A Novel Complex from Bovine Visual Cells of a 33 000-Dalton Phosphoprotein with β- and γ-Transducin: Purification and Subunit Structure†

Rehwa H. Lee,*,‡ Bernice S. Lieberman,§ and Richard N. Lolley‡,§

Department of Anatomy, School of Medicine, University of California at Los Angeles, Los Angeles, California 90024, and Developmental Neurology Laboratory, Veterans Administration Medical Center, Sepulveda, California 91343

Received August 28, 1986; Revised Manuscript Received February 24, 1987

ABSTRACT: Photoreceptors of mammalian retinas contain a 33-kDa (33K) protein that is phosphorylated, in vitro, by cyclic nucleotide dependent protein kinases. The 33K protein is phosphorylated in the dark, in situ, and dephosphorylated upon illumination. The soluble 33K protein from bovine retinas has been purified to near homogeneity by extraction at pH 5.7 and chromatography on ion-exchange, gel filtration, and hydroxylapatite columns. In the native conformation, the 33K protein is associated with a 37-kDa (37K) and a 10-kDa (10K) protein, forming a trimeric complex with a sedimentation coefficient of 4.9 S and an apparent molecular mass of 77 kDa. The 33K protein can be dissociated from the 37K/10K complex by centrifugation in the presence of high pH and high salt; the subunits reassociate to form the trimeric complex upon recentrifugation in an isotonic buffer with neutral pH. The 33K protein is phosphorylated rapidly by exogenous kinase, in vitro, whereas the 37K and 10K subunits remain unphosphorylated. The 37K and 10K subunits cross-react with antibodies prepared against the β- and γ-subunits, respectively, of bovine transducin, indicating that the 37K and 10K subunits are immunologically identical with β- and γ-transducin, respectively. No immuno-cross-reactivity was observed between the 33K protein and an antibody against the α-subunit of bovine transducin. The 33K–β-/γ-transducin complex exhibits striking similarity to transducin in its subunit structure and mode of subunit interaction, suggesting it may play an important role in the metabolism and function of rod photoreceptor cells.

Rod photoreceptor cells of mammalian retinas possess both membrane-bound and soluble phosphoproteins that may participate in the regulation of the visual process or in the integration of photoreceptor metabolism. The phosphorylation of the visual pigment rhodopsin occurs only after the pigment has absorbed radiation and has undergone conformational changes within the rod outer segment membrane (Kuhn & Dreyer, 1972; Frank et al., 1973). It is postulated that phosphorylated opsin is less efficient than opsin in catalyzing the light-triggered binding of transducin and, thereby, facilitates downregulation of the phosphodiesterase activation cascade (Kuhn, 1974; Liebman & Pugh, 1979; Sitaramayya & Liebman, 1983; Kuhn et al., 1984; Aton & Litman, 1984).

The major soluble phosphoprotein of mammalian rod visual cells is a 33-kDa (33K) protein (identified initially as 30K), which is found exclusively in visual cells of the retina (Lolley et al., 1977; McGinnis & Leveille, 1985). Its phosphorylation is catalyzed in vitro and probably in vivo by cyclic nucleotide dependent protein kinases (Lee et al., 1981a,b, 1982). The level of phosphorylated 33K is highest in dark-adapted retina or rod outer segments, and the 33K phosphoprotein is dephosphorylated during illumination (Lee et al., 1984).

In order to elucidate the regulation and function of the 33K protein, we have developed procedures to purify the 33K protein to near homogeneity. We find that in its native conformation 33K exists as a trimer composed of peptides of 33 kDa (33K), 37 kDa (37K), and 10 kDa (10K). The 33K subunit undergoes reversible dissociation and reassociation with the 37K/10K subunits, which remain complexed. Furthermore, the 37K and 10K subunits are identified immunologically as the β- and γ-subunits of transducin, respectively (Fung et al., 1981). The 33K–β-/γ-transducin complex exhibits striking similarity to transducin in its subunit structure and mode of subunit interaction (Gilman, 1984; Stryer, 1985), suggesting that it may play a regulatory role in the metabolism and function of rod photoreceptor cells.

Experimental Procedures

Materials

The catalytic subunit of the cAMP-dependent protein kinase (C)[1] was purified from rabbit skeletal muscle according to the procedure of Beavo et al. (1974). C was stored at 4 °C in the presence of 0.5 mg/mL bovine serum albumin and retained enzymatic activity for at least 6 months. [γ-^{32}P]ATP and ^{125}I-protein A were purchased from New England Nuclear. The purified transducin complex, alpha-transducin (Tα) and the antibody against the α-subunit (anti-T_α) or the β- and γ-subunits of transducin (anti-$T_{\beta\gamma}$) were gifts from Dr. Bernard K.-K. Fung, Jules Stein Eye Institute, UCLA School of Medicine. Alcohol dehydrogenase, bovine serum albumin, horseradish peroxidase, ovalbumin, myoglobin, soy bean trypsin

† This work was supported by National Institutes of Health Grant EY 00396 and National Science Foundation Grant BNS 83-19076 and by the Medical Research Service of the Veterans Administration.

* Correspondence should be addressed to this author at the Developmental Neurology Laboratory (151B9), VA Medical Center.

‡ *University of California at Los Angeles.*

§ Veterans Administration Medical Center.

[1] Abbreviations: IBMX, isobutylmethylxanthine; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; Mes, 2-(*N*-morpholino)ethanesulfonate; NP-40, nonidet P40; C, catalytic subunit of the cAMP-dependent protein kinase; ROS, rod outer segment(s); BSA, bovine serum albumin; T_α, T_β, and T_γ, α-, β-, and γ-subunit of transducin; Gpp(NH)p, guanosine 5′-(β,γ-imidotriphosphate); TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

0006-2960/87/0426-3983$01.50/0

inhibitor, aprotinin, pepstatin, and leupeptin were from Sigma. TPCK-trypsin was from Worthington.

Methods

Phosphorylation of 33K. During all steps of the purification the 33K protein was identified by its ability to accept γ-phosphate from [γ-^{32}P]ATP in a reaction catalyzed by C. The reaction mixture (final volume 100 μL) contained 50 mM Tris-HCl, pH 7.5, 0.1 mM IBMX, 5 mM $MgCl_2$, 60 μM [^{32}P]ATP (100–500 cpm/pmol), and 2–4 μg/mL C. The reaction was started by the addition of [^{32}P]ATP, and at the end of 10-min incubation at 30 °C, 19 μL of stopping solution containing 25% SDS, 25% 2-mercaptoethanol, 30% glycerol, and 0.1% bromophenol blue was added to the reaction mixture. The mixture was heated at 90–100 °C for 2 min and then subjected to electrophoresis on SDS–polyacrylamide gels, protein staining, and autoradiography to localize the ^{32}P-labeled 33K protein. The level of ^{32}P incorporation into 33K protein was determined either by densitometric scanning of the autoradiogram or by direct scintillation counting of the identified band.

SDS–Polyacrylamide Gel Electrophoresis and Autoradiography. Gel electrophoresis was carried out in 10 or 12.5% slab SDS–polyacrylamide gels in the presence of 2-mercaptoethanol according to Laemmli (1970). The proteins were detected with either Coomassie stain (for 1.5 mm thick gel) or the Bio-Rad silver stain (for 0.75 mm thick gel). The apparent molecular weights of the proteins were calibrated with standards from Pharmacia. Gels containing ^{32}P-labeled 33K were autoradiographed as described previously (Lee et al., 1984).

Immunotransblot. Immunoblots were carried out by the procedure of Towbin et al. (1979) with modification. Proteins were separated by SDS–polyacrylamide gel electrophoresis, and the gels were incubated with 20 mM Tris base, 150 mM glycine, 0.015% SDS, and 20% methanol for 20 min. The proteins were electrophoretically transferred to nitrocellulose membranes (Bio-Rad) at 60 V for 5 h. The nitrocellulose was dried and blocked with 4% BSA in 10 mM Tris-HCl, pH 7.4, 0.9% NaCl, and 0.02% NaN_3 for 4 h before being incubated overnight at 4 °C with affinity-purified antibody. The blot was then subjected to five 10-min washes as follows: two with 10 mM Tris-HCl–0.9% NaCl (washing buffer); two with 0.05% NP-40 in the washing buffer; one with the washing buffer. The washed nitrocellulose membranes were incubated for 1 h with ^{125}I-protein A (1×10^5 cpm/mL) in the washing buffer; the blot was air-dried and sealed in a sandwich bag before being exposed to X-ray film (Du Pont) with a lightening plus intensifying screen for 1–4 days at −70 °C.

Tryptic Treatment of T_α–Gpp(NH)p Complex. Purified T_α–Gpp(NH)p was treated with TPCK-trypsin as described by Fung and Nash (1983) to generate the 38- and 32-kDa tryptic peptides. Briefly, aliquots each containing 2.6 μg of T_α–Gpp(NH)p in 20 mM 4-morpholinepropanesulfonate, 2 mM $MgCl_2$, 1 mM DTT, and 200 mM NaCl, pH 7.5, were incubated with 0.1 μg of TPCK-trypsin at 0 °C for 40 min. At the end of the incubation, 1 μg of soy bean trypsin inhibitor was added to stop proteolysis before the samples were subjected to either SDS gel electrophoresis or phosphorylation assay.

Protein Determination. Protein was determined by the method of Lowry et al. (1951) as modified by Peterson (1983).

Sucrose Density Gradient Sedimentation. The sedimentation coefficient and subunit interaction of the 33K protein was studied by centrifugation on 5–20% linear sucrose density gradients (total volume 13.5 mL) prepared in either 5 mM Mes, pH 6.9 (25 °C), 0.1 M KCl, 0.2 mM EDTA, 0.1 mM PMSF, and 15 mM 2-mercaptoethanol (pH 6.9 buffer) or in 0.25 M Mes, pH 7.8 (25 °C), 0.2 mM EDTA, 0.1 mM PMSF, and 15 mM 2-mercaptoethanol (pH 7.8 buffer). Samples in 200-μL aliquots were applied to the gradients, and the centrifugation was carried out at 38000 rpm in a Beckman SW-40 Ti rotor at 3 °C for 40 h. The gradients were recovered as 500-μL fractions, and each was analyzed for 33K phosphorylation, protein staining, or immuno-cross-reactivity with anti-$T_{\beta\gamma}$.

Purification of Bovine 33K. Bovine eyes were obtained fresh from a local slaughter house, dark adapted on ice for 2 h before the retinas were dissected under dim red light, and stored at −20 °C until use. All purification steps were performed at 4 °C or in an ice bath. In most purification steps potassium phosphate buffers of various pHs and concentrations containing 5 mM DTT and 0.1 mM PMSF in the presence or absence of 1 mM EDTA were used. For the sake of simplicity, each potassium phosphate buffer will be called PEDP or PDP (when EDTA was omitted) buffer with its phosphate concentration and pH specified; for example, 30 mM PEDP buffer, pH 7.0, contains 30 mM potassium phosphate, pH 7.0, and 1 mM EDTA, 5 mM DTT, and 0.1 mM PMSF.

(*Step 1*) *Extraction.* Two hundred dark-adapted bovine retinas were distributed among 16 45-mL polypropylene centrifuge tubes, and each was suspended with 15 mL of ice-cold 1.13 density sucrose in 5 mM Tris-HCl, pH 7.5 (25 °C), 5 mM $MgCl_2$, and 62 mM NaCl. A loose-fitting Teflon pestle was inserted gently 5 times to detach the ROS, and the retinal suspension was centrifuged at 7000*g* for 12 min. The crude ROS suspension was saved, while the retinal pellets were resuspended in the same buffer and the same process was repeated 2 times. The crude ROS suspensions were pooled and immediately frozen with dry ice/ethanol and stored overnight at −70 °C. The frozen ROS suspension was thawed, and 2 volumes of the same Tris-HCl buffer (with no sucrose) were added before centrifugation at 30000*g* for 30 min. The supernatant was saved, while the ROS pellet was homogenized in 240 mL of the extraction buffer and recentrifuged, and the resulting extract was combined with the first supernatant. This crude extract was immediately precipitated with 474 g/L solid ammonium sulfate and centrifuged at 30000*g* for 20 min. The pellets were resuspended in 30 mM PEDP buffer, pH 7.0, and dialyzed overnight with one change of the same buffer.

(*Step 2*) *pH 5.7 Extraction.* The dialyzed sample was adjusted to pH 5.7 with 1 M acetic acid and centrifuged at 24000*g* for 15 min. The resulting supernatant was immediately adjusted to pH 7.0 with 1 M KOH.

(*Step 3*) *First DEAE-cellulose Column Chromatography.* The sample was made 110 mM in potassium phosphate by the addition of 1 M PEDP buffer, pH 7.0, before it was applied to a anion-exchange column (Whatman DE-52, 2.6 × 20 cm) preequilibrated in the same buffer. The column was eluted with 100 mL of the starting buffer followed by a 700-mL (total volume) linear gradient of 110–550 mM PEDP buffer, pH 7.0. The flow rate was 1 mL/min, and 5-mL fractions were collected. Individual fractions were phosphorylated by C in the presence of ^{32}P-labeled ATP, electrophoresed on SDS–polyacrylamide gels, stained for protein, and autoradiographed. The 33K protein, identified by both ^{32}P incorporation and protein staining, was eluted at about 180 mM potassium phosphate. The 33K fractions were pooled and concentrated to approximately 40 mL on a Amicon PM-10 filter, before dialysis overnight in 4 L of 10 mM PDP buffer, pH 6.8.

(*Step 4*) *Hydroxylapatite Column Chromatography.* The dialyzed 33K pool was centrifuged at 24000*g* for 10 min to

remove protein precipitate before it was applied to a hydroxylapatite column (Bio-Rad HTP, 2.6 × 12 cm) preequilibrated in 10 mM PDP buffer, pH 6.8. After sample application, the column was eluted with 100 mL of starting buffer followed by a linear gradient between 10 mM PDP buffer, pH 6.8, and 150 mM PEDP buffer, pH 7.0 (total volume 400 mL). The flow rate was 1.0 mL/min, and fractions of 4 mL were collected and assayed for 33K. The 33K that eluted as the major peak of absorbance at 280 nm at 75 mM potassium phosphate was collected.

(*Step 5*) *Second DEAE-cellulose Column Chromatography.* The 33K pool was adjusted to pH 8.0 with 1 M Tris base and diluted with water to bring the ionic strength to that of 250 mM Tris-HCl, pH 8.0 (25 °C), 1 mM EDTA, 5 mM DTT, and 0.1 mM PMSF. The sample was then applied to a second DEAE-cellulose column (1.5 × 12 cm) that was preequilibrated in the same Tris buffer and eluted with a linear gradient from 250 to 450 mM Tris-HCl (total volume 350 mL). The 33K again eluted as the major absorbance peak at 280 nm at about 375 mM Tris-HCl. The sample was pooled and concentrated on a Amicon PM-10 membrane to 1–2 mg of protein/mL and stored at −70 °C in the presence of 10 μg/mL leupeptin, pepstatin, and aprotanin. The 33K is stable for 2–3 weeks; afterward, detectable partial breakdown of the 33K band into polypeptides of 31 kDa and smaller molecular masses is observed. The 37K band, on the other hand, is quite resistant to proteolysis.

Results

Purification of 33K Protein. The 33K protein is a visual cell specific protein that was first detected in crude bovine outer segments (ROS) as the most prominent protein substrate for the endogenous cyclic nucleotide dependent protein kinase. Using the phosphorylation assay to identify the 33K protein, we have observed that the 33K protein is readily released from the visual cells during ROS detachment. A recent immunocytochemical study, using a monospecific antibody for 33K, localizes the 33K protein within both the inner and outer segments of rod visual cells (unpublished observation). In keeping with this localization, a hypotonic extract of bovine ROS contains a large amount of phosphorylatable 33K per milligram of protein, but the medium in which the ROS were detatched contains 8–10-fold more 33K protein than the ROS extract. Knowing that the 33K protein is found only in visual cells, the soluble and ROS pools of 33K were combined. This step increases the initial yield of 33K protein and provides a sample of 33K protein that is probably derived from both the inner and outer segments of the photoreceptor cells.

The 33K protein was purified to near homogeneity by ammonium sulfate precipitation, extraction at pH 5.7, DEAE-cellulose chromatography, and hydroxylapatite chromatography. Figure 1A shows the protein staining pattern, and Figure 1B shows the autoradiogram of phosphorylated 33K samples from the consecutive steps of the purification procedure. It was found that the 33K protein coeluted, from all columns, with a readily identified peptide of 37 kDa (37K) as well as a peptide of 10 kDa (10K), which can be more clearly shown by silver staining of 12.5% SDS–polyacrylamide gels that are overloaded with the 33K sample (Figure 2B and Figure 3A, lane 2). As shown below, the 33K protein and the 37K and 10K peptides constitute the subunits of a native trimeric 33K protein complex. The 33K protein is not a proteolytic product of the 37-kDa peptide, since the former is very labile while the latter is resistant to proteolysis. The 33K and 37K peptides also exhibted distinct protein staining characteristics with silver stain: the 37-kDa protein appears as a sharp dark brown band, whereas 33K appears as a broad, diffuse, grayish band. The nature of microheterogeneity in the 33K band is not known at this time. On the basis of the densitometric scanning of the protein stain, the 33K, 37K, and 10K peptides collectively represented at least 90–95% of the total protein in the final DEAE-cellulose eluate. The purified 33K protein complex was not contaminated by transducin (see later sections), cGMP phosphodiesterase, and cyclic nucleotide dependent and independent kinase activities.

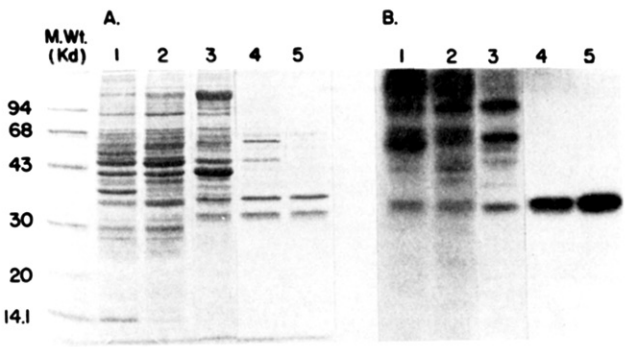

FIGURE 1: Purification of the 33K complex. The Coomassie staining pattern (A) and autoradiogram (B) of the 33K complex at progressive steps of purification were monitored by electrophoresis on a 10% SDS–polyacrylamide gel: (lane 1) 30 μg of crude extract (step 1); (lane 2) 24 μg of pH 5.7 supernatant (step 2); (lane 3) 15.7 μg of pool from first DEAE-cellulose column (step 3); (lane 4) 5.5 μg of pool from hydroxylapatite column (step 4); (lane 5) 4.5 μg of pool from second DEAE-cellulose column (step 5). Phosphorylation of the 33K protein was assayed with exogenous C as described under Methods. The molecular weights of proteins were calibrated by protein standards from Pharmacia.

The purification of 33K has been carried out at least 6 times with little variation in results. On the basis of the Lowry determination and protein staining, an average of 9 mg of the 33K/37K/10K protein complex was purified from 200 bovine retinas. The 33K protein was identified in the initial steps of purification by its ability to be phosphorylated by the catalytic (C) subunit of cyclic nucleotide dependent protein kinase in the presence of [^{32}P]ATP. The assay was carried out under conditions in which phosphate incorporation was linear with time and proportional to the amount of 33K sample. Elution of 33K from chromatographic columns was monitored by phosphorylation of individual fractions followed by SDS gel electrophoresis and autoradiography. After the first DEAE-cellulose column, the partially purified 33K and 37K peptides become identifiable by protein staining. In all subsequent chromatographic steps, the elution profiles of 33K was determined both by phosphorylation and by protein staining, with both profiles always coincident. A numeral value for the 33K recovery and yield during purification is not available now, since protein staining and 33K phosphorylation both are flawed for quantitation. Only the phosphorylation assay was able to track the 33K protein through each step of purification. This assay, however, is only qualitative, because it does not take into account the 33K population that is already phosphorylated nor the phosphoprotein phosphatase activity that contaminates the different steps of 33K purification. An antibody has been generated against the 33K protein, and accurate assessment of the yield and recovery will be achieved soon by radioimmunoassay.

Molecular Weight of Native 33K Protein. The molecular weight of the native 33K protein was determined by centrifugational study. The sedimentation coefficient ($s_{20,w}$) of the native 33K was determined in a 5–20% linear sucrose density gradient with alcohol dehydrogenase (7.2 S), malate dehydrogenase (4.3 S), bovine serum albumin (4.27 S), horse-

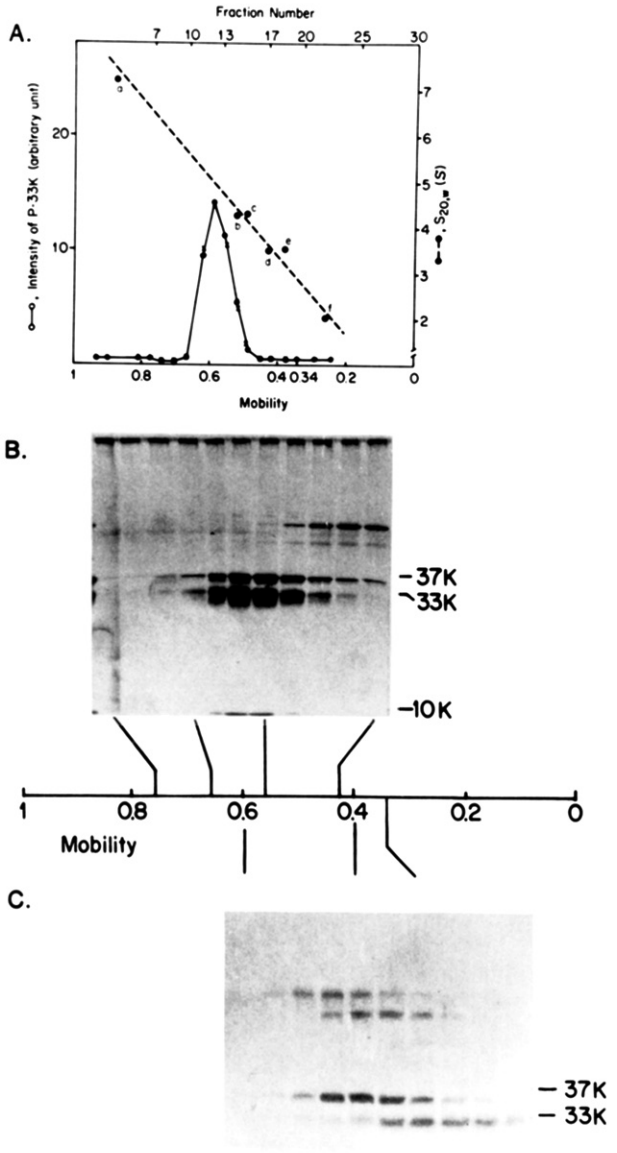

FIGURE 2: Sedimentation of the 33K protein complex on sucrose density gradient. The 33K protein complex purified up to the hydroxylapatite column was used in this study. The 33K sample, in 200-μL aliquots, was centrifuged on 5–20% linear sucrose density gradients of different pHs and ionic strengths as described under Methods (pH 6.9 gradients or pH 7.8 gradients). The gradients were recovered as 500-μL fractions, with 1 representing the most dense fraction. Each fraction was assayed by phosphorylation (for 33K complex) or by autoradiography (for ^{32}P-labeled 33K complex which was previously phosphorylated by C in the presence of [^{32}P]ATP). The content of 33K in each fraction was determined either by densitometric scanning of the autoradiograph or by protein staining. (A) Centrifugation profiles of the 33K complex (●) and ^{32}P-labeled 33K complex (×) on pH 6.9 gradients as determined by densitometric scanning of the ^{32}P-labeled 33K bands. The sucrose density gradient was calibrated (---) by alcohol dehydrogenase (a), BSA (b), malate dehydrogenase (c), horseradish peroxidase (d) ovalbumin (e), and myoglobin (f). The sedimentational mobilities of each standard protein and subunits of the 33K protein complex were determined as described in Table I and expressed in Svedberg units (S). (B) Centrifugation profile of the 33K complex on pH 6.9 gradients as determined by protein staining. (C) Centrifugation profile of the 33K complex on pH 7.8 gradients as determined by protein staining.

radish peroxidase (3.47 S), ovalbumin (3.53 S), and myoglobin (2.0 S) as marker proteins. Under our experimental conditions, these protein standards sedimented in a linear relationship to their $s_{20,w}$ values (Figure 2A). The native 33K sedimented with a mobility of 0.6 and a sedimentational coefficient of 4.9 S (Figure 2A). This corresponds to an apparent molecular mass of 77 kDa, as estimated by the method of Martin and Ames (1961). ^{32}P-Labeled 33K exhibited the same sedimentation characteristics (Figure 2A), suggesting that phosphorylation did not induce conformational changes that are detectable by the methodology used.

Subunit Structure of Native 33K Protein. During purification of the 33K protein, it became apparent that two polypeptides with molecular weights on SDS gels of 37 kDa (37K) and 10 kDa (10K), respectively, copurified with the 33K protein. With an estimated native molecular mass of 77 kDa, which approximately equals the sum of the molecular masses of the 33-kDa, 37-kDa, and 10-kDa peptides, we conclude that the native 33K protein is probably a trimeric complex, composed of the 33K, 37K, and 10K subunits.

To study the interaction between the proposed subunits, the 33K protein complex was centrifuged in 5–20% linear sucrose density gradients that were prepared in buffers of different pH and ionic strength. The sedimentational mobility of each individual subunit was monitored by SDS gel electrophoresis and protein staining of individual gradient fractions; the sedimentation coefficients and apparent molecular masses were estimated by the calibration curve in Figure 2A. Since the 33K, 37K, and 10K subunits exhibited the same electrophoretic mobilities on an SDS gel, the different sedimentational characteristics observed in different sucrose gradients must result from conformational changes, not from partial proteolysis, of the subunits. In the native conformation, the 33K, 37K, and 10K subunits cosedimented in a pH 6.9 gradient as a trimeric complex of 0.6 mobility and 77-kDa molecular mass (Figure 2B). However, when the centrifugation was carried out in a gradient prepared in the pH 7.8 buffer, the 33K protein complex dissociates into a 33K monomer and a 37K/10K dimer complex. All three subunits were absent in the fractions of 0.6 mobility (Figure 2C). The 33K peptide sedimented with a mobility of 0.34 and an apparent molecular mass of 31.5 kDa; the 37-kDa peptide sedimented with a mobility of 0.40 and an apparent molecular mass of 42.6 kDa. The demonstration by protein staining of cosedimentation of the 10K subunit with the 37K subunit is always tricky. As a small peptide that stains poorly, the 10K subunit is detected only when the larger subunits are applied to the SDS gels in such a large quantity as to overload the gel and to obscure their resolution. Figure 2C was chosen to demonstrate clearly the displacement of the two large subunits. On the other hand, the cosedimentation of the 37K and 10K subunits in the pH 7.8 gradient is clearly demonstrated in the immunoblot experiments (Figure 3B).

When fractions from the pH 7.8 gradient containing the 33K monomer and the 37K/10K complex, respectively, were recombined, dialyzed in the pH 6.9 buffer, concentrated, and recentrifuged on a pH 6.9 gradient, the 33K, 37K, and 10K peptides again cosedimented with a mobility of 0.55 and with an apparent molecular mass of 70 kDa, indicating reassociation of the subunits into the trimeric conformation. The sedimentation characteristics of the three subunits are summarized in Table I.

The 33K protein purified up to the hydroxylapatite column (step 4) was used in the experiments described above. As shown in Figure 1A, the hydroxylapatite-purified 33K contained a major contaminant of 58 kDa (lane 4). The sedimentation characteristics of the 58-kDa protein that is not a member of the complex provide a useful comparison with those of the subunits of the 33K/37K/33K complex. The 58-kDa protein sedimented with mobilities of 0.46 and 0.39 in the pH 6.9 and 7.8 gradients, respectively. When the pH of the

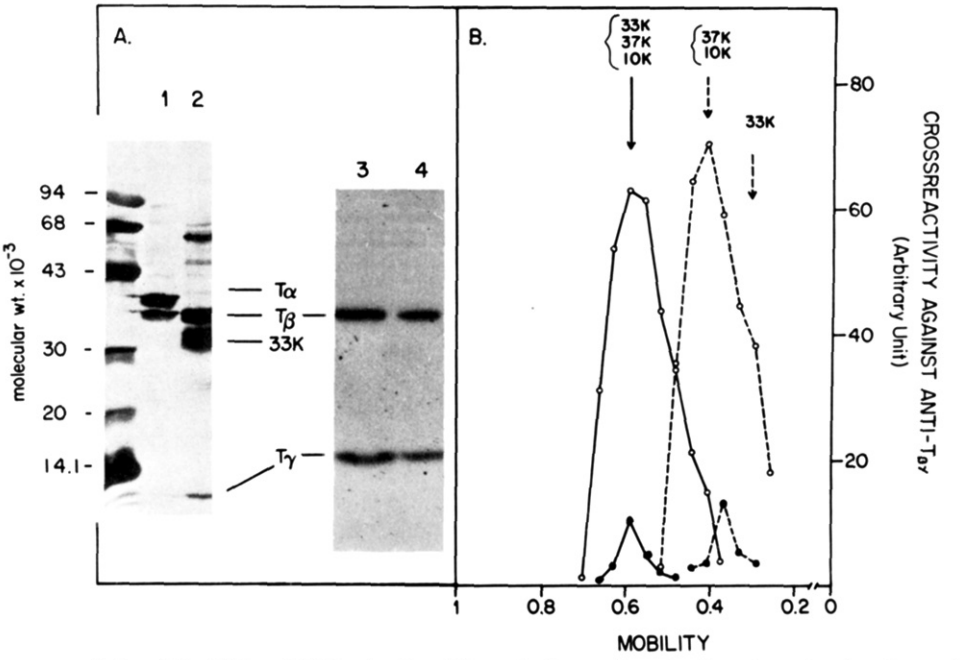

FIGURE 3: Immuno-cross-reactivity of the 37K and 10K subunits with antibodies against the β- and γ-subunits of transducin. (A) Purified transducin (gift from B. Fung) and 33K complex obtained from the hydroxylapatite column were subject to electrophoresis on a 12.5% SDS–polyacrylamide gel. Gel strips containing 1.9 μg of transducin (lane 1) and 2.4 μg of 33K (lane 2) were silver-stained and their molecular weights estimated by comparison with standards (left lane). Other gel strips containing 0.3 μg of 33K (lane 3) and 0.1 μg of transducin (lane 4), respectively, were electrophoresed separately and immunotransblotted against anti-$T_{\beta\gamma}$ before incubation with ^{125}I-protein A and autoradiography. The positions of T_α, T_β, T_γ, and 33K are indicated to aid comparison between the protein bands in lanes 1 and 2 and the radioactive bands in lanes 3 and 4. (B) Sedimentation characteristics of the immuno-cross-reactivity against anti-$T_{\beta\gamma}$. The 33K protein complex obtained from the hydroxylapatite column was centrifuged on sucrose density gradients prepared in pH 6.9 (—) and pH 7.8 (--) buffers as described under Methods. Gradient fractions were electrophoresed on duplicate 12.5% SDS gels. One of the gels was immunotransblotted against anti-$T_{\beta\gamma}$ and autoradiographed; the other was stained for protein. The sedimentation profile for anti-T_β cross-reactivity (open circle) and anti-T_γ cross-reactivity (closed circle) was determined by densitometric scanning of the autoradiograms. The profiles of 37K, 33K and 10K proteins were determined by scanning of the stained gels, and the peak fraction for each subunit is indicated by the arrows. The sedimentational mobility of each fraction was determined as described in Table I.

gradient was readjusted to 6.9 to allow reassociation of the 33K complex, the 58-kDa protein sedimented with the mobility of 0.46. As a monomer, the 58-kDa protein exhibits only a 15% decrease in mobility, which probably resulted from high pH and high salt induced conformational changes that are reversible upon returning to the pH 6.9 buffer. In contrast, the 33K and the 37K/10K subunits exhibited 44 and 33% decreases in mobility, respectively, as a result of subunit dissociation in the pH 7.8 gradient.

Identification of the 37K/10K Subunits as the β- and γ-Subunits of Transducin. The 37K and 10K subunits of the 33K complex migrate on SDS gels with the same electrophoretic mobilities as those of the β- (T_β) and γ-subunits (T_γ) of bovine transducin (Fung et al., 1981), respectively (Figure 3A, lanes 1 and 2). To investigate the identities of the 37K and 10K subunits, the 33K complex was applied to an SDS gel and immunotransblotted against affinity-purified antibodies to T_β and T_γ (anti-$T_{\beta\gamma}$). As shown in Figure 3A, the 37K and 10K subunits, but not the 33K subunits of the protein complex, exhibited two cross-reactive bands with anti-$T_{\beta\gamma}$, which comigrated with those of T_β and T_γ, respectively. Furthermore, the intensity for 0.3 μg of 33K protein complex (lane 3) is about 3 times that for 0.1 μg of transducin (lane 4). This suggests that the cross-reactivity is indeed due to the 37K/10K subunits and is not due to contamination of the 33K complex by a trace amount of $T_{\beta\gamma}$. To investigate further the cross-reactivity with the 10K subunit, the 33K complex was immunotransblotted against a monoclonal antibody against T_γ (monoclonal anti-T_γ), and the 10K subunit alone cross-reacted (resulted not shown), indicating that the 10K subunit is distinct from and is not a proteolytic product of the 37K subunit. Moreover, the 33K subunit shares no antigenic determinants with either T_β or T_γ.

To further confirm that the 37K/10K subunits cross-react with anti-$T_{\beta\gamma}$, we have investigated whether the cross-reactivity cosedimented with the 37K/10K subunits under conditions in which the 37K/10K subunits are in either trimeric (associated) or dimeric (dissociated) conformations. Samples from fractions of each sucrose density gradient were electrophoresed on duplicate SDS gels, followed by either protein staining or immunotransblotting against anti-$T_{\beta\gamma}$ and autoradiography. The amount of 37K/10K proteins and the intensity of the cross-reactivity with anti-T_β and anti-T_γ were estimated by densitometric scanning of the stained gels and of the autoradiograms. Under both pH conditions, the cross-reactivity with anti-$T_{\beta\gamma}$ cosedimented with the 37K/10K subunits, and the extent of cross-reactivity in each fraction corresponded to the amount of 37K/10K proteins (Figure 3B). These results conclusively showed that the 37K and 10K subunits of the 33K protein complex share antigenic determinants with T_β and T_γ, respectively.

Distinction between 33K Subunit and α-Transducin (T_α). The 33K subunit shares with T_α the ability to interact and to form a stable trimeric comlex with $T_{\beta\gamma}$. Fung and Nash (1983) reported that trypsin proteolysis of T_α under nondenaturing conditions generated 38- and 32-kDa fragments. The latter tryptic peptide is similar in size to the 33K subunit. In order to establish whether the 33K and the 32-kDa fragments of T_α are related, the 33K subunit, T_α, and its 32-kDa tryptic fragment were compared in terms of their electrophoretic mobility on SDS gels, cross-reactivity against anti-T_α, and ability to undergo phosphorylation. Figure 4B shows that T_α from either transducin or T_α–Gpp(NH)p (lanes b and c, re-

Table I: Dissociation and Reassociation of the Subunits of the 33K Protein Complex during Centrifugation on Sucrose Density Gradients[a]

sedimentation conditions	characteristics of protein bands identified on SDS gels			
		33K	37K	10K
native (pH 6.9)	mobility	0.60 (8)	0.60 (8)	0.60 (8)
	$s_{20,w}$	4.9	4.9	4.9
	molecular mass	77.0	77.0	77.0
dissociation (pH 7.8)	mobility	0.34 (3)	0.40 (3)	0.40 (3)
	$s_{20,w}$	2.7	3.3	3.3
	molecular mass	31.5	42.6	42.6
reassociation (pH 6.9)	mobility	0.55 (2)	0.55 (2)	0.55 (2)
	$s_{20,w}$	4.6	4.6	4.6
	molecular mass	70.0	70.0	70.0

[a] Purified 33K protein complex was applied to 5–20% linear sucrose density gradients prepared in either pH 6.9 (native) or pH 7.8 (dissociation) Mes buffer and centrifuged as described under Methods. In the reassociation experiments, fractions from the pH 7.8 gradient that contained dissociated 33K monomer or the 37K/10K complex were combined, dialyzed in pH 6.9 Mes buffer, and concentrated before being recentrifuged on a pH 6.9 gradient. Following centrifugation, each gradient was fractionated, and the sedimentational profiles of the 33K, 37K, and 10K polypeptides were determined by electrophoresis on 12.5% SDS–polyacrylamide gels, silver staining, and densitometric scanning. Sedimentational mobility is defined as follows: mobility = [(total no. of fractions) − (peak fraction)]/(total fractions). The sedimentation coefficient [$s_{20,w}$, in Svedberg unit (S)] was determined from the calibration curve shown in Figure 2A. The apparent molecular mass (kDa) of each peptide was estimated from the sedimentation coefficient according to Martin and Ames (1961). The numbers in the parentheses indicate the number of experiments carried out for the determination of sedimentational mobility.

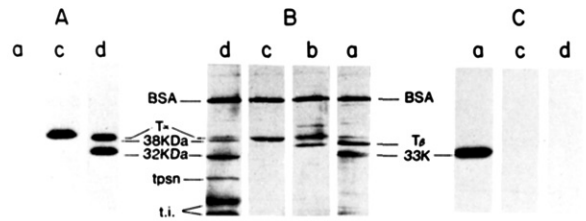

FIGURE 4: Immunological and phosphorylation characteristics of the 33K subunit and the α-subunit of transducin. Samples containing (a) 2.2 μg of purified 33K protein complex, (b) 2.9 μg of bovine transducin, (c) 2.6 μg of T_α–Gpp(NH)p, and (d) 2.6 μg of T_α–Gpp(NH)p that was previously trypsinized as described under Methods were electrophoresed on a 12.5% SDS–polyacrylamide gel. Panel A shows an autoradiograph for the transblotted samples after anti-T_α and protein A treatment. Panel B shows the protein pattern after silver staining of samples that were phosphorylated with [^{32}P]ATP, and panel C shows an autoradiograph of the same gel. The 38-kDa and 32-kDa proteins are proteolytic fragments that result from trypsin treatment of T_α–Gpp(NH)p (Fung & Nash, 1983). tspn, trypsin; t.i., trypsin inhibitor.

spectively) migrates as a 39-kDa polypeptide on 12.5% SDS gel; its 32-kDa tryptic fragment (lane d) exhibits essentially the same mobility as the 33K subunit (lane a). The immunoblot (Figure 4A) shows that T_α (lane c), as well as its 38- and 32-kDa tryptic peptides (lane d), cross-reacts with anti-T_α, but the 33K subunit does not (lane a). On the other hand, while the 33K subunit is readily phosphorylated by C (Figure 4C, lane a), neither T_α (lane c) nor the 38- and 32-kDa fragments (lane d) incorporate phosphate under the same conditions. The same result was observed whether T_α is in the trimeric or monomeric conformation, with or without bound guanine nucleotides. These observations show conclusively that the 33K subunit is distinct from T_α. The absence of any detectable cross-reactivity with anti-T_α further indicates that the 33K preparation is free of contamination by the transducin complex.

Discussion

Rod photoreceptor cells possess a variety of unique proteins. The appearance of these proteins in the visual cells is coordinated with their differentiation and preparation for visual function. Most of the visual cell specific proteins that have been identified and characterized, such as opsin, transducin, phosphodiesterase, rhodopsin kinase, and the 48K protein, probably are associated with the phototransduction mechanism (Stryer, 1985; Sitaramayya & Liebman, 1983; Kuhn et al., 1984; Aton & Litman, 1984). Rhodopsin and phosphodiesterase have been shown by immunocytochemical studies to be localized exclusively in the rod outer segments (Jan & Revel, 1974; Papermaster et al., 1978; Robb, 1974), whereas the transducin complex is found within both the inner and outer segments (Brann & Cohen, 1987; Navon et al., 1987). The 33K protein that is discussed in this paper has been shown by immunocytochemistry also to be localized in the inner and outer segments of rod visual cells (unpublished observation). Several observations have predicted this localization for 33K (Lolley et al., 1977; De Vries & Ferrendelli, 1982), but its association with the rod outer segments has been challenged (Shuster & Farber, 1984). Both the 33K protein and the $T_{\beta\gamma}$ complex of transducin are released rapidly from the visual cells during ROS detachment and isolation. In this work we analyzed the amount of 33K in each step of the procedure for preparation of ROS and pooled the fractions that were enriched with the 33K protein. Our aim was to maximize the yield of 33K and to achieve an appropriate sampling of the 33K pools that are found within the inner and outer segments.

The existence of a 33K/$T_{\beta\gamma}$ complex became evident during purification of the 33K protein. Whereas we have not demonstrated that this complex is found in situ, several observations suggest that the 33K/$T_{\beta\gamma}$ complex exists under physiological conditions. The 33K protein coelutes with the $T_{\beta\gamma}$ complex in all column chromatographies that fractionate proteins by charge, size, shape, hydrophobicity, or a combination of these characteristics. Neither the 33K nor the $T_{\beta\gamma}$ complex was observed to exist free of the complex. This complex is not formed during preparation of the soluble proteins. If either 33K or $T_{\beta\gamma}$ were to bind indiscriminately to proteins, we would find both uncomplexed pools of 33K and $T_{\beta\gamma}$ and hybrid complexs of each with other proteins. In the inital extraction, we find only the 33K complex and transducin. Thus, the $T_{\beta\gamma}$ association with 33K seems rather specific. The 33K/$T_{\beta\gamma}$ complex, either in the crude extract or in a highly purified preparation, exhibits the same molecular mass of about 77 kDa, which is consistent with a unit stoichiometry of the 33K, T_β, and T_γ subunits. The stoichiometry is usually apparent for the 33K and T_β subunits on the basis of their protein-staining intensities on the SDS gels. The T_γ subunit stains poorly, but a 1:1 stoichiometry between the T_β and T_γ subunits is consistent with the estimated 42.6-kDa molecular mass for the $T_{\beta\gamma}$ complex (Table II). The 33K/$T_{\beta\gamma}$ complex is joined by electrostatic forces; under appropriate conditions, the 33K peptide undergoes reversible dissociation and reassociation with the $T_{\beta\gamma}$ dimer. In the reassociation experiment, neither the 33K nor the $T_{\beta\gamma}$ is observed to form a hybrid complex with other proteins. The specificity for association of the 33K/$T_{\beta\gamma}$ complex, the strength of the complex association, and the absence of individual pools of 33K and $T_{\beta\gamma}$ dimer suggest that the 33K/$T_{\beta\gamma}$ complex is stable and, most likely, representative of an association that exists within the rod visual cells, in situ.

The 37K and 10K subunits have been shown by immunological techniques to share antigenic determinants with T_β and T_γ, respectively. Like T_β and T_γ, the 37K and 10K subunits

are not observed to exist alone in the absence of the other (Fung et al., 1981; Hildebrandt et al., 1984). Furthermore, each subunit exhibits identical molecular weight and protein-staining characteristics on SDS gels, respectively, with T_β and T_γ that were purified in association with the transducin complex. The β-transducin is a highly conserved polypeptide; barring microheterogeneity in T_γ, which will be revealed only by sequence analysis, 37K and 10K appear identical with T_β and T_γ, respectively (Hildebrandt et al., 1985).

The 33K subunit is distinct antigenically from the α-, β-, or γ-subunits of transducin as well as from the proteolytic fragments of T_α. The 33K protein is a soluble phosphoprotein that is readily released from the visual cells upon detachment of the rod outer segments, whereas most transducin remains with the rod outer segments. The subunits of $33K/T_{\beta\gamma}$ remain complexed during all chromatography conditions, whereas the proteolytic removal from T_α of even a small polypeptide segment of approximately 1000-Da promotes its dissociation from $T_{\beta\gamma}$ (Fung & Nash, 1983). The 33K subunit, unlike T_α, is not ADP-ribosylated (Abood et al., 1982; Manning et al., 1984) by pertussis toxin (unpublished observation). Moreover, none of the transducin subunits or their proteolytic fragments has been shown to be phosphorylated. These observations indicate that the 33K subunit is distinct from and not a proteolytic product of either T_α or T_β. However, the 33K subunit does share with T_α the ability to form with $T_{\beta\gamma}$ a stable trimeric complex that is analogous to the transducin complex. Furthermore, the 33K subunit, like T_α, undergoes reversible dissociation and reassociation with $T_{\beta\gamma}$, which remains complexed. Phosphorylation of the 33K subunit does not induce its dissociation from $T_{\beta\gamma}$, and the physiological conditions regulating the subunit interaction remain to be identified. We are currently investigating whether dissociation of the subunits of the $33K/T_{\beta\gamma}$ complex, like that of the transducin subunits, requires interaction with the ROS membranes.

The mammalian photoreceptor cell is a specialized neuron that absorbs radiation and translates that signal into membrane hyperpolarization (Hagins et al., 1970; Baylor & Hodgkin, 1970). Moreover, it adapts to illumination (Bownds & Brodie, 1975; Fain, 1976) and provides internal signals for the maintenance of cellular renewal and energy stores (Young, 1976; Matsumoto & Bok, 1984). The visual pigment rhodopsin acts as a receptor for light. The absorption of light, like the binding of a hormone to target cells, initiates a series of intracellular changes that utilize cyclic nucleotides and transducin, a member of the guanine nucleotide binding protein family, as the coupling agents (Gilman, 1984; Manning & Gilman, 1983). The visual response to light is triggered by bleached rhodopsin catalyzing a GTP–GDP exchange reaction on the transducin in complex (Fung et al., 1980). The T_α that contains the GTP binding site dissociates from $T_{\beta\gamma}$ to combine with and activate a unique phosphodiesterase, which hydrolyzes cyclic GMP (Fung et al., 1981; Stryer, 1985). Cyclic GMP is believed to interact with and regulate the ion channels of the rod plasmalemma, with reduced levels of cyclic GMP promoting closure of the channels and hyperpolarization (Yau & Nakatani, 1985; Fesenko et al., 1985).

The possibility exists that the discharged $T_{\beta\gamma}$ and the light-induced decrease in cyclic GMP levels might participate in reactions that branch from the phosphodiesterase activation cascade and that could regulate other metabolic or adaptive activities of the visual cells. In order to link components of the cascade with ancillary mechanisms, a coupling agent would need to interact with the transducin subunits and/or to be modulated by intracellular changes in the concentration of cyclic GMP. We have shown previously that the photoreceptor-specific 33K protein is, in vitro, the major endogenous substrate for phosphorylation by cyclic nucleotide dependent protein kinase and it, in situ, incorporates phosphate in the dark and becomes dephosphorylated upon illumination (Lee et al., 1982, 1984). Now, we demonstrate that the 33K protein forms a complex with $T_{\beta\gamma}$. It is tempting to speculate that the $33K/T_{\beta\gamma}$ complex may represent a branch point in the metabolic pathways that regulate the light-sensitive activities in the visual cells. The interaction between the $33K/T_{\beta\gamma}$ complex and α-transducin is a topic of our future study, and the availability of purified $33K/T_{\beta\gamma}$ should facilitate our investigation of its regulation and function.

Acknowledgments

We are grateful to Drs. Bernard Fung and Sam Navon of the Jules Stein Eye Institute, UCLA, for their helpful discussion and the generous gifts of purified transducin and the antibodies against transducin.

References

Abood, M. E., Hurley, J. B., Pappone, M. C., Bourne, H. R., & Stryer, L. (1982) *J. Biol. Chem. 257*, 10540–10543.

Antoniw, J. F., Nimmo, H. G., Yeaman, S. J., & Cohen, P. (1977) *Biochem. J. 162*, 423–433.

Aton, B., & Litman, B. J. (1984) *Exp. Eye Res. 38*, 547–559.

Baylor, D. A., & Hodgkin, A. L. (1970) *J. Physiol. (London) 207*, 77–92.

Beavo, J. A., Bechtel, P. J., & Krebs, E. G. (1974) *Methods Enzymol. 38*, 299–308.

Bitensky, M. W., Gorman, R. E., & Miller, W. H. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 561–562.

Bownds, D., & Brodie, A. E. (1975) *J. Gen. Physiol. 66*, 407–425.

Brann, M. R., & Cohen, L. V. (1987) *Science (Washington, D.C.) 235*, 585–587.

Cohen, P. (1982) *Nature (London) 296*, 613–620.

De Vries, G. W., & Ferrendelli, J. A. (1982) *Invest. Ophthalmol. Visual Sci. 22 (Suppl.)*, 186.

Fain, G. L. (1976) *J. Physiol. (London) 261*, 467–469.

Fesenko, E. E., Kolesnikov, S. S., & Lyubrarsky, A. L. (1985) *Nature (London) 313*, 310–313.

Frank, R. N., Cavanagh, H. D., & Kenyon, K. R. (1973) *J. Biol. Chem. 248*, 596–609.

Fung, B. K.-K., & Stryer, L. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2500–2504.

Fung, B. K.-K., & Nash, C. R. (1983) *J. Biol. Chem. 258*, 10503–10510.

Fung, B. K.-K., Hurley, J. B., & Stryer, L. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 152–156.

Gierschik, P. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 727–731.

Gilman, A. G. (1984) *Cell (Cambridge, Mass.) 36*, 577–579.

Hagins, W. A., Penn, R. D., & Yoshikami, S. (1970) *Biophys. J. 10*, 380–412.

Hildebrandt, J. D., Codina, J., Risinger, R., & Birnbaumer, L. (1984) *J. Biol. Chem. 259*, 2039–2042.

Hildebrandt, J. A., Codina, J., Rosenthal, W., Birnbaumer, L., Neer, E. J., Yamazaki, A., & Bitensky, M. W. (1985) *J. Biol. Chem. 260*, 14867–14872.

Jan, L. Y., & Revel, J. P. (1974) *J. Cell Biol. 62*, 257–273.

Kuhn, H. (1974) *Nature (London) 250*, 488–590.

Kuhn, H., & Deyer, W. J. (1972) *FEBS Lett. 20*, 1–6.

Kuhn, H., Hall, S. W., & Wilden, U. (1984) *FEBS Lett. 176*, 473–478.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lee, R. H., Brown, B. M., & Lolley, R. N. (1981a) *Biochemistry 20*, 7532–7538.
Lee, R. H., Brown, B. M., Lieberman, B. S., & Lolley, R. N. (1981b) *Abstr. Soc. Neurosci. 7*, 917.
Lee, R. H., Brown, B. M., & Lolley, R. N. (1982) *Biochemistry 21*, 3303–3307.
Lee, R. H., Brown, B. M., & Lolley, R. N. (1984) *Biochemistry 23*, 1972–1977.
Liebman, P. A., & Pugh, E. N. (1979) *Vision Res. 19*, 375–380.
Lolley, R. N., Brown, B. M., & Farber, D. B. (1977) *Biochem. Biophys. Res. Commun. 78*, 572–578.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–270.
Manning, D. R., & Gilman, A. G. (1983) *J. Biol. Chem. 258*, 7059–7063.
Manning, D. R., Fraser, B. A., Kahn, R. A., & Gilman, A. G. (1984) *J. Biol. Chem. 259*, 749–756.
Martin, R. O., & Ames, B. N. (1961) *J. Biol. Chem. 236*, 1372–1379.
Matsumoto, B., & Bok, D. (1984) *Invest. Ophthalmol. Visual Sci. 25*, 1–9.
McGinnis, J. F., & Leveille, P. J. (1985) *Curr. Eye Res. 4*, 1127–1135.
Navon, S., Lee, R. H., Lolley, R. N., & Fung, B. K.-K. (1987) *Exp. Eye Res. 44*, 115–125.
Papermaster, D. S., & Deyer, W. J. (1974) *Biochemistry 13*, 2438–2444.
Papermaster, D. S., Schneider, B. G., & Zorn, M. A. (1978) *J. Cell Biol. 77*, 196–210.
Peterson, G. J. (1983) *Methods Enzymol. 91 (Part I)*, 95–101.
Robb, R. M. (1978) *Invest. Opthalmol. Visual Sci. 17*, 476–480.
Roof, D. J., Applebury, M. L., & Sternweis, P. C. (1985) *J. Biol. Chem. 260*, 16242–16247.
Shuster, T. A., & Farber, D. B. (1984) *Biochemistry 23*, 515–521.
Sitaramayya, A., & Liebman, P. A. (1983) *J. Biol. Chem. 258*, 12106–12109.
Stryer, L. (1985) *Biopolymers 24*, 29–47.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Wilden, U., Hall, S. W., & Kuhn, H. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1174–1178.
Yau, K.-W., & Nakatani, K. (1985) *Nature (London) 317*, 252–255.
Young, R. W. (1976) *Invest. Ophthalmol. Visual Sci. 15*, 700–725.

Analysis of Long-Chain Bases in Sphingolipids by Positive Ion Fast Atom Bombardment or Matrix-Assisted Secondary Ion Mass Spectrometry†

Yoko Ohashi,‡ Masao Iwamori,‡ Tomoya Ogawa,§ and Yoshitaka Nagai*,‡

Department of Biochemistry, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113, Japan, and RIKEN (Institute of Physical and Chemical Research), Wako-shi, Saitama 351-01, Japan

Received October 8, 1986; Revised Manuscript Received February 19, 1987

ABSTRACT: The structures of long-chain bases are expressed as $[CH_2C(NH_2)=CHR]^+$ (Z^+) in the positive ion mode spectra obtained on fast atom bombardment (FAB) mass spectrometry or liquid-matrix-assisted secondary ion mass spectrometry (SIMS) [Benninghoven, A., Ed. (1983) *Ion Formation from Organic Solids*, Springer, Berlin]. This phenomenon is common to sphingolipids in general: glycosphingolipids [see reviews by Sweeley and Nunez [Sweeley, C. C., & Nunez, H. A. (1985) *Annu. Rev. Biochem. 54*, 765] and Kanfer and Hakomori [Kanfer, J. N., & Hakomori, S. (1983) *Handb. Lipid Res. 3*]] and phosphonosphingolipids [Hayashi, A., & Matsubara, T. (1982) in *New Vistas in Glycolipid Research* (Makita, A., Handa, S., Taketomi, T., & Nagai, Y., Eds.) p 103, Plenum, New York], inclusive. Phytosphingosine compounds show the same type of fragmentation without additional dehydration if a neutral matrix is used. A Z^+ ion is easily detected in the lower mass region (m/z 200–400) as an even mass number fragment ion, and confirmation is made by means of B/E constant and B^2/E constant linked scan techniques [Boyd, R. K., & Beynon, J. H. (1977) *Org. Mass Spectrom. 12*, 163; Boyd, R. K., & Shushan, B. (1981) *Int. J. Mass Spectrom. Ion Phys. 37*, 355; Macdonald, C. G., & Lacey, M. J. (1984) *Org. Mass Spectrom. 19*, 55]. [Principles of linked scannings are explicitly summarized by Jennings and Mason [Jennings, K. R., & Mason, R. S. (1983) in *Tandem Mass Spectrometry* (McLafferty, F. W., Ed.) p 197, Wiley, New York] besides the cited literature.]

Fast atom bombardment (FAB) mass spectrometry and secondary ion mass spectrometry (SIMS), especially in the negative ion mode, have been successfully applied to the identification of the saccharide chain in glycolipids (Arita et al., 1983a,b). However, the ceramide moiety in such glycosphingolipids has been so far described as one unit, the long-chain base and the fatty acid part inclusive, unless chemical cleavage and derivatization for electron ionization (EI) (Sweeley & Nunez, 1985) or desorption chemical ionization (DCI) [in-beam chemical ionization (in-beam CI)] (Markey & Wenger, 1974) mass spectrometry have been previously performed. Recently, Taketomi successfully applied FAB mass spectrometry to determine the long chain base size of chemically deacylated glycosphingolipids (Hara & Taketomi, 1986).

† This work was supported in part by a grant from the Ministry of Education, Science and Culture of Japan and the Special Coordination Funds for promoting science and technology from the Science and Technology Agency of Japan. Support by the Naito Foundation is also gratefully acknowledged.

* Author to whom correspondence should be addressed.
‡ University of Tokyo.
§ RIKEN.

0006-2960/87/0426-3990$01.50/0