a 4% stacking and a 10 or 12% resolving gel. Immunoreactive protein was detected using the B6–30N, rho 4D2, K42–41L, rho 1D4, or opi primary antibodies and horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG as the second antibody. The protein bands were visualized by chemiluminescence.

**Other Methods**—Enzyme-linked immunosorbent assays were carried out as described (9). COS-1 cell membranes were prepared by hypotonic lysis essentially as described (21). Membrane integration of wild-type opsin or the opsin fragments was determined by incubating the crude membrane preparations with 100 mM Na2CO3, pH 11.0, for 1 h at 4 °C (22). Protein determinations were done using the method of Peterson (23) with bovine serum albumin as the standard.

**RESULTS**

**Expression of the Opsin Fragments in COS-1 Cells**

The Singly Expressed Carboxyl-terminal Bovine Opsin Fragments—Cellular expression of the opsin fragments (Table I) was examined by immunoblotting whole cell detergent extracts with the rho 1D4, K42–41L, or opi primary antibodies. All of the singly expressed carboxyl-terminal fragments were stably produced in COS-1 cells (Fig. 2). A polypeptide of ~15 kDa was noted for the HF(233–280) fragment (Fig. 2A), which is considerably higher than the calculated molecular mass of ~5.5 kDa. Similarly, the EP(249–312) polypeptide (molecular mass = ~17 kDa) also migrated at a position much higher than the calculated molecular mass (~7.2 kDa). This most likely arises from an intrinsic property of many membrane proteins (and membrane protein fragments) to show anomalous migration on SDS-PAGE (9, 24). Importantly, both of these fragments required detergent for cellular extraction, suggesting that they are membrane integrated. Although the EF(233–348) and TH(241–348) fragments showed the appropriate size by SDS-PAGE, the corresponding fragments with Pro-267 mutations migrated at a slightly higher apparent molecular mass than their wild-type counterparts (Fig. 2, C and D). Replacement of Pro-267 by Gly and Leu presumably alters the conformation of the polypeptide fragment even in the presence of high concentrations of SDS. The spectral and functional properties of these Pro-267 mutations in context of the entire polypeptide chain have been previously reported (25–27). Notably, the Pro-267—Leu mutation is associated with autosomal dominant retinitis pigmentosa (26). Substitution of Trp-265 by Phe did not alter the migration of the EF(233–348) or TH(241–348) polypeptides. This mutation has been shown to have a profound effect on the spectral properties of rhodopsin (25, 28). The levels of carboxyl-terminal fragment production relative to wild-type opsin were estimated from enzyme-linked immunosorbent assays. With the exception of the HF(233–348) fragment, the carboxyl-ter-
minal fragments were present at levels equivalent to or higher than that of wild-type opsin (Table I).

The Coexpressed EF(1–232) Bovine Opsin Fragments—Stable expression of the EF(1–232) opsin fragment in cells transfected with the various carboxyl-terminal fragments was examined by immunoblotting of whole cell detergent extracts with the rho 4D2 or B6–30N primary antibodies. As shown previously (9), although single expression of the EF(1–232) fragment results in two faint polypeptides that are observed sporadically, coexpression of this opsin fragment with EF(233–348) results in stable and reproducible production of a high mannose complex relative to wild-type rhodopsin varied from 40%. Previous attempts to isolate a rhodopsin-like pigment composed of these fragments on immobilized rho 1D4 were unsuccessful (9). The reason(s) for the discrepancy between the wild-type fragment (Fig. 3A) Coexpression of EF(1–232) with TH(241–348) and EF(249–348) resulted in the appearance of two polypeptides at a level similar to that of coexpression with EF(233–348) (Fig. 2B and Table II). These findings suggest that the correct folding and membrane insertion of EF(1–232) does not depend on the presence of the third cytoplasmic loop (amino acids 233–240) and that this portion of the loop, if present, exerts a stabilizing effect on EF(1–232) expression. Surprisingly, introduction of the Pro-267 mutations into the TH(241–348) fragment resulted in the appearance of the two polypeptides at a level similar to that of coexpression with EF(233–348) (Fig. 2B and Table II). These findings suggest that the correct folding and membrane insertion of EF(1–232) does not depend on the presence of the third cytoplasmic loop (amino acids 233–240). Coexpression with HF(233–280) did not afford stable production of the EF(1–232) fragment (Fig. 2B). Similarly, removal of the third cytoplasmic loop and the sixth TM (FG(281–348)) also did not confer stable and reproducible production (Fig. 2C). However, the EF(249–312) fragment, which lacks the third cytoplasmic loop and carboxyl-terminal tail, resulted in significant EF(1–232) production (Fig. 2C and Table II). Taken together, the above results suggest that structural elements within the sixth and/or seventh TM influence the stable production of EF(1–232).

To further examine the role of the sixth TM in this process, EF(1–232) was coexpressed with EF(233–348) fragments containing Pro-267→ Gly/Leu or Trp-265→Phe mutations. As shown in Fig. 3D, the presence of the Pro-267 to Gly or Leu mutations in the EF(233–348) fragment essentially abolishes EF(1–232) expression. Surprisingly, introduction of the Pro-267 mutations into the TH(241–348) fragment resulted in the expression of EF(1–232) at levels similar to those of the wild-type fragment (Fig. 3E and Table II). Similarly, introduction of the Trp-265→Phe mutation into either the EF(233–348) and TH(241–348) fragments had essentially no effect on the level of EF(1–232) expression (Fig. 3F and Table II). These findings suggest that the Pro-267 mutations alter the conformation of the third cytoplasmic loop in the region extending from amino acids 233–240 and that this portion of the loop, if present, exerts a stabilizing effect on EF(1–232) expression.

Spectral Characterization of the Complexes Formed from the Coexpressed Opsin Fragments

The EF(1–232) + EF(233–348) fragment complex, like wild-type rhodopsin, shows a 500-nm chromophore after reconstitution with 11-cis-retinal (Fig. 4). The yield of this fragment complex relative to wild-type rhodopsin varied from ~25 to 40%. Previous attempts to isolate a rhodopsin-like pigment composed of these fragments on immobilized rho 1D4 were unsuccessful (9). The reason(s) for the discrepancy between the

| Opin or opsin fragment | Amino acids encoded | Restriction fragment(s) replaced | Opin or opsin fragment/10^6 cells |
|------------------------|--------------------|---------------------------------|---------------------------------|
| Wild type              | 1–348              |                                 |                                 |
| HF(233–280)            | 233–280            | EcoRI-PstI/NotI                 | 4.0                             |
| EF(233–348)            | 233–348            | EcoRI-PstI                     | 3.3                             |
| EF(233–348/W265F)      | 233–348            | EcoRI-PstI                     | 4.0                             |
| EF(233–348/P267G)      | 233–348            | EcoRI-PstI                     | 4.3                             |
| EF(233–348/P267L)      | 233–348            | EcoRI-MluI                     | 3.5                             |
| TH(241–348)            | 241–348            | EcoRI-MluI                     | 4.1                             |
| TH(241–348/P267G)      | 241–348            | EcoRI-MluI                     | 4.9                             |
| TH(241–348/P267L)      | 241–348            | EcoRI-MluI                     | 2.9                             |
| EF(249–312)            | 249–312            | EcoRI-MluI                     | 5.0                             |

* The values were obtained using the anti-rhodopsin rho 1D4, K42–41L, or opi antibodies. The data shown are averages from at least two independent determinations and are ± 0.2–0.5 μg.
indicated opsin polypeptides were solubilized in DM detergent, and equivalent amounts of protein (\(\mu g\)) in independent determinations and are B6–30N monoclonal antibodies. The data shown are averages from two present results and those reported earlier is not clear. Both the EF(1–232) + TH(241–348) and EF(1–232) + EF(249–312) fragment complexes formed chromophores to essentially the same level as the EF(1–232) + EF(233–348) complex (Fig. 4). Clearly, the complete absence of the third cytoplasmic loop does not appear to compromise the association of these fragments or their ability to bind retinal. This is consistent with the results of Franke et al. (29), who showed that a 13-amino acid deletion in the third cytoplasmic loop (positions 237–249) did not abolish chromophore formation. Although the EF(1–232) + TH(241–348)/P267G complex did not abolish chromophore formation. However, the EF(1–232) + TH(241–348)/P267L complex also formed the rhodopsin chromophore, the yield of regen

**Table II**

| Coexpressed opsin fragment | EF(1–232) opsin fragment/10^7 cells
|-----------------------------|--------------------------------|
| HF(233–280)                 | ND                            |
| EF(233–348)                 | 3.1                           |
| EF(233–348/W265F)           | 3.3                           |
| EF(233–348/P267G)           | ND                            |
| EF(233–348/P267L)           | ND                            |
| TH(241–348)                 | 3.4                           |
| TH(241–348/W265F)           | 3.1                           |
| TH(241–348/P267G)           | 2.8                           |
| TH(241–348/P267L)           | 3.0                           |
| EF(249–348)                 | 3.6                           |
| EF(249–312)                 | 3.4                           |
| FG(281–348)                 | ND                            |

*The values were obtained using the anti-rhodopsin rho 4D2 or B6–30N monoclonal antibodies. The data shown are averages from two independent determinations and are \(\pm 0.2–0.4 \mu g\), ND, no detectable expression.

The interactions that occur between different segments of a polypeptide chain during the folding and assembly process are the subject of numerous experimental and theoretical investigations. Although a variety of experimental strategies can be employed to gain valuable information about protein folding and assembly, we have utilized the approach of expressing fragments of a polypeptide to study this process in the integral membrane photoreceptor rhodopsin. Previous work from our and other laboratories has shown that many bovine opsin fragments contain sufficient information to fold independently, insert into a membrane, and assemble with a complementary fragment(s) in vivo to form a rhodopsin-like pigment (8, 9, 30, 31). However, not all opsin fragments are capable of folding independently and/or inserting into a membrane and appear to require other elements present in the corresponding complementary portion of the protein to adopt an appropriate conformation. Such is the case with the EF(1–232) opsin fragment, which does not fold to stable conformation unless it is coexpressed with its complementary fragment, EF(233–348). Presumably, the EF(1–232) fragment lacks the necessary information for proper folding and/or membrane insertion and is digested by cellular proteases that eliminate misfolded proteins from the cell. The purpose of this study was to examine whether truncated and/or mutated versions of EF(233–348) could stabilize expression of the EF(1–232) fragment.

Coexpression experiments with the various carboxyl-terminal fragments revealed that EF(1–232) production was independent of the third cytoplasmic loop and carboxyl-terminal tail but required both the sixth and seventh TM helices (Fig. 3, A–C). These findings suggest that helix-helix interactions between complementary TMs are sufficient to confer significant EF(1–232) fragment stabilization. However, mutations in the sixth TM showed that in some cases, the presence of the third cytoplasmic loop was detrimental to EF(1–232) expression (Fig. 4). Similarly, the EF(1–232) + TH(241–348)/P267L complex also formed a chromophore to a lesser degree than the P267L mutant. Coexpression of EF(1–232) with EF(233–348) resulted in a pigment with a blue-shifted chromophore (\(A_{\text{max}} \approx 480 \text{ nm}\)). This is the same absorbance maximum for the full-length opsin containing the Trp-265 → Phe mutation (27). Finally, the EF(1–232) + EF(249–312) fragment complex also bound retinal to form a 500-nm chromophore. After purification on immobilized concanavalin A (Fig. 4), the yield of the complex relative to that of wild-type rhodopsin was estimated to be \(\sim 30\%\). The lectin-purified pigment also showed a nearly-UV absorbing species, suggesting that a portion of the chromophore was linked as an unprotonated Schiff base. This has also been observed for the HG(1–312) fragment (9).

**DISCUSSION**

The interactions that occur between different segments of a polypeptide chain during the folding and assembly process are the subject of numerous experimental and theoretical investigations. Although a variety of experimental strategies can be employed to gain valuable information about protein folding and assembly, we have utilized the approach of expressing fragments of a polypeptide to study this process in the integral membrane photoreceptor rhodopsin. Previous work from our and other laboratories has shown that many bovine opsin fragments contain sufficient information to fold independently, insert into a membrane, and assemble with a complementary fragment(s) in vivo to form a rhodopsin-like pigment (8, 9, 30, 31). However, not all opsin fragments are capable of folding independently and/or inserting into a membrane and appear to require other elements present in the corresponding complementary portion of the protein to adopt an appropriate conformation. Such is the case with the EF(1–232) opsin fragment, which does not fold to stable conformation unless it is coexpressed with its complementary fragment, EF(233–348). Presumably, the EF(1–232) fragment lacks the necessary information for proper folding and/or membrane insertion and is digested by cellular proteases that eliminate misfolded proteins from the cell. The purpose of this study was to examine whether truncated and/or mutated versions of EF(233–348) could stabilize expression of the EF(1–232) fragment.

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chromophore with 11-cis-retinal (Fig. 4). These findings suggest that replacement of this highly conserved proline residue has a deleterious effect on a portion of the third cytoplasmic loop (amino acids 233–240) without dramatically altering the conformation and/or orientation of the sixth TM, a critical component of the retinal binding pocket. This is consistent with previous studies suggesting that Pro-267 mutations impair the kinetics of transducin activation by affecting the structure of the G-protein interaction site on the mutant protein (25, 27).

Site-directed spin labeling studies on cysteine substitution mutants of the sequence 232–249 indicate that this portion of the third cytoplasmic loop consists of solvent-exposed, regular secondary structure (32). Further, NMR studies on a synthetic peptide encompassing amino acids 231–252 suggests a turn-helix-turn motif for this segment of rhodopsin (33). Other models based on the available experimental data suggest that the 233–237 segment is in all probability a continuation of helical structure from the fifth TM, whereas residues 238–240 comprise a connecting turn to the sixth TM. Thus, it is not unreasonable to expect that addition of the “remainder” of the sequence, when in the appropriate conformation, helps stabilize the cytoplasmic end of the fifth TM. This conclusion is consistent with earlier work from Engelman and co-workers (34) on bacteriorhodopsin showing that a reduction in the length of the TM flanking sequences below a critical limit destabilizes the TM helix, thereby rendering it more susceptible to proteolysis. Despite this wealth of accumulated experimental and theoretical data on bovine opsin and related receptors, it is difficult to rationalize how the Pro-267 mutations exert their effects on the conformation of the third cytoplasmic loop. Although the third cytoplasmic loop is not critical for stabilization of the EF(1–232) fragment, when present, its conformation is important for establishing the proper contacts required for opsin folding, membrane insertion, assembly, and/or function. Future studies will focus on whether other site-directed or naturally occurring mutations in the TM helices also affect the conformation of the solvent exposed loop regions. For this purpose, the TOXCAT system recently developed by Ruas and Engelman (37) to examine helix-helix interactions in biological membranes may prove useful.

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