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Chemical Cross-linking of Bovine Retinal Transducin and cGMP Phosphodiesterase*

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The bifunctional reagents *para*-phenyldimaleimide and maleimidobenzoyl-*N*-hydroxysuccinimide ester were used to chemically cross-link the subunits of the transducin and cGMP phosphodiesterase (PDE) complexes of bovine rod photoreceptor cells. The cross-linked products were identified by Western immunoblotting using antisera against purified subunits of transducin (T_α and $T_{\beta\gamma}$) and PDE. Oligomeric cross-linked products of transducin subunits as large as $(T_{\alpha\beta\gamma})_3$ were observed in the latent form of transducin with bound GDP. In addition to the expected $T_{\alpha\beta}$ and $T_{\beta\gamma}$ cross-linked products, a $(T_{\alpha\gamma})_2$ structure was detected. The close proximity of T_α and T_γ suggests that T_γ may play a role in conferring the specificity of the interaction between T_α and rhodopsin. Most of the oligomeric cross-linked structures between T_α and $T_{\beta\gamma}$ were diminished in the activated form of transducin, with guanosine 5′-(β,γ-imidotriphosphate) (Gpp(NH)p) bound. However, cross-linking between T_β and T_γ was not altered. These results suggest that transducin exists as an oligomer in solution which dissociates upon the binding of Gpp(NH)p. To identify the possible interacting domains between the T_α, T_β, and T_γ subunits, the cross-linked products were subjected to limited tryptic proteolysis. Several cross-linked tryptic peptides of transducin subunits were found and include the cross-linked products of the N terminus 15-kDa fragment of T_β and the C terminus 5-kDa fragment of T_α, T_γ and the 12-kDa fragment of T_α, T_γ and the 15-kDa as well as the 23-kDa fragments of T_β, and an intra-T_α cross-linked product of the 2- and 21-kDa fragments. These results have allowed the construction of a topographical model for the transducin subunits. The organization of the subunits of PDE (P_α, P_β, and P_γ) was also studied. The formation of the high molecular size cross-linked products of PDE resulted in the concurrent loss of the P_β and P_γ subunits, suggesting that they are in close proximity. Finally, the interaction between transducin and PDE was examined by chemical cross-linking of transducin-Gpp(NH)p and PDE. Two additional cross-linked products of 180 and 210 kDa were obtained which could be due to the cross-linking of T_α or T_β with $P_{\alpha\beta}$ subunits. These results suggest that the activation of PDE may involve a direct interaction between T_α·Gpp(NH)p and $T_{\beta\gamma}$ with the $P_{\alpha\beta}$ subunit of PDE.

Visual excitation in vertebrate retinal rod cells is mediated by a cGMP enzyme cascade (for a review, see Liebman *et al.*, 1987; Hurley, 1987; Stryer, 1986; Stryer and Bourne, 1986; Chabre, 1985; Fung, 1985; Applebury and Hargrave, 1986). Three major protein components are involved in the signal transduction process: the photoreceptor molecule (rhodopsin), a GTP-binding protein called transducin (T),[1] and a cGMP phosphodiesterase (PDE). Both transducin and PDE are heterotrimeric complexes containing three different polypeptide chains. Transducin can be separated into two subunits: T_α (40 kDa) and $T_{\beta\gamma}$ (37 and 8 kDa). The T_α subunit containing the GTP-binding site functions as the signal carrier, and the $T_{\beta\gamma}$ subunit mediates the interaction of T_α with rhodopsin. The PDE complex is a latent enzyme. The $P_{\alpha\beta}$ subunit (88 and 84 kDa) contains the catalytic site for cGMP hydrolysis, and P_γ (14 kDa) functions as an inhibitory peptide which suppresses the hydrolytic activity until photoactivation of the enzyme cascade. The excitation occurs in a two-stage amplification cascade (Yee and Liebman, 1978; Liebman and Pugh, 1979, 1982; Fung and Stryer, 1980; Hurley and Stryer, 1982; Fung, 1983). In the dark-adapted state, transducin exists in its latent form where T_α·GDP is associated with $T_{\beta\gamma}$. Photolyzed rhodopsin catalyzes the exchange of bound GDP for GTP in hundreds of transducin molecules (Fung and Stryer, 1980). The subunits of the activated transducin then dissociate from the rod outer segment membrane as T_α·GTP and $T_{\beta\gamma}$ (Kuhn, 1980). T_α·GTP activates the latent PDE complex by removing the inhibitory constraints imposed by P_γ upon the $P_{\alpha\beta}$ catalytic site (Fung *et al.*, 1981), which results in the rapid hydrolysis of intracellular cGMP in the rod outer segment. This transient decrease in cGMP leads to the closure of cGMP-sensitive cation channels in the plasma membrane of the rod outer segment (Fesenko *et al.*, 1985; Yau and Nakatani, 1985) and the subsequent hyperpolarization of the photoreceptor cell (Hagins *et al.*, 1970). The cascade is shut off via a combined reaction of the hydrolysis of transducin-bound GTP (Wheeler and Bitensky, 1977; Yee and Liebman, 1978) and the phosphorylation of the photolyzed rhodopsin by rhodopsin kinase (Liebman and Pugh, 1980; Sitaramayya and Liebman, 1983).

The amplification mechanism for the photoactivation sig-

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[1] The abbreviations used are: T, transducin; Gpp(NH)p, guanosine 5′-(β,γ-imidotriphosphate); PDE, cGMP phosphodiesterase; SDS, sodium dodecyl sulfate; MOPS, 4-morpholinepropanesulfonic acid; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; MBS, maleimidobenzoyl-*N*-hydroxysuccinimide ester; NHS-ASA, *N*-hydroxysuccinimidyl-4-azidosalicylic acid; *p*-PDM, *para*-phenyldimaleimide; *o*-PDM, *ortho*-phenyldimaleimide; APB, *p*-azidophenylacyl-bromide.

nal is largely based on the association and dissociation of the protein components of the cGMP cascade at various stages of the activation process. Information pertinent to the relationship between the subunits of transducin and PDE with respect to their organization and interactions is essential in elucidating the molecular mechanism of visual transduction. In this paper, we report the results obtained from chemical cross-linking studies of transducin and PDE. The use of bifunctional reagents as molecular bridges in the determination of the quaternary structure of oligomeric protein complexes in solution has been quite successful, especially in revealing the transient interactions or migratory behavior of proteins that occur in order for proteins to express their physiological function (for a review, see Ji, 1979; Das and Fox, 1979; Peters and Richards, 1977). The aim of this study on the retinal cGMP cascade is 1) to gain new insights into the subunit organization of the transducin and PDE complexes in solution and the changes which occur upon activation by bound Gpp(NH)p; 2) to establish the interacting domains between the T_α, T_β, and T_γ subunits of transducin by integrating the cross-linking approach with limited tryptic digestion studies; and 3) to probe the interactions between $T_\alpha \cdot$Gpp(NH)p and PDE which are relevant to the activation mechanism.

EXPERIMENTAL PROCEDURES

Materials—Bovine retinas were obtained from Brown Packing Co. (South Holland, IL) and stored at −70 °C until used. The chemical cross-linkers dimethyl adipimidate, dimethyl suberimidate, maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS), *N*-hydroxysuccinimidyl-4-azidosalicylic acid (NHS-ASA), and *p*-azidophenylacyl bromide (APB) were from Pierce Chemical Co. *para*-Phenyldimaleimide (*p*-PDM) was purchased from Aldrich; *ortho*-phenyldimaleimide (*o*-PDM) was from ICN Pharmaceuticals, and *para*-phenyl diisothiocyanate was from Eastman. Hexyl-agarose and ω-amino octyl-agarose were products of Miles Laboratories, Inc. GTP and Gpp(NH)p were from Pharmacia LKB Biotechnology Inc. TPCK-trypsin was from Boehringer Mannheim, and soybean trypsin inhibitor was purchased from Cooper Biomedical, Inc. Radioactive ^{125}I-iodinated protein A was obtained from ICN Radiochemicals. Nitrocellulose paper (0.1-μm pore size) was from Vangard International Inc. All other reagents were of the highest purity available.

Protein Isolation—Rod outer segment disc membrane was isolated from bovine retina by the sucrose flotation method, and rhodopsin in reconstituted membrane was prepared by the detergent dialysis method (Hong and Hubbell, 1973). Retinal PDE and transducin were extracted from rod outer segment membrane with low ionic strength buffer (10 mM Tris, 1 mM dithiothreitol, 1 mM $MgCl_2$, pH 7.5) in the absence and presence of 0.1 mM GTP, respectively. Transducin was purified by hexyl-agarose column chromatography according to Fung (1983). The transducin-Gpp(NH)p complex was prepared by incubating purified transducin with 500 μM Gpp(NH)p in the presence of 10 μM photolyzed rhodopsin in reconstituted membrane and then purified by hexyl-agarose chromatography. The purified transducin-Gpp(NH)p complex contained 0.95 mol of Gpp(NH)p/mol of transducin. The T_α and $T_{\beta\gamma}$ subunits were separated by ω-amino octyl-agarose column chromatography (Ho and Fung, 1984). PDE was purified by a modified procedure of Hurley and Stryer (1982) using DEAE-Sephadex, ω-amino octyl-agarose, and gel filtration chromatography (Fung, 1983). Purified PDE is a latent enzyme which can be activated by trypsin treatment or in the presence of transducin-Gpp(NH)p complexes. All purified proteins were stored in 40% glycerol at −20 °C. SDS-polyacrylamide gel electrophoresis with subsequent Coomassie Blue staining revealed that the purified transducin contained three polypeptides of 40, 37, and 8 kDa and that PDE contained three polypeptides of 88, 84, and 14 kDa.

Preparation of Antisera—Antisera against T_α, $T_{\beta\gamma}$, and PDE were obtained by immunizing 10-week-old New Zealand rabbits with the purified proteins. In general, 150 μg of protein was mixed with complete Freund's adjuvant (Difco). The mixture was injected subcutaneously under the limbs and behind the neck of the animal. Two additional boosters were administered at 10 and 30 days after the first injection. Rabbits were bled 5 days after the last booster shot. Serum was prepared from blood samples and stored at −70 °C. The titer and specificity of the antisera were examined by solid-phase radioimmunoassay in which purified proteins were coated on microtiter plates and then reacted with specific antiserum. The existence of the antibody-antigen complex was detected by ^{125}I-protein A (Tsu and Herzenberg, 1980). There was no significant cross-reactivity among these three antisera which were used directly in Western immunoblotting. At a dilution of 1:100 for the anti-T_α antiserum and 1:500 for the anti-$T_{\beta\gamma}$ and anti-PDE antisera, the detection limit in Western immunoblotting was found to be approximately 1 ng of antigen.

Condition for Chemical Cross-linking—Prior to chemical cross-linking, the purified proteins, transducin and PDE, were eluted through a P-6 column to remove the glycerol and exchange them into the cross-linking buffer. The protein concentration was determined and adjusted to 0.5–1.0 mg/ml for transducin (~10 μM) and 0.2–0.5 mg/ml for PDE (~3 μM) by the addition of the cross-linking buffer. For cross-linking with dimethyl adipimidate and dimethyl suberimidate, the buffer used was 250 mM triethanolamine hydrochloride, 2 mM $MgCl_2$, pH 8.5. A buffer containing 10 mM MOPS, 200 mM NaCl, 2 mM $MgCl_2$, pH 8.0, was used in experiments with other cross-linkers. All cross-linkers were added as 100 mM stock solutions in the following solvents. Dimethyl adipimidate and dimethyl suberimidate were dissolved in distilled water; MBS and APB were dissolved in acetone; and *o*-PDM, *p*-PDM, *para*-phenyl diisothiocyanate, and NHS-ASA were dissolved in dimethylformamide. They were added to the protein sample in a 10–100-fold molar excess. The cross-linking reaction was allowed to proceed for 1 h at room temperature. The reactions were quenched with either dithiothreitol or diethylamine, and the protein samples were immediately prepared for SDS-polyacrylamide gel electrophoresis. For the photoactivated cross-linkers such as APB and NHS-ASA, the reaction was carried out in the dark for 1 h, and then the sample was photolyzed under ultraviolet light (UVP, Inc. Model R-52G) for 5 min at a distance of 10 cm.

Identification of Cross-linked Products—Identification of the cross-linked products was initially based on molecular size determinations. The cross-linked proteins were first separated on SDS-polyacrylamide gels and stained with protein-binding dye. Since the molecular size of the cross-linked product is the sum of the molecular sizes of the subunits that are being cross-linked, a simple calculation is generally used to identify the composition of the cross-linked adduct. However, the major subunits of transducin (T_α and T_β) and PDE (P_α and P_β) have similar molecular sizes, which generates ambiguity in the interpretation of the results. To circumvent this problem, the Western immunoblotting method (Burnett, 1981), using antisera against T_α, $T_{\beta\gamma}$, and PDE, was applied in order to identify the composition of the cross-linked products. The cross-linked samples were first separated on SDS-polyacrylamide gels in duplicate or triplicate and then electrophoretically transferred onto nitrocellulose paper (0.1-μm pore size) in a Hoeffer Transphor apparatus for a 4–6-h period at 0.3 A. The nitrocellulose blots were placed in 5% bovine serum albumin overnight to block the nonspecific binding sites and then incubated with specific antisera against T_α, $T_{\beta\gamma}$, and PDE. The cross-reactive bands were revealed by treatment of the nitrocellulose blots with ^{125}I-protein A and subsequent autoradiography on Kodak XAR-5 x-ray film with a Du Pont-New England Nuclear Lightning Plus intensifying screen. The relative mobility of the bands were determined by densitometric scanning of the autoradiograms using a Biomed Instruments laser scanning densitometer.

Analytical Methods—Protein concentrations were determined by the method of Coomassie Blue binding (Bradford, 1976) with γ-globulin from Bio-Rad as the standard. SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970). The following prestained molecular weight standards (from Sigma) were used for molecular weight calibration: triose-phosphate isomerase (26,600), lactic dehydrogenase (36,500), fumarase (48,500), pyruvate kinase (58,000), fructose-6-phosphate kinase (84,000), β-galactosidase (116,000), and α_2-macroglobulin (180,000).

RESULTS

Selection of Chemical Cross-linkers

The basic principle underlying chemical cross-linking is the use of bifunctional reagents to covalently link protein components which exist as close neighbors in solution. Several factors influence the formation of a cross-linked product; these include the availability of the appropriate amino acid

residues on the proteins, the chemical specificity of the cross-linker, and the reaction conditions. Negative results in chemical cross-linking experiments do not conclusively demonstrate that two protein components are not close to each other. A paucity of cross-linked products may be a result of a lack either of spatial proximity or of appropriate reactive groups on the adjacent polypeptide chains. The first step in this investigation was to select suitable chemical cross-linkers and to establish the optimum reaction conditions. We screened a battery of commercially available bifunctional reagents with different cross-linking distances and reactive groups for their efficiency in cross-linking the subunits of transducin and PDE. The reaction conditions were also systematically examined with respect to pH, reaction time, and protein and cross-linker concentration and ratio. The results are summarized as follows. Reagents that cross-link amino groups, such as the bifunctional imido esters dimethyl adipimidate (with an 8.6-Å cross-linking bridge) and dimethyl suberimidate (11 Å) as well as *para*-phenyl diisothiocyanate (12 Å), were found to be poor cross-linkers for transducin and PDE. Sulfhydryl-cross-linking reagents such as *o*-PDM (9 Å) and *p*-PDM (12 Å), the heterobifunctional MBS (13 Å, which links amino and sulfhydryl groups), and APB, (13 Å, which contains a sulfhydryl-reactive and a photoactivated azido group) were shown to be most effective in cross-linking subunits of transducin and PDE. Another heterobifunctional reagent, NHS-ASA (13 Å, with an amino-reactive and an azido group), was again a poor cross-linker. It is of interest that each of the effective reagents is capable of reacting with at least one sulfhydryl group. The cross-linked products of transducin generated by cross-linking with *o*-PDM, *p*-PDM, APB, and MBS were similar. Therefore, for detailed studies, *p*-PDM and MBS were chosen as representative reagents due to their known specificity and their high efficiency in cross-linking transducin.

In order to eliminate the possibility that the high molecular size cross-linked products were generated by simple chemical modification that led to protein denaturation and aggregation, monofunctional reagents such as methyl imidate and *N*-ethylmaleimide were used as controls. These agents alone had no effect on the mobility of the subunits during SDS-polyacrylamide gel electrophoresis (data not shown). Therefore, the high molecular size products generated with bifunctional reagent treatment were all due to subunit cross-linking.

Characterization of Antisera against T_α and $T_{\beta\gamma}$

A major goal of this chemical cross-linking study was to identify the sites of interaction between the transducin subunits. Proteolysis experiments have led to the generation of the linear peptide map of the transducin subunits (Fung and Nash, 1983; Medynski *et al.*, 1985; Lochrie *et al.*, 1985; Hingorani and Ho, 1987b). The approach we took was to first cross-link the transducin subunits and then treat the cross-linked products with TPCK-trypsin to generate covalently linked peptide fragments which could be identified by Western immunoblotting with anti-T_α and anti-$T_{\beta\gamma}$ sera. In order to interpret the results, it was essential to characterize the antigenicity of the peptide fragments of T_α and $T_{\beta\gamma}$ toward the antisera used in this study. The results are shown in Fig. 1. *Panel A* shows the tryptic fragmentation pattern of transducin with Coomassie Blue staining and is similar to that obtained by Fung and Nash (1983). Upon treatment with TPCK-trypsin, T_α is first cleaved at Lys^{18} and 2 kDa is removed from the N terminus, resulting in a 38-kDa peptide.[2] A second cleavage at Arg^{310} removes a 5-kDa fragment from the C terminus to generate a transient 33-kDa fragment which is finally cleaved at Arg^{204} into a 21- and a 12-kDa fragment. This final cleavage is blocked when Gpp(NH)p is bound to T_α, thereby causing the accumulation of the 33-kDa fragment. T_β has a single tryptic cleavage site at Arg^{129} and results in an N-terminal 15-kDa and a C-terminal 23-kDa peptide. T_γ is not specifically cleaved by trypsin, and prolonged incubation leads to a very small loss of the peptide.

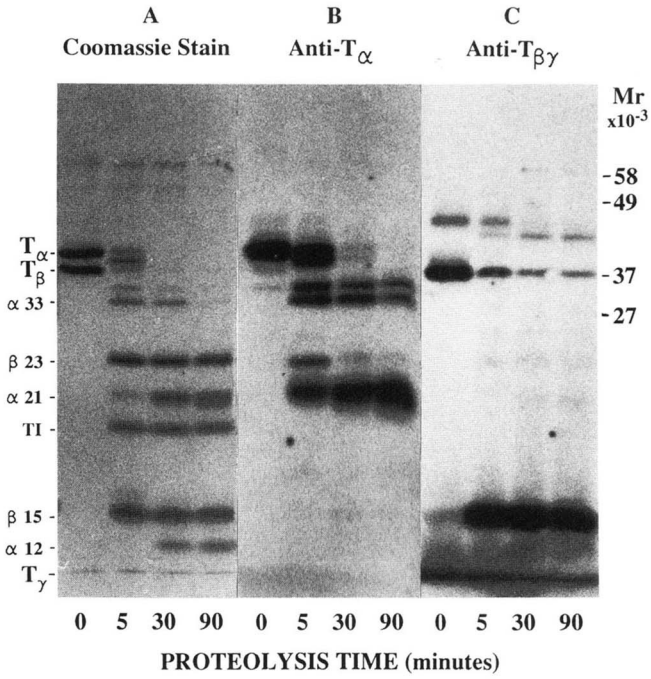
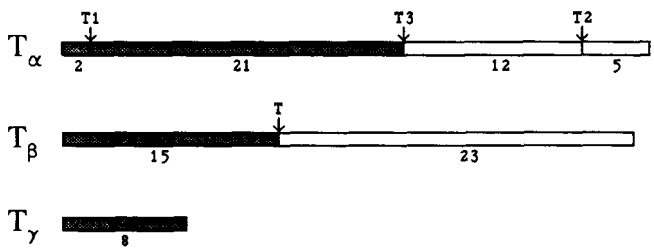


FIG. 1. **Specificity of antisera against T_α and $T_{\beta\gamma}$ toward tryptic peptides of transducin.** Limited tryptic digestion was carried out according to Fung and Nash (1983). Transducin at a concentration of 1 mg/ml was digested at 0 °C with TPCK-trypsin at a protein:protease ratio of 25:1. The reaction was stopped at the indicated time points with a 10-fold excess of soybean trypsin inhibitor, and the resulting peptide fragments were separated on a 13% SDS-polyacrylamide gel. *Panel A* was stained with Coomassie Blue. *Panels B* and *C* are immunoblots reacted with antisera against purified T_α and $T_{\beta\gamma}$, respectively. The reactive bands were observed after binding of ^{125}I-protein A and autoradiography as described under "Experimental Procedures."

Panels B and *C* in Fig. 1 are immunoblots of the gel in *Panel A* using anti-T_α and anti-$T_{\beta\gamma}$ sera, respectively. The antisera used in these experiments demonstrated specificity toward certain peptide fragments of the transducin subunits. The antiserum against T_α was found to be strongly reactive against the 21-kDa fragment. All the other reactive bands, such as the 38-, 34-, and 33-kDa fragments, are known to contain this peptide within their sequence. Since the nitrocellulose used in these experiments is unable to retain fragments of less than 2 kDa, it is difficult to comment on the antigenicity of the N terminus. However, since characterization of several monoclonal antibodies against T_α has shown that the N terminus is highly antigenic (Navon and Fung,

[2] Tryptic fragments of transducin subunits were identified according to Fung and Nash (1983). The molecular sizes of the tryptic fragments from T_α (1, 23, 9, and 6 kDa) and T_β (14 and 24 kDa) were determined from relative mobility on SDS-polyacrylamide gel electrophoresis. Since the exact tryptic sites and sequences of the proteins have been elucidated, the calculated molecular sizes of the fragments of T_α (2, 21, 12, and 5 kDa) and T_β (15 and 23 kDa) are used in this report.

1988), it is likely that the polyclonal antiserum is also reactive toward this peptide. We do not have experimental evidence that the 2-kDa fragment is not further degraded after removal from T_α. However, since the 2-kDa fragment is not involved in any intersubunit cross-linking, this does not alter the interpretation of the results. The 0.1-μm nitrocellulose paper used in this study is capable of retaining the 5-kDa fragment. This fragment contains the pertussis toxin ADP-ribosylation site at Cys^{347} (Watkins *et al.*, 1985). Retention after electroblotting was confirmed by transfer of the [^{32}P]ADP-ribosylated 5-kDa fragment (data not shown). The 5- and 12-kDa fragments of T_α showed absolutely no reactivity toward the antibodies. It is clear from *Panel C* that both the N terminus 15-kDa fragment of T_β and the T_γ subunit are reactive toward the antiserum raised against purified $T_{\beta\gamma}$. The C-terminal 23-kDa peptide of T_β is non-antigenic. The band at 46 kDa seen in the undigested lane (0 min) of *Panel C* which only cross-reacted with the antiserum against $T_{\beta\gamma}$ is likely to be an aggregate of T_β and T_γ which does not completely dissociate under the denaturing conditions of the gel. Control experiments using monofunctional reagents such as *N*-ethylmaleimide and phenyl isothiocyanate showed that chemical modification does not affect the antigenicity or the tryptic cleavage pattern of transducin. Therefore, all possible cross-linked tryptic peptides with their specific cross-reactivity toward the antisera can be predicted. These are listed in Fig. 2 and serve as a key to identify intersubunit cross-linking of the transducin complex.



Peptide Composition of Proteolyzed Cross-linked Products

Composition	M.W.(kDa)	Anti-T_α	Anti-$T_{\beta\gamma}$
α2•γ	10	Yes	Yes
α5•γ	13	No	Yes
α2•β15	17	Yes	Yes
α5•β15	20	No	Yes
α12•γ	20	No	Yes
β15•γ	23	No	Yes
α2•β23	25	Yes	No
α12•β15	27	No	Yes
α5•β23	28	No	No
α21•γ	29	Yes	Yes
β23•γ	31	No	Yes
α12•β23	35	No	No
α21•β15	36	Yes	Yes
α21•β23	44	Yes	No

FIG. 2. **Linear peptide map of transducin subunits and characterization of possible cross-linked tryptic fragments.** The linear arrangement of the tryptic fragments of transducin is shown (*upper panel*). The *arrows* indicate the sites of tryptic cleavage, and the *shaded regions* represent tryptic fragments which are reactive toward the antisera. The molecular size of each fragment is shown under the peptide map. Possible cross-linked peptides are indicated (*lower panel*), along with the expected molecular size of the complex and the antigenicity toward antisera against purified T_α and $T_{\beta\gamma}$.

Cross-linking of Transducin Subunits with p-PDM

Transducin both in its latent conformation, with bound GDP, and in its activated form, with bound Gpp(NH)p, was used in the *p*-PDM cross-linking experiment. The results from the Western immunoblotting of the cross-linked products of transducin and the subsequent tryptic proteolysis are shown in Fig. 3 (*left panel*). The 0-min lane in each panel is a result of cross-linking without subsequent trypsin digestion. The 5-, 30-, and 90-min lanes represent the time course of tryptic proteolysis. In general, the cross-linking pattern of transducin was different from that of the transducin-Gpp(NH)p complex. Several high molecular size products were observed with transducin which were diminished in the transducin-Gpp(NH)p sample. To facilitate the discussion, the molecular size, the antigenicity, and the possible subunit composition of the cross-linked products are summarized in Table I and are discussed as follows.

Band T_α'—The cross-linking of both transducin and transducin-Gpp(NH)p results in a 35-kDa band which reacts with only the antiserum against T_α. This product, called T_α', is likely to be a result of intramolecular cross-links within the T_α subunit which prevent unfolding of the protein in the presence of SDS and therefore migrates with a lower apparent molecular size in the polyacrylamide gels.

Band T1—A 46-kDa fragment seen only to react with the $T_{\beta\gamma}$ antiserum is a result of the cross-linking of the T_β subunit to T_γ. This product is also unaffected by the presence of Gpp(NH)p.

Band T2—Band T2, with an apparent molecular size of 80 kDa, reacts with both T_α and $T_{\beta\gamma}$ antisera and is the cross-linked product of either the heterotrimer T_α-T_β-T_γ or T_α'-T_β-T_γ.

Band T3—Since this 95-kDa cross-linked products is seen to react strongly with the T_α antiserum but only weakly with antiserum against $T_{\beta\gamma}$, a dimer of T_α-T_γ is suggested.

Band T4—Band T4 with an apparent molecular size of 104 kDa, cross-reacts with both antisera. The high molecular size of T4 suggests that it is composed of two copies of either T_α or T_β and may have a stoichiometry of $(T_\alpha')_2$-T_β or T_α'-$(T_\beta)_2$.

Bands T5–T8—Several high molecular size products can be observed in the cross-linking of transducin which can only be formed if transducin exists in solution as a multisubunit oligomer. T5, with a molecular size of 117 kDa, may be a cross-linked product of either $(T_\alpha)_2$-T_β or T_α-$(T_\beta)_2$. T6 (158 kDa) and T7 (170 kDa), which seem to travel as a single band in Fig. 2, can be clearly distinguished as two distinct bands with a shorter exposure of the autoradiograms (data not shown). From the molecular size determinations, one may conclude that T6 is a result of the cross-linking of a dimer of the transducin complex $(T_\alpha'$-T_β-$T_\gamma)_2$, whereas T7 is the product of the dimer $(T_\alpha$-T_β-$T_\gamma)_2$. T8 represents a structure larger than the trimer of transducin such as $(T_\alpha$-T_β-$T_\gamma)_3$ or $(T_\alpha$-$T_\gamma)_4$. The formation of tetramers of transducin in solution could easily result in the high molecular size cross-linked products which are labeled T8 (~230 kDa). Although the expected molecular size of a tetramer would be 340 kDa, the likelihood of every subunit being cross-linked to another is decreased as more cross-linking sites are involved. As can be seen in Fig. 3, the amount of T8 formed is much less than that of T6 and T7. Furthermore, higher molecular size cross-linked products would be unable to enter the polyacrylamide gel matrix.

Are the high molecular size cross-linked products truely representative of the oligomeric structure of transducin in solution or are they products of nonspecific cross-linking (*i.e.* an aggregation of irreversibly cross-linked proteins that combined through random collision)? The answer is provided by

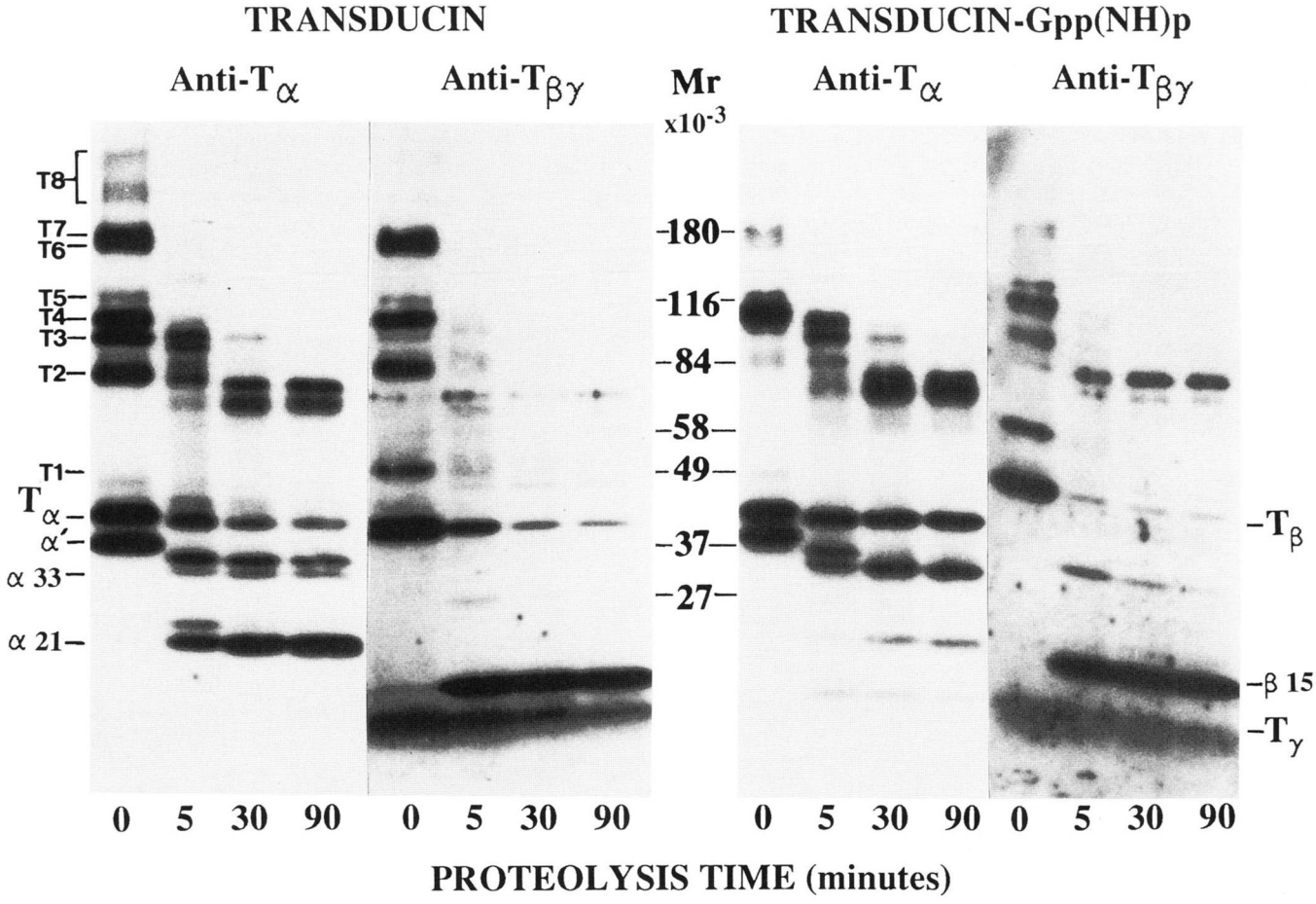


FIG. 3. **Tryptic digestion of transducin cross-linked with *p*-PDM in presence and absence of bound Gpp(NH)p.** Transducin and transducin-Gpp(NH)p at a concentration of 1 mg/ml were cross-linked with a 10-fold molar excess of *p*-PDM as described under "Experimental Procedures." The cross-linked products were digested with trypsin as described for Fig. 1 and separated on a 5–20% gradient SDS-polyacrylamide gel. The cross-linked products were detected by immunoblotting. *Left panel,* immunoblots of the transducin sample; *right panel,* immunoblots of the transducin-Gpp(NH)p sample. The time course represents the tryptic digestion time. Each panel was reacted with antisera against purified T_α and $T_{\beta\gamma}$ as shown. The major cross-linked products (Bands T1–T8) are also shown. The characterization of the cross-linked products is summarized in Table I.

TABLE I

Characterization of the cross-linked products of transducin

Band	Molecular size	Cross-linker	Subunit composition	Diminished with Gpp(NH)p	Reactivity	
					Anti-α	Anti-$\beta\gamma$
	kDa					
T'_α	35	MBS, *p*-PDM	T_α-intramolecular	No	+	−
T1	46	MBS, *p*-PDM	T_β-T_γ	No	−	+
T2	80	*p*-PDM	T'_α-T_β-T_γ, T_α-T_β-T_γ, T_α-T_β	Yes	+	+
T3	95	*p*-PDM	$(T_\alpha\text{-}T_\gamma)_2$	Yes	+	Slight
T4	104	MBS, *p*-PDM	$(T'_\alpha)_2$-T_β, T'_α-$(T_\beta)_2$	No	+	+
T5	117	MBS, *p*-PDM	$(T_\alpha)_2$-T_β, T_α-$(T_\beta)_2$	Slight	+	+
T6	158	*p*-PDM	$(T'_\alpha\text{-}T_\beta\text{-}T_\gamma)_2$	Slight	+	+
T7	170	*p*-PDM	$(T_\alpha\text{-}T_\beta\text{-}T_\gamma)_2$	Slight	+	+
T8	~230	*p*-PDM	$(T_\alpha\text{-}T_\beta\text{-}T_\gamma)_3$, $(T_\alpha\text{-}T_\gamma)_4$	Yes	+	+

comparing the cross-linking patterns of transducin and the transducin-Gpp(NH)p complex as shown in Fig. 3. It has been shown that when Gpp(NH)p is incorporated into transducin, the T_α and $T_{\beta\gamma}$ subunits dissociate from each other and from the membrane (Fung *et al.*, 1981; Fung, 1983). This dissociation of T_α from $T_{\beta\gamma}$ would be expected to greatly reduce the probability of forming cross-linked products between the T_α and $T_{\beta\gamma}$ subunits, but intramolecular cross-linking within a subunit should remain unchanged. The results of the cross-linking of the transducin-Gpp(NH)p complex are shown in Fig. 3 (*right panel*). All of the high molecular size cross-linked products (T8, T7, T6, T3, and T2), with the exception of T4 and T5, were diminished. On the other hand, the production of T_α' and T1, both of which are results of intrasubunit cross-linking, remained the same. These observations are in agreement with the known characteristics of subunit association and dissociation of transducin with bound Gpp(NH)p. We conclude that the latent transducin molecules with bound GDP aggregate as oligomers in solution. Bands T4 and T5, which are insensitive to Gpp(NH)p binding, could be products of the nonspecific cross-linking of denatured proteins present in the sample or may be generated during the cross-linking

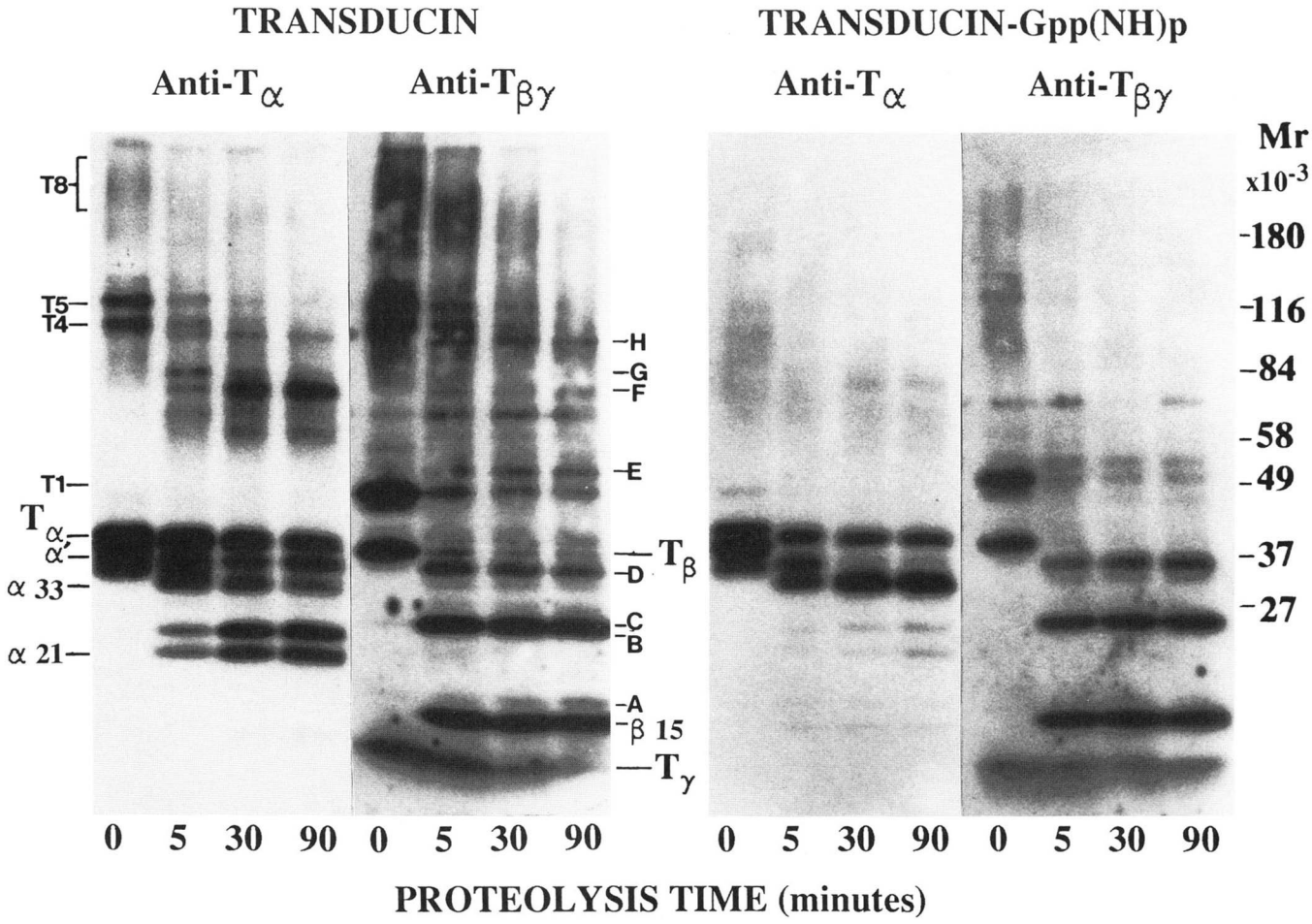


FIG. 4. **Tryptic digestion of transducin cross-linked with MBS in presence and absence of bound Gpp(NH)p.** Transducin and transducin-Gpp(NH)p at a concentration of 1 mg/ml were cross-linked with a 10-fold molar excess of MBS. Subsequent tryptic digestion and immunoblotting were the same as described for Fig. 3. *Left panel,* immunoblots of transducin alone; *right panel,* immunoblots of transducin with bound Gpp(NH)p. Each panel was reacted with antisera against purified T_α and $T_{\beta\gamma}$ as shown.

TABLE II

Peptide fragments generated by tryptic digestion of cross-linked transducin

Band	Molecular size	Cross-linker	Subunit composition	Diminished with Gpp(NH)p	Reactivity	
					Anti-α	Anti-$\beta\gamma$
	kDa					
A	19	MBS	T_α(5 kDa)-T_β(15 kDa), T_α(12 kDa)-T_γ	Yes	−	+
B	23	MBS	T_α(2 kDa)-T_α(21 kDa)	Yes	+	−
C	24	MBS	T_β(15 kDa)-T_γ	No	−	+
D	35	MBS	T_β(23 kDa)-T_γ	No	−	+
E	51	MBS	T_α(5 kDa)-$T_{\beta\gamma}$	Yes	−	+
F	74	MBS, *p*-PDM		Yes	+	−
G	82	MBS, *p*-PDM		Yes	+	−
H	98	MBS, *p*-PDM	$(T_\alpha)_2$-T_β(15 kDa)	Yes	+	−

reaction. All the cross-linked peptide fragments generated by the proteolysis of *p*-PDM-treated transducin were also found when MBS was used and are therefore discussed below.

Cross-linking of Transducin Subunits with MBS

The results of the cross-linking of transducin and transducin-Gpp(NH)p with MBS and the subsequent tryptic proteolysis of the cross-linked samples are shown in Fig. 4. In comparison with the results of *p*-PDM cross-linking, fewer high molecular size products were seen prior to digestion, and these included T4, T5, and T8. However, the intrasubunit cross-links T_α' and T1 were more intense. After prolonged tryptic digestion, a small amount of T1 remains intact. This may be due to the cross-linking of T_γ to both peptide fragments of the T_β subunit (*i.e.* the T_γ-T_β-(15 kDa)-T_β(23 kDa) product), thus preventing complete dissociation in SDS gel electrophoresis. Since the two cross-linkers have different chemical properties with respect to reactive groups and cross-linking distance, it is not surprising that T2, T6, and T7 were missing with the MBS cross-linking. The properties of the cross-linked products are summarized in Table I.

We turn our focus to the interpretation of the results of the proteolytic digestion of the cross-linked transducin subunits. A careful inspection of the results obtained from the two

cross-linking reagents revealed that all the cross-linked fragments found with *p*-PDM were also found in the MBS experiment. A striking difference is that several additional cross-linked fragments can be seen with MBS. The fragments produced by the digestion of the cross-linked products generated in the presence of MBS are labeled Bands A–H in Fig. 4, and their molecular sizes and antigenicity toward the anti-T_α and anti-$T_{\beta\gamma}$ sera are summarized in Table II. The properties of these bands are discussed as follows.

Band A—Band A, with a molecular size of 19 kDa, reacts with only the $T_{\beta\gamma}$ antiserum. It is greatly diminished in the presence of Gpp(NH)p, which indicates that it is a cross-linked product of the T_α and $T_{\beta\gamma}$ subunits. Since only the T_γ and T_β(15 kDa) peptides are antigenic, the composition of Band A must be either T_α(5 kDa)-T_β(15 kDa) or T_α(12 kDa)-T_γ. Since both the 5- and 12-kDa fragments of T_α are non-antigenic, it is difficult to distinguish between these two possibilities. Biochemical evidence suggests that the 12-kDa peptide of T_α is primarily involved in GTP binding and is homologous to other GTP-binding proteins such as elongation factor Tu and ras-p21 (Halliday, 1984), whereas the 5-kDa peptide has been implicated in interaction with rhodopsin and the $T_{\beta\gamma}$ subunit.

Band B—Band B, with a molecular size of 23 kDa, is only reactive with the antiserum against T_α. Since the 23-kDa peptide is the only non-antigenic fragment of the $T_{\beta\gamma}$ subunit, the possibility that Band B contains any fragment of $T_{\beta\gamma}$ is ruled out. The only remaining possibility is that it is a cross-linked product of the 2- and 21-kDa fragments of T_α which is generally seen as a transient and low yield fragment in the digestion of transducin (Fig. 1) and is eventually cleaved into its component fragments. However, after cross-linking with MBS, Band B accumulates due to cross-linking of the 2- and 21-kDa fragments. As expected, Band B is absent in the transducin-Gpp(NH)p sample since the tryptic cut necessary to produce the 21-kDa fragment is hindered by the bound Gpp(NH)p. Instead of the accumulation of the 23-kDa Band B, a 35-kDa product composed of the 2-, 21-, and 12-kDa peptides of T_α should appear. As can be seen in Fig. 4 (*right panel*), a 35-kDa band does accumulate with the tryptic digestion of the MBS-cross-linked transducin-Gpp(NH)p complex. The primary sequence of T_α shows that the N terminus 2-kDa peptide contains no cysteine residues (Medynski *et al.*, 1985; Yatsunami and Khorana, 1985; Tanabe *et al.*, 1985; Ovchinnikov *et al.*, 1985). In order for MBS to cross-link these two fragments, 1 of the lysine residues on the N terminus 2-kDa peptide must be covalently linked to 1 of the 2 cysteine residues on the 21-kDa fragment (Cys^{62} and Cys^{135}). Thus, in the T_α subunit, either Cys^{62} or Cys^{135} is within 13 Å of the N terminus peptide.

Bands C and D—Two cross-linked peptides, Bands C and D, have molecule sizes of 24 and 35 kDa, respectively. Both only react with the $T_{\beta\gamma}$ antiserum, are unaffected by the presence of bound Gpp(NH)p, and are most likely products of intramolecular cross-linking of the $T_{\beta\gamma}$ subunit. They are derived from the tryptic digestion of T1, the 46-kDa T_β-T_γ cross-link. From the intensity of staining and the apparent molecular size, one can conclude that Band C is composed of the 15-kDa fragment of T_β cross-linked to T_γ and Band D is composed of the 23-kDa fragment of T_β cross-linked to T_γ. This also suggests that both peptide fragments of T_β are involved in the interaction with the T_γ peptide and may explain the observation that a small amount of T1 seems to remain even after prolonged tryptic digestion. This could be due to the formation of multiple cross-links between T_γ and the 15- and 23-kDa peptides of T_β which would result in a product of the same molecular size as T1 but would no longer dissociate after tryptic digestion.

Band E—Band E, with an approximate molecular size of 51 kDa, shares similar properties with Band A. It is only reactive with the antiserum against $T_{\beta\gamma}$ and is diminished in the presence of the bound Gpp(NH)p. The most likely explanation for this product is that T_β is cross-linked to T_γ as well as to the 5-kDa fragment of T_α. Since this peptide of T_α is non-antigenic, the product reacts only with anti-$T_{\beta\gamma}$ serum but is sensitive to the subunit dissociation induced by Gpp(NH)p incorporation. This result reinforces the earlier conclusion that the site of interaction of the T_β subunit with T_α is at the C terminus 5-kDa peptide. The digestion of Band E over a 90-min period is much slower than the digestion of the T_β subunit which has not been cross-linked (Fig. 1*C*), implying that the site of proteolysis (Arg^{129}) is sterically hindered by the presence of the cross-linked T_α(5 kDa) peptide. Moreover, Band A (T_α(5 kDa)-T_β(15 kDa)) is probably derived from the digestion of Band E.

Bands F–H—Three high molecular size cross-linked peptides, Bands F (74 kDa), G (82 kDa), and H (98 kDa), were shown to be sensitive to Gpp(NH)p incorporation. All of them are reactive to both T_α and $T_{\beta\gamma}$ antisera. It is difficult to determine the composition of these high molecular size bands. They are likely to be the proteolytic products of the oligomeric structures T6, T7, and T8. In the digestion of the *p*-PDM-cross-linked products (Fig. 3), two transient 100- and 94-kDa bands and their final proteolytic products of molecular size 80 and 70 kDa were only reactive to anti-T_α serum and were not sensitive to Gpp(NH)p incorporation. They may have originated from T4, but their peptide composition cannot be identified conclusively.

Cross-linking of cGMP PDE Subunits with p-PDM and MBS

We have carried out chemical cross-linking of the purified PDE complex with *p*-PDM and MBS. It was found that the degree of cross-linking is largely dependent on the concentration of cross-linker used during the reaction. A 10- and 100-fold excess of cross-linking agent were used routinely in all studies with PDE. The cross-linked products were again identified by Western immunoblotting using antiserum against purified PDE. The polyclonal antiserum of PDE strongly reacts with $P_{\alpha\beta}$ subunit and weakly with the P_γ peptide. The result is shown in Fig. 5. MBS (*lanes 3* and *5*) was far more effective in cross-linking the subunits than was *p*-PDM (*lanes 2* and *4*). The autoradiogram was exposed long enough to allow the P_γ subunit (14 kDa) to be detected, resulting in overexposure and a loss in the resolution of the P_α and P_β subunits which are close in molecular size (88 and 84 kDa). The molecular sizes of the cross-linked products and the possible subunit compositions are listed in Table III.

Band P1—When a low concentration of cross-linker (Fig. 5, *lanes 2* and *3*) was used in the experiment, P1 with a molecular size of 97 kDa could be identified easily. It is likely to be the product of either the P_α or P_β subunit cross-linked to the P_γ peptide. Judging from the concurrent disappearance of P_β and P_γ, one may conclude that P1 is composed of P_β and P_γ. If the inhibitory action of P_γ is to directly block the catalytic site of PDE, this observation suggests that the cGMP hydrolytic site may be located on P_β.

Bands P2 and P3—When a higher cross-linker concentration (Fig. 5, *lanes 4* and *5*) was used, several higher molecular size products were formed. P2 of 150 kDa is probably a cross-linked product of P_α and P_β, and P3 of 190 kDa could be the cross-linked heterotrimer P_α-P_β-P_γ.

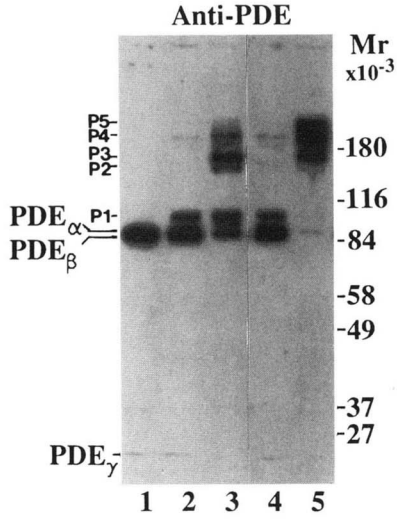


FIG. 5. **Cross-linking of cGMP phosphodiesterase with *p*-PDM and MBS.** PDE at a concentration of 0.5 mg/ml was cross-linked with *p*-PDM and MBS, and the products were separated on an 8% SDS-polyacrylamide gel and immunoblotted with antisera against purified PDE as described under "Experimental Procedures." *Lane 1,* untreated PDE; *lanes 2* and *4,* cross-linked with *p*-PDM at a 10- and 100-fold excess of cross-linker, respectively; *lanes 3* and *5,* cross-linked with MBS at a 10- and 100-fold excess of cross-linker, respectively. The subunits of PDE as well as the major cross-linked products are indicated as Bands P1–P5. The characterization of the cross-linked products is summarized in Table III.

TABLE III

Subunit composition of cross-linked products of PDE

Band no.	Molecular size	Cross-linker	Subunit composition
	kDa		
P1	97	MBS, *p*-PDM	P_β-P_γ
P2	150	MBS	P_α-P_β
P3	190	MBS	P_α-P_β-P_γ
P4	>220	MBS, *p*-PDM	
P5	>240	MBS	

Bands P4 and P5—Several higher molecular size cross-linked products larger than P3 can be seen in Fig. 5. Their subunit composition and mass are uncertain due to the unavailability of antisera toward the purified subunits of PDE and lack of information on the proteolytic digestion pattern of PDE. There are a number of possibilities to explain the formation of P4 and P5. They may represent the oligomeric structure of the PDE complex in solution, or the latent PDE complex may contain multiple copies of certain subunits possessing a structure such as P_α-P_β-$(P_\gamma)_2$.

Cross-linking of Transducin and PDE

The results from the experiments on the cross-linking of the individual proteins are necessary for the interpretation of the results of the cross-linking of transducin to PDE. When the latent form of transducin with bound GDP is mixed with PDE, the two protein complexes do not seem to interact with each other as judged by the lack of cGMP hydrolysis activity. Therefore, the cross-linking pattern of a mixture of transducin and PDE should be the same as that of the individual proteins. However, when transducin-Gpp(NH)p is mixed with PDE, transducin-Gpp(NH)p is capable of removing the inhibitory constraint of the P_γ subunit and activates the cGMP hydrolytic activity (Fung *et al.*, 1981). Under this experimental condition, transducin-Gpp(NH)p caused a 15% activation of PDE as compared to the trypsin-activated level. The interaction between transducin-Gpp(NH)p and PDE could result in the formation of additional cross-linked products. By comparing the difference between the two cross-linking results, one may be able to identify the interacting subunits of transducin-Gpp(NH)p and the PDE.

The results of this experiment are shown in Fig. 6. The cross-linked products in this case were identified by three immunoblots using antisera against T_α, $T_{\beta\gamma}$, and PDE. Fig. 6 (*left panel*) is the result of cross-linking transducin with PDE. The cross-linked products for both transducin and PDE were indeed identical to those seen in the experiments with the individual enzymes (Figs. 3–5). This indicates that transducin in its latent form does not interact with PDE. Fig. 6 (*right panel*) shows the cross-linking results of transducin-Gpp(NH)p and PDE. In addition to the individual subunit cross-linked products, two new high molecular size bands, TP1 (180 kDa) and TP2 (210 kDa), were observed (*right panel, lanes C*). In contrast to the other transducin cross-linked products which were diminished with transducin-Gpp(NH)p, TP1 and TP2 were formed only in the presence of both transducin-Gpp(NH)p and PDE. Although these two bands cannot be clearly identified in the PDE immunoblot due to the existence of the P4 and P5 cross-linked products of PDE, one may still conclude that both TP1 and TP2 are likely to be cross-linked products of the transducin and PDE subunits. From the molecular size and antigenic characteristics, they may represent the cross-linking between T_α or T_β with either P_α or P_β. It has been suggested that in rod outer segment membrane, a small fraction of GTP-activated transducin remains membrane-bound and can efficiently activate PDE (Wensel and Stryer, 1985). This could imply that *in situ*, the activation of PDE may involve the formation of a transducin-PDE complex. Although it has been suggested that the mechanism of activation of PDE requires the removal of the P_γ subunit from $P_{\alpha\beta}$ by $T_\alpha \cdot$Gpp(NH)p (Deterre *et al.*, 1986; Yamazaki *et al.*, 1983), we were not able to detect the cross-link products which would demonstrate $T_\alpha \cdot$Gpp(NH)p and P_γ interaction. The formation of a T_α-P_α or T_α-P_β cross-linked product could suggest that $T_\alpha \cdot$Gpp(NH)p may interact directly with the $P_{\alpha\beta}$ subunits in the course of activating the PDE. Although $T_\alpha \cdot$Gpp(NH)p alone can activate PDE, this does not preclude the possibility that $T_{\beta\gamma}$ may also bind to the PDE complex. Since the lack of cross-linked product does not necessarily mean a lack of subunit interaction, further experiments are needed to resolve the nature of the interaction between $T_\alpha \cdot$Gpp(NH)p and PDE.

DISCUSSION

The involvement of the photoactivated cGMP cascade in visual signal transduction in the rod photoreceptor cell has been well established. The availability of all the purified protein components of the system and the development of specific assays for individual steps of the cascade cycle have led to the elucidation of the coupling mechanism (Fung *et al.*, 1981; Fung, 1983). The coupling between the receptor (the photolyzed rhodopsin) and effector (the cGMP PDE) is mediated by a GTP-binding protein, transducin, which modulates the information transfer by the binding of guanine nucleotides. It has been demonstrated that the binding of transducin to rhodopsin requires a 1:1 ratio of the T_α and $T_{\beta\gamma}$ subunits (Fung, 1983). T_α functions as an activator which dissociates from rhodopsin and $T_{\beta\gamma}$ upon the binding of GTP and activates the latent PDE. The $T_{\beta\gamma}$ subunit acts as a modulator which mediates the binding of T_α to rhodopsin. The molecular structure of the transducin subunits, especially the T_α subunit, has been studied extensively by biochemical

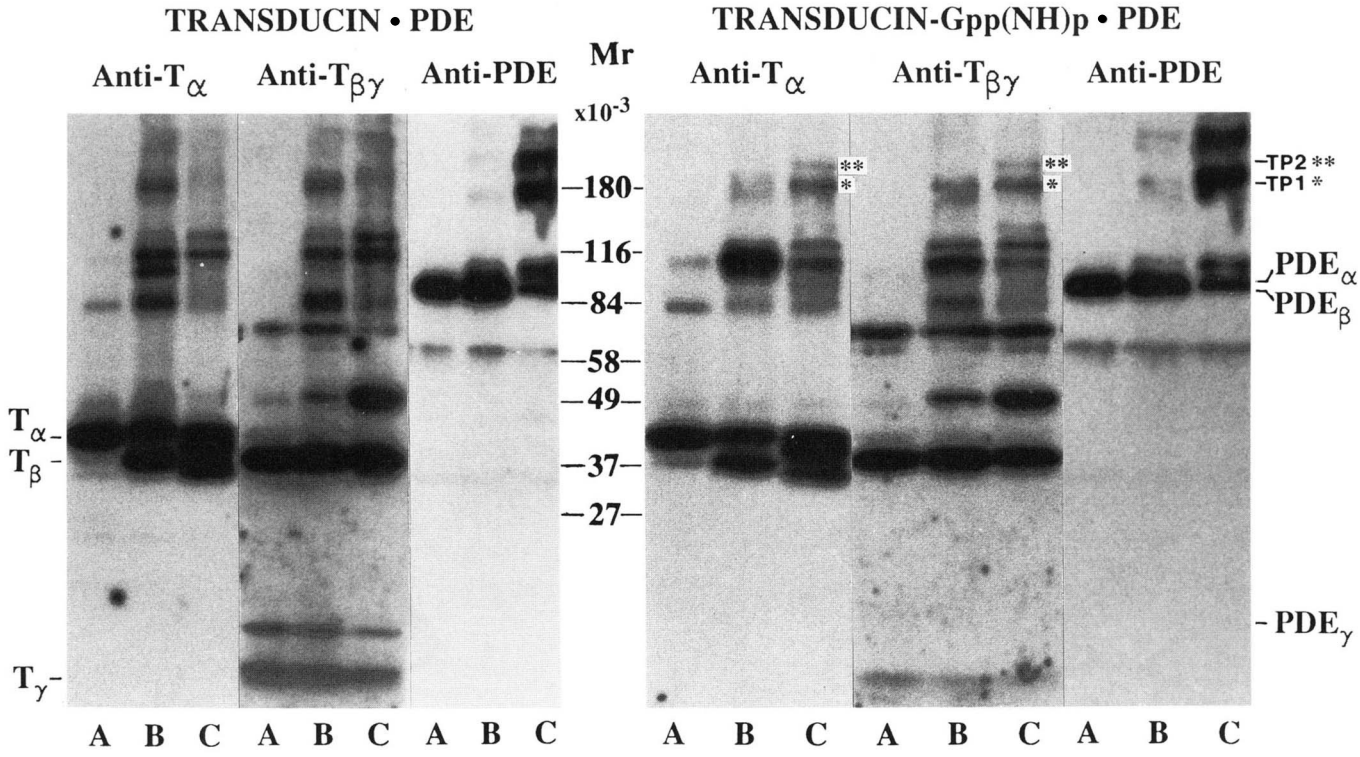


FIG. 6. **Chemical cross-linking of cGMP phosphodiesterase with transducin and transducin-Gpp(NH)p.** Transducin and transducin-Gpp(NH)p at a concentration of 1 mg/ml were mixed with PDE at a concentration of 0.5 mg/ml. The mixtures were cross-linked with a 10-fold molar excess of *p*-PDM and MBS as described for Fig. 5. The cross-linked products were separated on a 5–20% gradient SDS-polyacrylamide gel. *Left panel,* immunoblots of transducin alone; *right panel,* immunoblots of transducin with bound Gpp(NH)p. Each panel contains three immunoblots which were reacted with antisera against purified T_α, $T_{\beta\gamma}$, or PDE as described under "Experimental Procedures." In each immunoblot, *lane A* is the control of the untreated sample, and *lanes B* and *C* are cross-linked samples with *p*-PDM and MBS, respectively. The cross-linked products of the transducin-PDE sample were essentially the same as those obtained by cross-linking of individual protein. Only the additional cross-linked products, TP1 and TP2, in the transducin-Gpp(NH)p-PDE sample are indicated.

techniques such as chemical modification and proteolysis (Ho and Fung, 1984; Hofmann and Reichert, 1985; Hingorani and Ho, 1987a; Fung and Nash, 1983), molecular cloning (Medynski *et al.*, 1985; Yatsunami and Khorana, 1985; Hurley *et al.*, 1984; Fong *et al.*, 1986), and structural homology analysis with the other signal coupling GTP-binding proteins (the G-proteins) (Halliday, 1984; for a review, see Stryer and Bourne, 1986; Gilman 1987). The general picture emerging from these studies suggests that the T_α molecule contains three functional domains: one interacts with rhodopsin-$T_{\beta\gamma}$ which involves the amino- and carboxyl-terminal peptides of T_α, a second domain contains the GTP-binding site which resides on the 12-kDa tryptic fragment, and the third domain participates in PDE activation which is located on the 23-kDa fragment. A three-dimensional model of the T_α molecule based on its structural homology to other GTP-binding proteins has been proposed (Masters *et al.*, 1986; Hingorani and Ho, 1987b). In spite of these efforts in modeling transducin and other G-proteins, direct experimental evidence is needed to verify the suggested subunit interaction sites among the transducin and PDE complexes. The combined approach of chemical cross-linking and subsequent tryptic proteolysis has allowed us to identify the neighboring tryptic fragments of the transducin subunits. It is of interest to compare this structural information with that obtained from biochemical and modeling studies.

A topographical map of the cross-linked tryptic peptides of transducin can be constructed and is shown in Fig. 7. The map is in total agreement with the current understanding of

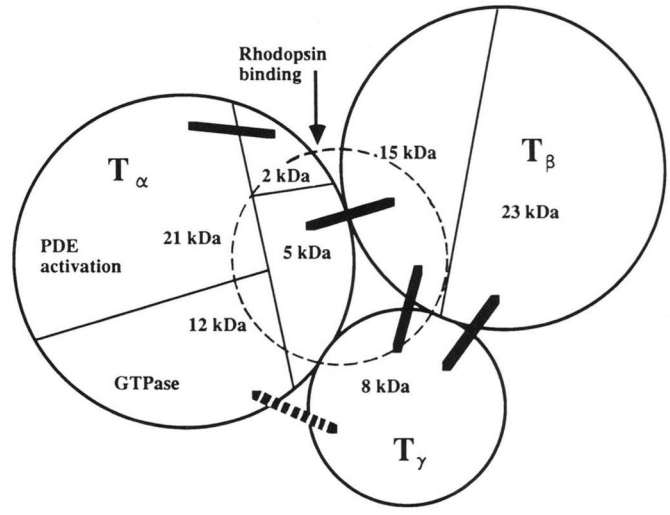


FIG. 7. **Schematic diagram of topological map cross-linked products of transducin subunits.** The molecular sizes represent the tryptic fragments for each subunit. The *heavy black lines* indicate the observed cross-linking between various fragments of the subunits. The relative locations of the functional domains on the T_α subunit for interactions with PDE, GTP, and rhodopsin-$T_{\beta\gamma}$ were based on the structural model proposed by Hingorani and Ho (1987b).

the functional domains of the transducin subunits; and in addition, several new features are revealed that may have functional significance. It has been suggested that the amino and carboxyl termini of the T_α subunit are responsible for

interaction with the photolyzed rhodopsin and $T_{\beta\gamma}$. The cross-linking results which indicate that the 5-kDa C terminus fragment of T_α directly interacts with the N terminus 15-kDa fragment of T_β are consistent with this interpretation. The role of the T_γ subunit of transducin in the coupling mechanism is still unclear. It has been shown that like T_α, T_γ is a rod outer segment-specific protein (Navon *et al.*, 1987). Different γ subunits have been identified among the various G-proteins. This suggests that T_γ may in some manner interact with either rhodopsin or the T_α subunit, or both. Beside the cross-linked products of T_β and T_γ, the data also suggest the possible interaction between T_α and T_γ. In latent transducin with GDP bound, possible cross-links between the T_γ and T_α 12-kDa tryptic fragments and a cross-linked dimer of T_α-T_γ were implied. These observations suggest that T_γ is in contact with both the T_α and T_β subunits and may be involved in the formation of the rhodopsin-binding site that includes regions of the amino and carboxyl termini of T_α and the 15-kDa tryptic fragment of T_β.

As noted earlier, all the cross-linked products described in this study involved sulfhydryl groups. This finding was useful in determining the spatial distribution of cysteine residues and in generating the topographical map. An intramolecular cross-linked product of the fragments of T_α (Band B) was seen. This product was formed by the cross-linking of the N terminus 2-kDa peptide to either Cys^{62} or Cys^{135} on the 21-kDa fragment, suggesting a distance of a few angstroms between these regions. All the successful cross-links between the transducin subunits were found to be those between 2 cysteines or 1 cysteine and 1 lysine. This is not surprising when one considers the number of cysteine residues present in transducin. T_α contains 9 cysteine residues of which 3 can be modified with *N*-ethylmaleimide (Ho and Fung, 1984). T_β contains 14 cysteines of which 10 are in conserved positions on the molecule in regions with a 4-fold internal homology (Fong *et al.*, 1986), and T_γ has 3 cysteines. The close proximity of some of these sulfhydryls may play a significant role in regulating the subunit interactions.

This study has served to corroborate some of the earlier studies on subunit interactions using other methods. The high affinity of T_β for the T_γ subunit under all conditions is seen by the production of T1, the aggregate of these two molecules. The interaction between T_α and $T_{\beta\gamma}$ is also demonstrated by the production of high molecular size cross-linked products which reacted with antisera against both subunits. This association was shown to be dependent on the nature of the guanine nucleotide bound to the T_α subunit. In the presence of bound GDP, the interaction between T_α and $T_{\beta\gamma}$ was strong and resulted in the high yield of the T_α-$T_{\beta\gamma}$ cross-linked products. When the GDP was replaced by Gpp(NH)p, a dissociation of the T_α and $T_{\beta\gamma}$ subunits was apparent and was seen as a reduction in yield and number of the cross-linked products. However, cross-linked products between T_β and T_γ remained unchanged. Moreover, the high molecular size cross-linked products in the latent transducin sample suggested that transducin may exist as an oligomer in solution. From molecular size estimation, the oligomeric structure of transducin is at least $(T_\alpha\text{-}T_{\beta\gamma})_3$. Since the cross-linking efficiency of higher oligomers decreases as multiple cross-linking sites are involved, the solution structure of transducin could be a tetramer of $(T_\alpha\text{-}T_{\beta\gamma})_4$. The equilibrium between the monomeric and oligomeric forms of transducin cannot be accurately studied by chemical cross-linking since cross-linking generates a covalently linked dead-end product. Sedimentation should be used to understand the solution properties of transducin (Baehr *et al.*, 1979). The interaction between the oligomers of transducin probably occurs through the $T_{\beta\gamma}$ subunits as is evidenced by the existence of $T_{\beta\gamma}$ dimers. The 4-fold internal homology seen in the T_β molecule may be essential in providing the symmetry needed for oligomer formation. The oligomer of transducin may play a functional role in the cascade cycle. Kinetic studies have suggested the possibility that a single rhodopsin may interact with four transducin molecules at once (Bennett and Dupont, 1985). Furthermore, an allosteric activation of transducin by photolyzed rhodopsin has been reported (Wessling-Resnick and Johnson, 1987). The observed allosteric effect can be explained in terms of the existence of transducin oligomers.

The role of transducin in the cGMP cascade is the activation of PDE. It has been suggested that $T_\alpha \cdot$Gpp(NH)p removes P_γ from the catalytic $P_{\alpha\beta}$ subunits, which leads to the activation of PDE and the rapid hydrolysis of cGMP (Yamazaki *et al.*, 1983; Deterre *et al.*, 1986). We attempted to test this using chemical cross-linking. The cross-linking of the PDE subunits alone showed that P_γ probably interacts with the P_β subunit. When PDE cross-linking was carried out in the presence of $T_\alpha \cdot$Gpp(NH)p, two new high molecular size products (180 and 210 kDa) were detected which were not seen with cross-linking either protein alone. These are probably a result of the direct interaction of transducin with the $P_{\alpha\beta}$ subunits. No cross-linked products between T_α and P_γ or the release of P_γ in the presence of $T_\alpha \cdot$Gpp(NH)p were observed, possibly due to a lack of the appropriate reactive residues. This result is consistent with the suggestion that $T_\alpha \cdot$Gpp(NH)p interacts directly with $P_{\alpha\beta}$, forming a complex with altered P_γ interaction which allows the hydrolysis of cGMP to occur (Sitaramayya *et al.*, 1986).

One of the advantages of using purified protein to carry out biochemical studies is that the concentration of the protein can be easily controlled. For example, in cross-linking transducin-Gpp(NH)p with PDE, a molar ratio of approximately 2:1 of transducin-Gpp(NH)p to PDE was used in order to increase the yield of the cross-linked products which otherwise cannot be detected in rod outer segments. We have carried out *in situ* cross-linking studies on rod outer segment disc membranes. Only part of the cross-linked products described above were observed and include Bands T1, T3, T4, T6, and P1 (data not shown). The extensive cross-linking of transducin to rhodopsin in the disc membrane resulted in large aggregates of cross-linked products which did not enter the polyacrylamide gel and, hence, limited our interpretation. Although the existence of all the cross-linked products cannot be verified in rod outer segments, the results described in this report provide new insights into the subunit interactions of the components of the cGMP cascade and may be useful in understanding the role of signal coupling G-proteins in general.

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REFERENCES

Applebury, M. L., and Hargrave, P. A. (1986) *Vision Res.* **26,** 1881–1895

Baehr, W., Devlin, M. J., and Applebury, M. L. (1979) *J. Biol. Chem.* **254,** 11669–11677

Bennett, N., and Dupont, Y. (1985) *J. Biol. Chem.* **260,** 4156–4168

Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254

Burnett, W.-N. (1981) *Anal. Biochem.* **112,** 195–203

Chabre, M. (1985) *Annu. Rev. Biophys. Chem.* **14,** 331–360

Das, M., and Fox, F. (1979) *Annu. Rev. Biophys. Bioeng.* **8,** 165–193

Deterre, P., Bigary, H. J., Robert, M., Pfister, C., Kuhn, H., and Chabre, M. (1986) *Protein* **1,** 188–193

Fesenko, E. E., Kolesnikov, S. S., and Lyubarsky, A. L. (1985) *Nature* **313,** 310–313
Fung, B. K.-K. (1985) in *Molecular Mechanisms of Transmembrane Signalling* (Cohen, P., and Houslay, M. D., eds) pp. 183–214, Elsevier Science Publishers, New York
Fung, B. K.-K. (1983) *J. Biol. Chem.* **258,** 10495–10502
Fung, B. K.-K., and Nash, C. R. (1983) *J. Biol. Chem.* **258,** 10503–10510
Fung, B. K.-K., and Stryer, L. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 2500–2504
Fung, B. K.-K., Hurley, J. B., and Stryer, L. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 152–156
Fong, H. K. W., Hurley, J. B., Hopkins, R. S., Miake-Lye, R., Johnson, M. S., Doolittle, R. F., and Simon, M. I. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 2162–2166
Gilman, A. G. (1987) *Annu. Rev. Biochem.* **56,** 615–649
Hagins, W. A., Penn, R. D., and Yoshikami, S. (1970) *Biophys. J.* **10,** 380–412
Halliday, K. R. (1984) *J. Cyclic Nucleotide Protein Phosphorylation Res.* **9,** 435–448
Ho, Y.-K., and Fung, B. K.-K. (1984) *J. Biol. Chem.* **259,** 6694–6699
Hingorani, V. N., and Ho, Y.-K. (1987a) *Biochemistry* **26,** 1633–1639
Hingorani, V. N., and Ho, Y.-K. (1987b) *FEBS Lett.* **220,** 15–22
Hofmann, K. P., and Reichert, J. (1985) *J. Biol. Chem.* **260,** 7990–7995
Hong, K., and Hubbell, W. L. (1973) *Biochemistry* **12,** 4517–4527
Hurley, J. B. (1987) *Annu. Rev. Physiol.* **49,** 793–812
Hurley, J. B., and Stryer, L. (1982) *J. Biol. Chem.* **257,** 11094–11099
Hurley, J. B., Fong, H. K. W., Teplow, D. B., Dreyer, W. J., and Simon, M. I. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 6948–6952
Ji, T. H. (1979) *Biochim. Biophys. Acta* **559,** 39–69
Kuhn, H. (1980) *Nature* **283,** 587–589
Laemmli, U. K. (1970) *Nature* **227,** 680–685
Liebman, P. A., and Pugh, E. N., Jr. (1979) *Vision Res.* **19,** 375–380
Liebman, P. A., and Pugh, E. N., Jr. (1980) *Nature* **287,** 734–736
Liebman, P. A., and Pugh, E. N., Jr. (1982) *Vision Res.* **22,** 1475–1480
Liebman, P. A., Parker, K. R., and Dratz, E. A. (1987) *Annu. Rev. Physiol.* **49,** 765–791
Lochrie, M. A., Hurley, J. B., and Simon, M. I. (1985) *Science* **228,** 96–99
Masters, S. B., Stroud, R. M., and Bourn, H. R. (1986) *Protein Engineering* **1,** 47–54
Medynski, D. C., Sullivan, K., Smith, D., Van Dop, C., Chang, F. H., Fung, B. K.-K., Seeburg, P. H., and Bourne, H. R. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 4311–4315
Navon, S. E., and Fung, B. K.-K. (1988) *J. Biol. Chem.* **263,** 489–496
Navon, S. E., Fung, B. K.-K., Lee, R., and Lolley, R. N. (1987) *Exp. Eye Res.* **44,** 115–125
Ovchinnikov, Y. A., Lipkin, V. M., Shuvaeva, T. M., Bogachuk, A. P., and Shemyakin, V. V. (1985) *FEBS. Lett.* **179,** 107–110
Peters, K., and Richards, F. M. (1977) *Annu. Rev. Biochem.* **46,** 523–551
Sitaramayya, A., and Liebman, P. A. (1983) *J. Biol. Chem.* **258,** 1205–1209
Sitaramayya, A., Harkness, J., Parkes, J. H., Gonzalez-Oliva, C., and Liebman, P. A. (1986) *Biochemistry* **25,** 651–656
Stryer, L. (1986) *Annu. Rev. Neurosci.* **9,** 87–119
Stryer, L., and Bourne, H. R. (1986) *Annu. Rev. Cell Biol.* **2,** 391–419
Tanabe, T., Nukada, T., Nishikawa, Y., Sugimoto, K., Suzuki, H., Takahashi, H., Noda, M., Haga, T., Ichiyama, A., Kangawa, K., Minamino, N., Matsuo, H., and Numa, S. (1985) *Nature* **315,** 242–245
Tsu, T.-T., and Herzenberg, L. A. (1980) in *Selected Methods in Cellular Immunology* (Mishell, B. B., and Shigi, S. M., eds) pp. 373–397, Freeman Publications, San Francisco
Watkins, P. A., Burns, D. L., Kanaho, Y., Liu T.-Y., Hewlett, E. L., and Moss, J. (1985) *J. Biol. Chem.* **260,** 13478–13482
Wensel, T. G., and Stryer, L. (1985) *J. Cell Biol.* **101,** 221 (Abstr.)
Wessling-Resnick, M., and Johnson, G. L. (1987) *J. Biol. Chem.* **262,** 3696–3706
Wheeler, G. L., and Bitensky, M. W. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 4238–4242
Yamazaki, A., Stein, P. J., Chernoff, N., and Bitensky, M. W. (1983) *J. Biol. Chem.* **258,** 8188–8194
Yatsunami, K., and Khorana, H. G. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 4316–4320
Yau, K.-W., and Nakatani, K. (1985) *Nature* **313,** 579–582
Yee, R., and Liebman, P. A. (1978) *J. Biol. Chem.* **253,** 8902–8909