Studies on Light Transduction by Bacteriorhodopsin and Rhodopsin

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The visual photoreceptor pigments in vertebrates and invertebrates all use retinal (vitamin A aldehyde) as the light-absorbing molecule. Recently, Stoeckenius et al. (1979) discovered bacteriorhodopsin (bR) in the purple membrane of the extreme halophile, Halobacterium halobium, which also contains all-trans retinal as the chromophore. bR carries out light-dependent proton translocation from the inside to the outside of the H. halobium cell. Since the discovery of bR, H. halobium has been found to elaborate three more retinal-based light-transducing proteins. These are halorhodopsin, a chloride ion pump, and sensory rhodopsins I and II (Spudich and Bogomolni 1988).

The visual pigments as well as bR are all transmembrane proteins that are presumed to traverse the membrane seven times. The sole function of light in these retinal-based pigments appears to cause the isomerization of the protein-bound retinal molecules, 11-cis → all-trans for visual pigments and all-trans → 13-cis for bR. A series of structural changes ensues in the proteins, as evidenced by the photo intermediates (Wald 1968). We are carrying out structure-function studies of bacteriorhodopsin, bovine rhodopsin, and related proteins primarily by the technique of recombinant DNA; we summarize below the results we have obtained recently.

BACTERIORHODOPSIN

Bacteriorhodopsin as a Transmembrane Protein

bR contains a single polypeptide chain of 248 amino acids, whose sequence is known (Dunn et al. 1983). The protein is synthesized in the H. halobium cell as a precursor, with extra sequences of 13 amino acids at the amino terminus and an aspartic acid residue at the carboxyl terminus. The amino-terminal presequence appears to be removed cotranslationally, but the processing of the carboxy-terminal aspartic acid is not understood. The orientation of the mature protein in the membrane is such that the carboxyl terminus is at the cytoplasmic side with about 20 amino acid residues protruding into the cytoplasm, whereas the amino terminus is at the outside of the cell membrane (Fig. 1). All-trans retinal is attached as a Schiff base to Lys-216 in the putative helix G. From the mode of cross-linking of a photoactivatable retinal analog to the protein, the retinal appears to be oriented in the bilayer pointing toward the extracellular side of the protein (Huang et al. 1982). The latter result is not different from that obtained by other methods (Heyn et al. 1988).

The purple membrane can be solubilized to give bR as monomers in detergents such as Triton X-100. The bR monomers can be completely freed from endogenous phospholipids (Huang et al. 1980). The delipidated bR can be reconstituted into vesicles without denaturation or loss of the bound retinal. bR usually acquires inverted orientation in reconstituted vesicles irrespective of the phospholipids or the procedures used (Huang et al. 1980). However, largely right-side-out vesicles can be prepared from the H. halobium cell envelopes (MacDonald and Lanyi 1975). The most remarkable property of bR is its rapid and virtually quantitative renaturation following complete denaturation (Huang et al. 1981). Thus, the denatured apoprotein, bacterio-opsin (bO), refolds correctly and quantitatively to its native structure, binds retinal, and regenerates the bR chromophore. Purified proteolytic fragments of bR also reassociate to form the native bR-like structure (Liao et al. 1984).

Structure of bR and Intermediates in the Photocycle

A secondary structure model consistent with the electron and neutron diffraction data, the primary structure, and proteolytic cross-linking data is shown in Figure 1. Many uncertainties and questions remain in the model; for example, (1) correlation of the helices in the diffraction pattern with those in the primary structures; (2) the number of amino acids in the individual α helices; (3) the sizes and structures of the loops that connect the successive helices; and (4) the specific interactions between the membrane-embedded helices.

The photointermediates observed in the bR photocycle have been intensively studied (Stoeckenius et al.
An incisive synthesis of a great deal of conflicting work on the bR photocycle has been accomplished recently by Kouyama et al. (1988). Their model is consistent with the translocation of one proton per photocycle.

The photointermediates may reflect changes in the protein, internal motions, or conformational changes that correspond to steps in proton translocation. A number of questions arise; e.g., is the transient deprotonation of the Schiff base at the M state directly coupled to H⁺ translocation? The deprotonation-protonation of other groups such as tyrosine and aspartic acid side chains has been demonstrated recently (Braiman et al. 1988 and in prep.). The availability of defined mutants described below makes it possible to identify the specific amino acid residues involved.

Structure-function Studies by Amino Acid Replacements

The central question is the mechanism of proton translocation by bR. In particular, does it involve proton conductance along a hydrogen-bonded chain in which the side chains of certain specific amino acids participate? Second, the absorption characteristics of the chromophore in bR and the visual pigments display a large bathochromic shift relative to retinal Schiff base. How is this shift determined by protein-retinal interactions? Third, what is the nature of the specific interactions among the membrane-embedded helices and between the protein and the phospholipids that stabilize the folded structure of bR?

One approach to the above questions is specific amino acid replacements. Site-specific mutagenesis at the gene level provides a versatile approach for this purpose. The bR gene in H. halobium has been characterized (Dunn et al. 1981). To express the gene, two broad approaches can be considered. The first is transformation of H. halobium with a suitable vector carrying the gene or its mutants. This approach is currently under investigation. The second approach is the use of heterologous hosts such as Escherichia coli for expression of the bR gene and its mutants. This approach, which has been used in the present work, involves the following steps: (1) the use of a totally synthetic bR gene containing suitably placed unique restriction sites (Nassal et al. 1987) and the substitution of amino acids by replacement of restriction fragments encoding the native amino acid sequence by synthetic counterparts containing the altered codon(s); (2) development of a relatively efficient expression system for bO (Karnik et al. 1987); (3) rapid and efficient purification of the expressed bO mutants in the denatured form (Braiman et al. 1987); and (4) refolding of the bO mutants and regeneration of the bR-like chromophores in the presence of retinal.

To test if the H⁺ translocation mechanism involves the participation of amino acids with side chains capable of forming H bonds, substitutions of single amino acids, such as Tyr→Phe, were carried out (Mogi et al. 1987).

Single Amino Acid Substitutions

The total substitutions made are shown in Table 1 (Fig. 1). All the mutants regenerated bR-like chromophores. However, the rates of chromophore regeneration for the different mutants varied widely (Table 2). Some mutants were very slow (e.g., Asp-212→Glu) in regenerating the chromophore, whereas an occasional mutant regenerated faster than native bO (e.g., Asp-85→Glu). Furthermore, on the basis of their effects on the absorption spectrum, the mutants could be divided into two classes (Table 3). Class I showed chromophores with λmax values very similar to that of the wild-type bR. Examples are 8 of the 11 Tyr→Phe replacements (Mogi et al. 1987) and several Asp→Asn, Arg→Gln, and Pro→Ala substitutions. The chromophores in class II mutants showed significant to marked shifts in the λmax from that of the wild type. Trp-86, -137, -182, and -189→Phe substitutions gave relatively large (20–70 nm) blue shifts (T. Mogi, unpubl.). The substitutions Pro-186→Leu (Val) and Thr-89→Val also gave large (70 nm) blue shifts, whereas some other substitutions (Asp-115→Glu, Asp-212→Ala) gave smaller (~20 nm) blue shifts. In contrast, the substitutions Asp-85→Asn, Asp-212→Glu, and Arg-82→Cys or Gln gave large (30–35 nm) red shifts.

We conclude that the spectral shifts in the mutants are caused by the substitution of those amino acids that interact with retinal in some way, whereas the amino acids corresponding to class I substitutions do not interact with the chromophore, at least in the ground state. It is noteworthy that the amino acids whose replacements cause spectral shifts are inside the membrane in the secondary structure model (Fig. 1).
Table 1. Single Amino Acid Substitution Mutants of bR

<table>
<thead>
<tr>
<th>Amino acid substitution</th>
<th>Positions in bR sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg → Gin</td>
<td>7, 82, 134, 164, 175, 225, 227</td>
</tr>
<tr>
<td>Asp → Asn</td>
<td>36, 38, 85, 96, 102, 104, 115, 212</td>
</tr>
<tr>
<td>XXX → Cys</td>
<td>Gly-72, Arg-82, Thr-90, Leu-92, Ser-169, Lys-216</td>
</tr>
<tr>
<td>Glu → Gin</td>
<td>9, 74, 161, 166, 194, 204</td>
</tr>
<tr>
<td>Pro → Ala</td>
<td>50, 91, 186</td>
</tr>
<tr>
<td>Ser → Ala</td>
<td>183, 193</td>
</tr>
<tr>
<td>Thr → Val</td>
<td>17, 24, 46, 51, 55, 89, 90, 107, 121, 128, 142, 157, 178, 205</td>
</tr>
<tr>
<td>Trp → Phe</td>
<td>10, 12, 80, 86, 137, 138, 182, 189</td>
</tr>
<tr>
<td>Tyr → Phe</td>
<td>26, 43, 57, 64, 79, 83, 131, 133, 147, 150, 185</td>
</tr>
</tbody>
</table>

Data from Hackett et al. (1987), Mogi et al. (1987, 1988), and T. Mogi et al. (unpubl.).

*Every amino acid of this type has been substituted as indicated.
Other substitutions have also been made at this position.

Table 2. Chromophore Regeneration Rates of Selected Single Amino Acid Substitution Mutants of bR

<table>
<thead>
<tr>
<th>Substitution</th>
<th>Regeneration rate 1/2 (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Wild type)</td>
<td>0.6</td>
</tr>
<tr>
<td>Arg-82 → Gin</td>
<td>15</td>
</tr>
<tr>
<td>Asp-85 → Asn</td>
<td>20</td>
</tr>
<tr>
<td>Asp-85 → Glu</td>
<td>0.1&gt;</td>
</tr>
<tr>
<td>Asp-212 → Asn</td>
<td>38</td>
</tr>
<tr>
<td>Asp-212 → Glu</td>
<td>31</td>
</tr>
<tr>
<td>Pro-91 → Ala</td>
<td>29</td>
</tr>
<tr>
<td>Thr-89 → Val</td>
<td>54</td>
</tr>
<tr>
<td>Tyr-57 → Phe</td>
<td>22</td>
</tr>
<tr>
<td>Tyr-185 → Phe</td>
<td>11</td>
</tr>
</tbody>
</table>

Table 3. Absorbance Maxima of the Chromophores of Selected Single Amino Acid Substitution Mutants of bR

<table>
<thead>
<tr>
<th>Substitution</th>
<th>λ_max (light adapted) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Wild type)</td>
<td>561</td>
</tr>
<tr>
<td>Arg-82 → Gin</td>
<td>580</td>
</tr>
<tr>
<td>Asp-85 → Asn</td>
<td>594</td>
</tr>
<tr>
<td>Asp-85 → Glu</td>
<td>560</td>
</tr>
<tr>
<td>Asp-212 → Asn</td>
<td>548</td>
</tr>
<tr>
<td>Asp-212 → Glu</td>
<td>581</td>
</tr>
<tr>
<td>Pro-186 → Ala</td>
<td>567</td>
</tr>
<tr>
<td>Thr-99 → Val</td>
<td>479</td>
</tr>
<tr>
<td>Trp-182 → Phe</td>
<td>493</td>
</tr>
<tr>
<td>Trp-189 → Phe</td>
<td>491</td>
</tr>
<tr>
<td>Tyr-185 → Phe</td>
<td>521</td>
</tr>
<tr>
<td>Tyr-185 → Phe</td>
<td>573</td>
</tr>
</tbody>
</table>

Table 4. Proton Pumping Activity of Selected Single Amino Acid Substitution Mutants of bR

<table>
<thead>
<tr>
<th>Substitution</th>
<th>Pumping rate * (Wild type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Wild type)</td>
<td>1.00</td>
</tr>
<tr>
<td>Asp-85 → Asn</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Asp-85 → Glu</td>
<td>0.32</td>
</tr>
<tr>
<td>Asp-96 → Asn</td>
<td>0.03</td>
</tr>
<tr>
<td>Asp-96 → Glu</td>
<td>0.82</td>
</tr>
<tr>
<td>Asp-212 → Asn</td>
<td>0.15</td>
</tr>
<tr>
<td>Asp-212 → Glu</td>
<td>0.06</td>
</tr>
<tr>
<td>Pro-186 → Ala</td>
<td>1.09</td>
</tr>
<tr>
<td>Pro-186 → Val</td>
<td>0.32</td>
</tr>
<tr>
<td>Trp-86 → Phe</td>
<td>0.26</td>
</tr>
<tr>
<td>Trp-137 → Phe</td>
<td>0.34</td>
</tr>
<tr>
<td>Tyr-185 → Phe</td>
<td>0.36</td>
</tr>
</tbody>
</table>

*Fraction of wild-type proton pumping rate.

Amino Acid Substitutions That Affect Proton Pumping

Examples where proton pumping seemed to be partially affected were found (Table 4). Thus, in Tyr-185 → Phe, it was 40% of normal. The meaning of partial blocks in proton pumping is not clear. Multiple interpretations are possible. Additional mutations were found that had strong effects on proton pumping. Replacement of Asp-85 or Asp-96 by asparagine abolished proton pumping completely or almost completely (Mogi et al. 1988). Asp-212 mutants (Asp-212 → Glu and Asp-212 → Asn) showed 10–20% proton pumping, whereas the mutant Asp-212 → Ala was unstable to light and showed no detectable pumping. Therefore, it is certain that Asp-85 and Asp-96 in helix C (Fig. 1) are involved in proton translocation. Similarly, Asp-212 may also participate in proton translocation, or it may regulate protonation-deprotonation of the Schiff base. These results show for the first time that proton translocation occurs by a conductance pathway involving the side chains of several amino acids. An immediate task is the characterization of the photocycles of the above mutants. Fourier transform infrared spectroscopy and UV difference spectroscopy of the Tyr-185 → Phe mutant have shown that the Tyr-185 residue normally changes its protonation state during the br → K step and again during the L → M step (Ahl et al. 1988; Braiman et al. 1988). These and other time-resolved experiments (T. Marinetti et al., unpubl.) have begun to reveal steps at which Asp-85, -96, and -212 gain or lose protons.

Projection Model for Bacteriorhodopsin

The projection model (Fig. 2) (Mogi et al. 1988) shows rough spatial relationships between the amino...
Figure 2. Helical wheel model of bacteriorhodopsin showing the relationships between the aspartic acid mutations that affect proton pumping and the amino acids that are believed to interact with the chromophore.

acids relevant to proton translocation. (Those amino acids that are believed to interact with retinal are shown in ovals.) One possible starting point for proton movement could be Asp-96. Another possible sequence of proton transfers could begin with the protonated Schiff base, which undergoes rapid deuterium/hydrogen exchange with water in the dark and perhaps maintains tight association with a water molecule (Hi!-debrandt and Stockburger 1984). If proton translocation begins at the Schiff base, how is the proton subsequently restored to this site? Is there a gated pore that makes the Schiff base transiently accessible to water from the cytoplasmic side? Such a light-dependent channel seems reasonable because of the known light-dependent bleaching of the protonated Schiff base by NH$_2$OH as well as its light-dependent reduction by NaBH$_4$.

Further Work

Although the results on mutants affecting proton translocation are encouraging, clearly we need to know a great deal more to understand the mechanism of the process. Site-specific mutagenesis can be taken further and, in addition, chemical and biophysical studies of the different groups of mutants must be comprehensive. Additional chemical approaches need to be tapped for understanding bR as a membrane protein. Furthermore, an essential requirement is the solution of the three-dimensional structure. This is most urgent, since we need to bring about a synthesis of the different types of results. Finally, we need to understand the energetics of coupling in the H$^+$ translocation process in bR.

VISUAL RHODOPSIN

Rhodopsin is the photoreceptor in the rod cell of the visual system. It is an integral membrane protein for which a secondary structure model (Fig. 3) has been proposed (Dratz and Hargrave 1983). The chromophore, 11-cis-retinal, is linked to Lys-296 as a Schiff base. The isomerization of retinal, 11-cis to all-trans, causes structural changes in the protein that result in the formation of a series of transient intermediates (Wald 1968). One intermediate, meta-rhodopsin II, seems to activate a guanine nucleotide-binding protein, transducin (T), and undergoes phosphorylation by rhodopsin kinase (Stryer 1986). Following activation, T exchanges bound GDP for GTP and dissociates from the $\gamma$ subunits to form $\gamma$-GTP. The latter then activates the rod cell cGMP phosphodiesterase. This results in a decrease in cGMP levels, which causes closure of plasma membrane cation channels, with consequent hyperpolarization of the rod cell. The above cascade is modulated by the intrinsic GTPase activity of $\gamma$-GTP, by rhodopsin phosphorylation, and possibly by 48K protein binding to the phosphorylated rhodopsin.

Rhodopsin is an example par excellence of a large number of receptors (e.g., adrenergic, muscarinic, and neural peptide hormone receptors) containing seven transmembrane segments that undergo structural changes by light or ligand binding and activate G-binding proteins and are subsequently desensitized by phosphorylation and, possibly, other mechanisms (Sibley et al. 1987). Rhodopsin and related pigments of the visual system are attractive models for studies of the above general and important classes of signal transduction systems.

Orientation of Retinal in Rhodopsin

At one end, retinal is tethered to Lys-296. However, its orientation and the nature of its binding interactions within the protein are not known. One approach is the use of a retinal analog that carries a photoactivatable group. Following photolysis, the specific amino acid(s) to which the analog becomes cross-linked could be determined. Rhodopsin was reconstituted with the $^3$H-labeled analog, 1,5-dimethyl-3-trifluoromethyl diazirinophenyl 11-cis-retinal (I).

\[ \text{(I)} \]

A rhodopsin-like pigment with $\lambda_{\text{max}}$ at 458 nm was obtained. The linkage of the analog to Lys-296 via Schiff base was confirmed. Furthermore, on illumination, the reconstituted rhodopsin activated transducin in a light-dependent manner and also underwent phosphorylation by rhodopsin kinase. Photolysis of the reconstituted pigment at 365 nm ($-15^\circ\text{C}$) led to the cross-linking of the analog to the opsin. After photolysis and separation of the resulting peptides, two radioactive peptides were obtained. Sequence analysis showed that...
the major cross-linking occurred within the sequences Gly-114-Glu-150 (putative helix C) and the minor within Ser-202-Gly-232 (putative helix E). Thus, the β-ionone ring of retinal appears to orient toward helices C and E from its point of attachment in helix G. Identification of sites of cross-linking should provide insight into specific interactions of the retinal with amino acid side chains that determine the spectral properties of the pigment.

Expression of a Rhodopsin Gene in COS-1 Cells

The first requirement for structure-function studies of rhodopsin is the satisfactory expression of rhodopsin in a functional form. We have previously reported on the total synthesis of a gene for rhodopsin (Ferretti et al. 1986). The chief motivation, as with bR, was site-specific mutagenesis by cassette replacement.

The vector for the expression of the rhodopsin gene in the monkey kidney cell line, COS-1, has been described elsewhere (Oprian et al. 1987). Isolation of more than 80% pure rhodopsin from the cells was accomplished by the use of an immunoaffinity procedure. Rhodopsin thus prepared showed the characteristic UV/Vis absorption spectrum and activated bovine transducin in a light-dependent manner. The specific activity of COS cell rhodopsin was identical to that of purified bovine rhodopsin.

Expression of Rhodopsin in Xenopus Oocytes and Coupling to Endogenous Channels

Xenopus oocytes provide a complementary expression system in which receptors and channels can be assayed in situ (Dascal 1987). The expression of the synthetic gene encoding bovine rhodopsin, its SP6 transcripts, and total retinal mRNA was demonstrated in Xenopus oocytes. Immunoprecipitation showed the presence of three forms of opsin, with an apparent relative molecular weight of 41K, 35K, and 30K. The 30K protein was unglycosylated opsin, whereas the higher-molecular-weight proteins, the major products, were differentially glycosylated forms. Addition of 11-cis-retinal to the cells expressing the above proteins regenerated rhodopsin, which, after purification by immunoaffinity chromatography, showed the expected UV/Vis absorption spectrum and characteristic light-dependent activation of T. Oocytes expressing rhodopsin exhibited light-dependent ionic currents that were detected by voltage clamp techniques (Fig. 4).

In Vivo Phosphorylation of Rhodopsin in COS Cells

Light-dependent desensitization of visual transduction resulting in impaired G-protein activation is thought to involve receptor phosphorylation (Kuhn et al. 1973; Liebman and Pugh 1980). Phosphorylation of
rhodopsin as expressed in COS cells was tested as follows. The transfected COS cells were incubated with inorganic $^{32}$P after the addition of 11-cis-retinal. Samples were either left in the dark or exposed to room light. Rhodopsin was then purified. SDS-PAGE, followed by silver-staining and autoradiography, showed that rhodopsin regenerated with 11-cis-retinal and exposed to light in COS cells was phosphorylated, whereas controls (dark, COS cell opsins lacking 11-cis-retinal) were not phosphorylated (Fig. 5). The results indicate light-dependent phosphorylation of rhodopsin in COS cells by endogenous receptor kinases such as β-adrenergic receptor kinases (Benovic et al. 1986). Furthermore, purified COS cell rhodopsin, following incorporation into phospholipid vesicles, was also phosphorylated in a light-dependent manner by rod outer segment extracts enriched for rhodopsin kinase activity.

Role of Cysteine Residues in Rhodopsin Structure-Function

The role of cysteine residues in integral membrane proteins has been studied by a number of workers, but clear-cut conclusions are largely lacking. Bovine rhodopsin contains ten cysteine residues in the primary structure (Fig. 3). These residues are conserved in human and bovine rhodopsin (Applebury and Hargrave 1986). The cysteines appear to be distributed into three topographical groups as follows: three cysteines (Cys-110, Cys-185, and Cys-187) are in the intradiskal loops, four cysteines (Cys-140, Cys-167, Cys-222, and Cys-264) are membrane-embedded, and three cysteines (Cys-316, Cys-322, and Cys-323) are located in the carboxy-terminal tail (Dratz and Hargrave 1983). Conflicting conclusions have been drawn by different workers regarding the involvement of different cysteine residues in disulfide bond formation.

We have constructed ten mutants of the opsin gene in which cysteines were replaced by serines as shown in Table 5. All of the mutants (CysI-CysVII) were expressed in COS cells. An immunoblot of the product is shown in Figure 6. The band patterns obtained with CysII (replacements of Cys-140, -167, -222, and -264), CysIII (replacements of Cys-316, -322, and -323), and CysV (replacements as in CysII and CysIII) are typical of the pattern obtained with the wild-type rhodopsin gene. However, the mutant groups (CysI, CysIV, CysVI, and CysVII) gave patterns that were similar to each other, but radically different from that obtained in...
Figure 5. Light-dependent in vivo phosphorylation of rhodopsin in COS cells. The rhodopsin gene was expressed in COS cells; $^{32}$P inorganic phosphate and 11-cis-retinal were added. A portion of the cells were exposed to light and the remainder was kept in the dark. After purification by immunoaffinity absorption, the cells were subjected to SDS-PAGE and autoradiography. (Lane 1) Rhodopsin purified from COS cells incubated with $^{32}$P but without 11-cis-retinal; (lane 2) rhodopsin from cells preincubated with 11-cis-retinal and $^{32}$P and kept in the dark; (lane 3) rhodopsin from 11-cis-retinal-treated cells, labeled with $^{32}$P under light exposure.

with the wild type. In each, there were faster-migrating bands, suggesting altered levels of glycosylation. The area above the band corresponding to the "normally" glycosylated rhodopsin was, in each case, strikingly devoid of slower-moving proteins (Fig. 7). In addition, whereas the class B (Table 5) mutants all regenerate the chromophore with 11-cis-retinal and activate transducin in a light-dependent manner, none of the class A (Table 5) mutants regenerate the chromophore or activate transducin.

Further study with single Cys→Ser substitution mutants showed (Table 5) that replacement of Cys-110 or Cys-187 conferred on these mutants the phenotype of class A mutants (CysI) (Fig. 7), whereas the substitution of Cys-185 gave the wild-type protein pattern.

These results show that, although all the other cysteine residues in rhodopsin are dispensable, Cys-110 and Cys-187 are necessary. Presumably, the formation of a specific disulfide bond between Cys-110 and Cys-187 is required for the formation of the correct tertiary structure. This enables the binding of 11-cis-retinal and the subsequent activation of transducin.

Transducin Interaction with Photolyzed Rhodopsin

To probe the rhodopsin domain(s) that interacts with transducin, rhodopsin mutants with alterations on the cytoplasmic surface have been prepared (Franke et al. 1988). The following three mutants in loop EF (Fig. 3) were prepared: mutant 1, Glu-239→Gln; mutant 2, Lys-248→Leu; and mutant 3, Glu-247→Gln, Lys-248→Leu, and Glu-249→Gln. Following expression in COS cells, all of the mutant proteins displayed wild-type visible absorption spectra. However, as seen in Figure 7, only mutants 1 and 3 stimulated the light-dependent GTPase activity of transducin. Mutant 2 failed to activate transducin. These results show that the loop EF is involved in some way in the interaction with transducin.

Deletions in the Intradiskal Loops

A series of mutants with varying deletions in the loops BC, DE, and FG were prepared. The amino acid sequences deleted were as follows: (1) 102–109, (2) 177–182, (3) 181–197, (4) 189–197, and (5) 280–283.
and 8K, respectively. Although the primary structures of the three subunits via molecular cloning are known (Medynski et al. 1985; Sugimoto et al. 1985; Yatsunami and Khorana 1985; Yatsunami et al. 1985), no information is available concerning the topological organization of the functional domains and their interactions during the transduction process. We are employing chemical cross-linking techniques to identify specific amino acid residues that play a role in subunit-subunit interactions.

The ability of cupric phenanthroline (CuPh) to catalyze the formation of disulfide bonds in transducin was investigated. Incubation of transducin with CuPh produced a cross-linked species that migrated on electrophoresis under nonreducing conditions with an apparent molecular weight of 43K. The latter species was not observed when the sample was treated with β-mercaptoethanol before electrophoresis. Again, the 43K band was absent if the incubation mixture was treated with N-ethylmaleimide and EDTA prior to the addition of CuPh. Further experiments show that the 43K product results from disulfide bonds induced between T and T. Identification of the specific cysteines involved is under investigation.

**Synthesis and Expression of a Gene for the α Subunit of Transducin**

A gene for the α subunit of transducin (T) was synthesized. By design, the synthetic gene contains 38 unique restriction sites, so as to facilitate site-specific mutagenesis by restriction fragment replacement. The 1076-bp gene was assembled by enzymatic joining of 44 synthetic oligonucleotides. The oligonucleotides were first joined to form four fragments of roughly equal lengths. These were purified and then cloned to assemble the entire gene (Sakmar and Khorana 1988). The synthetic T gene was expressed in transiently transfected COS cells using the expression vector (pMT3) described previously (Franke et al. 1988). The level of the synthetic T gene expression was comparable to the level of the corresponding cDNA expression, indicating that the codon changes introduced in the synthetic gene

**Figure 7.** Rhodopsin mutant 2 (Lys-248→Leu) did not enhance the GTPase activity of transducin. A time course of GTP hydrolysis by transducin is shown for COS-1 rhodopsin and for each mutant. (A) Rhodopsin purified from COS-1 cells (wild-type); (B) mutant 1; (C) mutant 2; (D) mutant 3. Reactions were performed in the light and in the dark. Mutant 2 did not activate transducin. The specific activities of the other 2 mutants (1.6 min⁻¹) were similar to that of wild-type COS-1 cell rhodopsin (1.0 min⁻¹).

**Figure 8.** Immunoblot analysis of the α subunit of transducin was expressed in COS cells. (Lane 5) Purified bovine holotransducin (100 ng); (lane 1) mock transfected COS cells; (lane 2) COS cells transfected with expression vector without insert; (lane 3) COS cells transfected with vector containing synthetic gene; (lane 4) COS cells transfected with vector containing cDNA. Endogenous COS cell G-protein 36 kD β subunit cross-reacts with anti-holotransducin antibody.

**References**

- Kuhn, et al. (1982).

**Regions of Contact between β and γ Subunits**

Transducin is a heterotrimer composed of α, β, and γ subunits with molecular weights of about 40K, 36K, and 8K, respectively. Although the primary structures of the three subunits via molecular cloning are known (Medynski et al. 1985; Sugimoto et al. 1985; Yatsunami and Khorana 1985; Yatsunami et al. 1985), no information is available concerning the topological organization of the functional domains and their interactions during the transduction process. We are employing chemical cross-linking techniques to identify specific amino acid residues that play a role in subunit-subunit interactions.

The ability of cupric phenanthroline (CuPh) to catalyze the formation of disulfide bonds in transducin was investigated. Incubation of transducin with CuPh produced a cross-linked species that migrated on electrophoresis under nonreducing conditions with an apparent molecular weight of 43K. The latter species was not observed when the sample was treated with β-mercaptoethanol before electrophoresis. Again, the 43K band was absent if the incubation mixture was treated with N-ethylmaleimide and EDTA prior to the addition of CuPh. Further experiments show that the 43K product results from disulfide bonds induced between T and T. Identification of the specific cysteines involved is under investigation.

**Synthesis and Expression of a Gene for the α Subunit of Transducin**

A gene for the α subunit of transducin (Tα) was synthesized. By design, the synthetic gene contains 38 unique restriction sites, so as to facilitate site-specific mutagenesis by restriction fragment replacement. The 1076-bp gene was assembled by enzymatic joining of 44 synthetic oligonucleotides. The oligonucleotides were first joined to form four fragments of roughly equal lengths. These were purified and then cloned to assemble the entire gene (Sakmar and Khorana 1988). The synthetic Tα gene was expressed in transiently transfected COS cells using the expression vector (pMT3) described previously (Franke et al. 1988). The level of the synthetic Tα gene expression was comparable to the level of the corresponding cDNA expression, indicating that the codon changes introduced in the synthetic gene

**Figure 7.** Rhodopsin mutant 2 (Lys-248→Leu) did not enhance the GTPase activity of transducin. A time course of GTP hydrolysis by transducin is shown for COS-1 rhodopsin and for each mutant. (A) Rhodopsin purified from COS-1 cells (wild-type); (B) mutant 1; (C) mutant 2; (D) mutant 3. Reactions were performed in the light and in the dark. Mutant 2 did not activate transducin. The specific activities of the other 2 mutants (1.6 min⁻¹) were similar to that of wild-type COS-1 cell rhodopsin (1.0 min⁻¹).

**Figure 8.** Immunoblot analysis of the α subunit of transducin was expressed in COS cells. (Lane 5) Purified bovine holotransducin (100 ng); (lane 1) mock transfected COS cells; (lane 2) COS cells transfected with expression vector without insert; (lane 3) COS cells transfected with vector containing synthetic gene; (lane 4) COS cells transfected with vector containing cDNA. Endogenous COS cell G-protein 36 kD β subunit cross-reacts with anti-holotransducin antibody.
did not affect translation. Furthermore, levels of the endogenous COS cell G-protein 36-kD β subunit did not appear to be affected by overexpression of Tα. The 36-kD β subunit of various G proteins has been shown to be immunologically identical to Tβ (Mumby et al. 1986). An immunoblot analysis using an anti-holotransducin antibody is shown in Figure 8. Purification and characterization of the Tα expressed in COS cells are in progress.

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