G Protein $\beta\gamma$ Subunit: Physical and Chemical Characterization†

Thomas C. Thomas,‡ Theresa Sladek,‡ Fei Yi,‡ Temple Smith,§ and Eva J. Neer*,‡

Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115, and Biomolecular Engineering Research Center, Boston University, Boston, Massachusetts 02215

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ABSTRACT: The $\beta\gamma$ subunits of heterotrimeric G proteins play a central role in regulating the function of the G protein α subunits and in modulating the activity of several enzymes and ion channels. We have used the signature tryptic cleavage pattern of native $\beta\gamma$ from bovine brain as a starting point for our analysis of its physical and chemical properties. Digestion of bovine brain $\beta\gamma$ with trypsin yields only 2 β-derived fragments, with relative mobilities on SDS–PAGE of 14 kDa (amino terminal) and 27 kDa (carboxyl terminal), despite the presence of 32 potential tryptic cleavage sites in the β_1 subunit. Trypsin-cleaved $\beta\gamma$ remains in a complex that has the same apparent sedimentation coefficient as intact $\beta\gamma$, and retains its ability to associate functionally with the α_o subunit. Comparison of the incorporation of [^{14}C]iodoacetamide into reduced denatured β and unreduced denatured β showed that there are no disulfide bonds in the molecule to hold the complex together. The brain β and γ subunits can be cross-linked by 1,6-bis(maleimido)hexane to form a 46-kDa product on SDS–PAGE, and trypsin cleavage of cross-linked $\beta\gamma$ shows that γ is cross-linked to the 14-kDa amino-terminal fragment of the β subunit. On the basis of its primary sequence, the β subunit is predicted to form a repetitive structure encompassing the 27-kDa fragment and part of the 14-kDa fragment. Analysis of the thermal denaturation of trypsin-cleaved $\beta\gamma$ supports this prediction and confirms that both fragments retain stable tertiary structures following tryptic cleavage. The stability of the $\beta\gamma$ subunit may reflect strong noncovalent interactions among repetitive structural units of β.

Heterotrimeric GTP binding proteins (G proteins),[1] made up of α and $\beta\gamma$ subunits, play a central role in many types of transmembrane signaling pathways initiated by hormones, neurotransmitters, and light [reviewed by Neer and Clapham (1988) and Spiegel et al. (1992)]. Both the α and the $\beta\gamma$ subunits are able to activate intracellular effector enzymes and ion channels. The β and γ subunits do not dissociate from each other and behave as a functional monomer. Effectors regulated by $\beta\gamma$ subunits include cardiac K^+ channels (Logothetis et al., 1987, 1988; Kim et al., 1989; Ito et al., 1992), retinal phospholipase A_2 (Jelsema & Axelrod, 1987), adenylyl cyclase (Tang & Gilman, 1991), phospholipase C (Boyer et al., 1992; Camps et al., 1992a,b; Katz et al., 1992), and β-adrenergic and muscarinic receptor kinase (Haga & Haga, 1992; Pitcher et al., 1992). In yeast, $\beta\gamma$ activates an unidentified effector critical for the mating pheromone response (Whiteway et al., 1989; Blinder et al., 1989). In order to understand how $\beta\gamma$ mediates the transduction of these signals, it is necessary to define the structure of $\beta\gamma$.

The amino acid sequence of the β subunit suggests that it is made of two different types of structures. The 39 amino acid long N-terminal region of mammalian β is predicted to form an amphipathic α helix that might be involved in coiled-coil interactions (Lupas et al., 1991). The remainder of the protein is made up of 7 repeating units of about 43 amino acids each (Fong et al., 1986). The deduced amino acid sequences of the four identified mammalian β subunit isoforms, as well as β subunits from yeast, *Drosophila melanogaster* and *Caenorhabditis elegans*, are extremely similar. The γ subunits are much less similar to each other. Retinal γ_1 is only 38% identical to brain γ_2 and is modified by a different isoprenyl group from γ_2 (Lai et al., 1990; Yamane et al., 1990; Spiegel, 1992). Pairs of β and γ subunits with identical β subunits, but different γ subunits, have different functions [for example, see Cerione et al. (1987), Logothetis et al. (1988), and Tang and Gilman (1991)]. Thus, the β subunit may define the overall structure of the $\beta\gamma$ complex, while the γ subunit determines effector specificity.

Although the β_1 subunit has 32 potential tryptic cleavage sites, only the arginine at position 129 of the native protein is accessible to trypsin (Fong et al., 1986). We have used the signature tryptic cleavage pattern of the $\beta\gamma$ complex as the starting point for our analysis of $\beta\gamma$ structure. Tryptic cleavage of native $\beta\gamma$ complexes from rod outer segments or from brain generates an N-terminal β fragment with an apparent molecular weight on SDS–PAGE of 14 000 and a C-terminal β fragment of M_r 27 000 (Fung & Nash, 1983; Winslow et al., 1986). These fragments are quite resistant to further degradation by trypsin [see, for example, Winslow et al. (1986)]. The γ subunit seems not to be cleaved by trypsin (Fung & Nash, 1983; this laboratory, unpublished results). The site of cleavage of β_1 falls near the boundary between the second and third repeating units. Thus, the 14-kDa fragment contains the putative N-terminal α helix and two repeating units, while the 27-kDa fragment is entirely made up of repeating units. The accessibility of only one tryptic cleavage

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* To whom correspondence should be addressed at the Cardiovascular Division, Brigham and Women's Hospital, 75 Francis St., Boston, MA 02115. Telephone: (617) 732-5866. FAX: (617) 732-5132.

‡ Brigham and Women's Hospital and Harvard Medical School.

§ Boston University.

[1] Abbreviations: G protein, heterotrimeric guanine nucleotide binding protein; G_o, G protein heterotrimer $\alpha_o\beta\gamma$; the separated subunits are designated α_o and $\beta\gamma$; BMH, 1,6-bis(maleimido)hexane; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; CA, carbonic anhydrase.

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site suggests that this site may mark the boundary between two independent structural domains of β. The repetitive sequence of β, however, suggests that it forms a single structure stabilized by repetitive interactions. Our analysis of the function of the cleaved protein, and of the physical and chemical characteristics of both the βγ subunit and the 14- and 27-kDa tryptic fragments of β, indicates that the β subunit forms a compact structure that is not stabilized by disulfide bonds, but rather is firmly held together by strong noncovalent interactions.

MATERIALS AND METHODS

Preparation of G_o, α_o, and βγ.[1] G_o, α_o, and βγ were prepared as described previously (Neer et al., 1984). Samples of βγ predominantly contained the 36-kDa β_1 isoform and some 35-kDa β_2. Many of the samples were further fractionated on a DEAE-Sephacel and/or a Sepharose 6B column. The buffers used in these additional steps were 50 mM Tris-HCl, pH 8, 6 mM $MgCl_2$, 75 mM sucrose, 1 mM dithiothreitol, and 1 mM EDTA (TMSDE), with additional NaCl for anion-exchange chromatography. In addition, 3 mM benzamidine was present in some buffers. The α_o sample used for ribosylation experiments contained 0.9% cholate. The βγ sample used for ribosylation experiments contained TMSDE and 0.4% cholate. All other samples were in 0.6% Lubrol PX. Samples were concentrated as necessary using either a stirred cell apparatus or a Centricon centrifugation filter (Amicon), and then equilibrated in 50 mM HEPES, pH 8, 6 mM $MgCl_2$, 75 mM sucrose, and 1 mM EDTA (HMSE) by application to a NAP column (prepacked Sephadex G-25, Pharmacia) equilibrated in the same buffer. This removed DTT, Tris, and benzamidine from the purified protein preparations.

Trypsin Digestions. Protein samples were treated with N^{α}-tosylphenylalanine chloromethyl ketone (TPCK)-treated trypsin (Cooper Biomedical, Inc.) at concentrations stated in the text below or in the figure legends. Unless otherwise stated, the samples were incubated at 30 °C for 30 min. Digestions were stopped by addition of either benzamidine or soybean trypsin inhibitor at final concentrations described below and in the figure legends (Sigma).

ADP-Ribosylation by Bordetella Pertussis Toxin. The βγ subunit was incubated for 30 min at 30 °C with a 5:1 molar ratio of βγ to trypsin. The reaction was stopped with 1 pmol/μL each of soybean and limabean trypsin inhibitors and 3 mM benzamidine. The α_o subunit (0.2 μg) was incubated with 0.01–0.5 μg of either trypsin-cleaved or uncleaved βγ. Pertussis toxin-catalyzed ADP-ribosylation was carried out as described by Neer et al. (1984) for 45 min at 37 °C with 0.1% Lubrol PX in the buffer. Each reaction contained 1 μCi of [32P]NAD. The proteins were then separated by SDS–PAGE on an 11% acrylamide gel (Laemmli, 1970), stained with Coomassie Blue R-250. The dried gels were exposed to Kodak XAR5 film at −70 °C for 24–48 h with one enhancing screen.

Sucrose Density Gradient Centrifugation. Trypsin-cleaved βγ was prepared by incubation of βγ with trypsin at 0.05 pmol/μL at 30 °C for 10 min. The reaction was quenched with 8.3 mM benzamidine and then mixed with uncleaved βγ at a ratio of 2:1 (cleaved:uncleaved ratio) and additional markers. Aliquots of 150 μL were loaded onto 4-mL 5–20% sucrose gradients prepared in either H_2O or D_2O containing 50 mM HEPES, pH 7.8, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.1% (w/v) Lubrol PX, and 2 mM benzamidine. Gradients in H_2O were spun at 54 000 rpm for 16 h in an SW60 Ti rotor (Beckman) at 4 °C. Gradients in D_2O were spun at 59 000 rpm for 17.5 h. Fractions were collected after puncturing the bottom of each tube, analyzed by SDS–PAGE in 15% acrylamide gels (Laemmli, 1970), and stained with Coomassie Blue, and the relative intensities of specific bands were determined by densitometry.

Cross-linked βγ was prepared by treating βγ with 2 mM 1,6-bis(maleimido)hexane (prepared as described below) at 4 °C for 20 min. The reaction was stopped by addition of 20 mM DTT. Cross-linked samples of 100 μL each were loaded onto 5–20% sucrose gradients prepared in 50 mM HEPES, pH 7.8, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1% cholate, and 2 mM benzamidine and spun at 54 000 rpm for 16 h in an SW60 Ti rotor (Beckman) at 4 °C. Fractions were collected and analyzed as described in the above paragraph.

Modification of Cysteines with [^{14}C]Iodoacetamide. Samples containing G_o were applied to a NAP column as described above in order to remove DTT. Aliquots of these samples were dried in a Speed Vac concentrator (Savant Instruments) under vacuum for 1 h. Samples were then denatured and alkylated with [^{14}C]iodoacetamide (23.9 mCi/mmol, 72 mM, ICN Radiochemicals) according to one of three protocols. *Protocol 1*: The sample was resolubilized in 8 M urea, 2.5 mM DTT, and 50 mM HEPES, pH 7.8, and incubated at 50 °C for 1–1.5 h. [^{14}C]Iodoacetamide in 8 M urea/50 mM HEPES, pH 7.8, was then added to a final concentration of 10 mM and the reaction incubated at 50 °C for an additional 1–1.5 h. At this stage, the concentration of DTT had been diluted to 1 mM. *Protocol 2*: The sample was resolubilized in 8 M urea, 1–1.5 mM [^{14}C]iodoacetamide, and 50 mM HEPES, pH 7.8, and incubated at 50 °C for 1–1.5 h. *Protocol 3*: The sample was resolubilized in 8 M urea/50 mM HEPES, pH 7.8, and incubated at 50 °C for 1–1.5 h. [^{14}C]Iodoacetamide in 8 M urea/50 mM HEPES, pH 7.8, was added to a final concentration of 1–1.5 mM, and the reaction was incubated at 50 °C for an additional 1–1.5 h. Alkylation reactions were quenched by addition of Laemmli sample buffer containing β-mercaptoethanol at a final concentration of 5% (v/v), and samples were subjected to SDS–PAGE (Laemmli, 1970). Proteins were then transferred to nitrocellulose as described below. The β subunit was transferred with an efficiency of 86%. Blots were stained with Ponceau S (0.33% in 5% trichloroacetic acid and 5% sulfosalicylic acid), and the location of the β subunit was marked with a pencil. Blots were then destained and air-dried. The bands containing β subunits were cut out, and the quantity of ^{14}C was determined by scintillation counting in 5 mL of EcoScint scintillation cocktail (counting efficiency = 89%; National Diagnostics).

Cross-Linking with 1,6-Bis(maleimido)hexane. The cross-linking reagent 1,6-bis(maleimido)hexane (BMH) is a homobifunctional reagent with an effective cross-linking distance of 16.1 Å that reacts with cysteines (Pierce Chemical Co.).[2] It was prepared fresh as a 50 mM stock in dimethyl sulfoxide. Samples were incubated on ice with 1.5–5 mM BMH for 30–60 min. Cross-linking reactions were quenched with a 2–6-fold molar excess of DTT. Control un-cross-linked samples were prepared either by addition of DTT prior to addition of BMH or by substitution of buffer for BMH.

Western Blotting. After separation by SDS–PAGE (as described above), proteins were blotted onto nitrocellulose membranes (Schleicher & Schuell) using an LKB semidry transfer apparatus (Towbin et al., 1979). The membranes were blocked overnight in phosphate-buffered saline (Sigma) containing 5% goat serum, 1% bovine serum albumin, and

[2] Information from Pierce Chemical Co.

0.03% sodium azide at 4 °C, and then incubated overnight at 4 °C with rabbit antiserum R7 to the β subunit (diluted 1000-fold with the above buffer). This β antibody has been described previously by Winslow et al. (1986) from this laboratory. The blot was then washed, incubated with ^{125}I-labeled goat anti-rabbit IgG (ICN Radiochemicals) (100 000 cpm/mL), and washed again. The dried membrane was then exposed to Kodak XAR5 film with intensifying screens at −70 °C overnight to 1 week.

Labeling of Subunits with ^{125}I-Labeled Bolton–Hunter Reagent. For thermal stability studies, ^{125}I-labeled Bolton–Hunter reagent [*N*-succinimidyl 3-(4-hydroxyphenyl)propionate, ICN Radiochemicals], which reacts primarily with the ε-amino group of lysines, was obtained in benzene, dried under nitrogen, and taken up in 100–200 μL of 2 mM Bolton–Hunter reagent in dimethyl sulfoxide. The specific activity was adjusted to 300–500 cpm/pmol by dilution with nonradioactive Bolton–Hunter reagent for cross-linking and digestion studies. For other studies, βγ was labeled with ^{125}I-labeled Bolton–Hunter by adding sample directly to dried reagents and incubating on ice for 1-2 h. Labeling was quenched by addition of 6–9 mM glycine. In those samples that were cross-linked after iodination, unincorporated label was removed by gel filtration over a Sephadex G50 column (Pharmacia) in HMSE buffer. Blue Dextran 2000 (Pharmacia) was added to the sample to mark its elution position.

Thermal Stability of the 14- and 27-kDa Fragments of the β Subunit. The temperature dependence of denaturation of the 14- and 27-kDa tryptic fragments was evaluated in two ways:

(1) Accessibility of Lysine Residues to Modification by ^{125}I-Labeled Bolton–Hunter Reagent. Samples of βγ were digested with 0.2 pmol of trypsin/μL for 30 min at 30 °C. Further digestion was stopped by the addition of soybean trypsin inhibitor to a final concentration of 1.2 pmol/μL. Trypsin-cleaved samples were incubated for 30 min at 0–62 °C, and then equilibrated to 30 °C for 5 min. ^{125}I-Labeled Bolton–Hunter reagent was then added to a final concentration of 0.1 mM, and the reaction was allowed to proceed for 15 min at 30 °C. The fragments of βγ were separated by SDS–PAGE (Laemmli, 1970) with 0.1 M sodium acetate in the lower reservoir buffer (Christy et al., 1989). Dried gels were exposed to Kodak XAR5 film for 5–16 h at −70 °C with intensifying screens. The radioactive bands were cut out of the gel and counted in an LKB γ-counter.

(2) Exposure of Tryptic Cleavage Sites. The βγ subunits were incubated at temperatures ranging from 0 to 60 °C for 30 min, and then equilibrated at room temperature for 10 min. Trypsin was added to a final concentration of 0.4 pmol/μL, and samples were incubated at 30 °C for 30 min. Digestions were quenched by the addition of soybean trypsin inhibitor to a final concentration of 1.4 pmol/μL. Samples were analyzed by SDS–PAGE, and the relative amounts of the Coomassie Blue stained 14- and 27-kDa fragments were determined by densitometry with an LKB laser densitometer.

RESULTS

The Native 14- and 27-kDa Fragments of the β Subunit Are Tightly Associated. In order to determine whether the two tryptic fragments of β could be separated, trypsin-cleaved βγ was mixed with intact βγ and subjected to centrifugation through 5–20% sucrose gradients containing Lubrol PX. Figure 1A compares the mobilities of the 14- and 27-kDa fragments and uncleaved βγ in a single gradient. All three comigrated with apparent sedimentation coefficients of 2.2 S (n = 3; SD

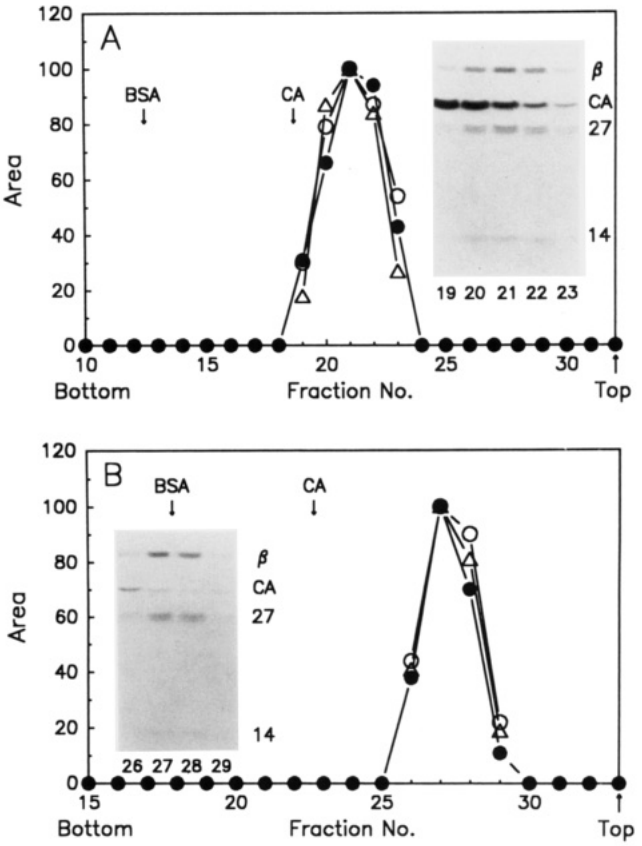

FIGURE 1: Comigration of 14- and 27-kDa tryptic fragments of β in sucrose density gradients prepared with either H_2O or D_2O. Trypsin-cleaved βγ was mixed with uncleaved βγ and fractionated on a 5–20% sucrose density gradient prepared in either H_2O (Panel A) or D_2O (Panel B) as described under Materials and Methods. The relative amounts in each fraction of β (○), the 14-kDa fragment (△), and the 27-kDa fragment (●) are plotted as a percentage of the area of the peak fraction (determined by SDS–PAGE and densitometry). The stained gel has been inset for comparison, and the positions of the β subunit, carbonic anhydrase (CA), and the 14- and 27-kDa tryptic fragments of β are indicated. Base-line values are only plotted for the 27-kDa fragment. Fractions are numbered from the bottom to the top of the gradient, and the positions of the sedimentation markers, bovine serum albumin (BSA) and carbonic anhydrase (CA), are indicated. The results presented are from single gradients, and in each case, similar results were observed in two additional gradients.

from 0.05 to 0.15 for the different proteins). Brain βγ is a hydrophobic protein with a partial specific volume in Lubrol PX that is much higher than that of a typical hydrophilic protein (Huff et al., 1985). Because of its hydrophobicity, βγ sediments more slowly relative to hydrophilic marker proteins in D_2O than in H_2O sucrose gradients. Since the two fragments of βγ were not likely to be equally hydrophobic, we attempted to separate them on sucrose gradients prepared in D_2O. As shown in Figure 1B, the sedimentation rates of native βγ and the tryptic fragments shifted identically. Ion-exchange chromatography on DEAE-cellulose or Mono Q FPLC (Pharmacia), hydrophobic interaction chromatography, and gel filtration also failed to separate the tryptic fragments of β (data not shown). The cosedimentation and coelution of the 14- and 27-kDa tryptic fragments of β with undigested βγ suggest that these fragments form a single stable structure indistinguishable from native βγ.

The β Subunit Does Not Contain Disulfide Bonds. One explanation for the failure to separate the 14- and the 27-kDa fragments was the potential existence of one or more disulfide bonds linking the fragments. A previous report by Ho and Fung (1984) presented indirect evidence that the β subunit

of transducin, which is identical to the major β subunit in brain, contains three intrasubunit disulfide bonds. We attempted to confirm the existence of these disulfides by titrating the free thiols in reduced and nonreduced β subunits with [^{14}C]iodoacetamide. The total number of cysteines present in the β subunit was determined by first denaturing and reducing samples of $\beta\gamma$ with 8 M urea and 1 mM DTT and then alkylating with [^{14}C]iodoacetamide (protocol 1). The number of free thiols in the β subunit was determined by two different methods. In the first, samples of $\beta\gamma$ were simultaneously denatured and alkylated with 8 M urea and 1.5 mM iodoacetamide (protocol 2). Under these conditions, those cysteines involved in disulfide bonds should not be available for reaction with iodoacetamide. In the second method, samples of $\beta\gamma$ were first denatured with 8 M urea and subsequently alkylated with 1.5 mM iodoacetamide (protocol 3). Figure 2 presents the results from four separate experiments. Protocol 2 gave an average level of [^{14}C]iodoacetamide incorporation that was 99.7% ± 7.2% (SD) relative to the average level obtained by protocol 1 (100 ± 5.5%, SD). The essentially equal levels of iodoacetamide labeling obtained with protocols 1 and 2 suggest that there are no disulfide bonds in the β subunit.

Since β_1 contains a total of 14 cysteines, the presence of 1, 2 or 3 disulfide bonds in β would result in 14.3%, 28.6%, or 42.9%, respectively, lower levels of labeling by protocol 2 (free thiols) than by protocol 1 (total thiols). Our data were, therefore, analyzed to determine the statistical probability that a true difference of 14.3% could have been missed due to the variance in the data (Friedman et al., 1982). This analysis indicated that the probability of missing a difference as great as 14.3% was only 0.6%,[3] which supports our conclusion that the tryptic fragments of β are not held together by intrasubunit disulfide bonds.

The incorporation of iodoacetamide was substantially lower in protocol 3 than in protocols 1 or 2 (25.8 ± 3.8% relative to protocol 1). In protocol 2, the free cysteines were alkylated with [^{14}C]iodoacetamide at the same time that the subunits were being denatured, while in protocol 3 the subunits were denatured first and then alkylated. This difference resulted in a 74% lower level of alkylation by protocol 3 than by protocol 2. It appears that in protocol 3, free cysteines, that were previously held apart by the three-dimensional structure of the protein, formed mixed disulfides. Protocol 2 prevented this from occurring by immediately alkylating free thiols that became exposed as the protein denatured. This conclusion is further supported by the observation that additional cysteines could be labeled after treating the alkylated samples from protocol 3 with a reducing agent (data not shown).

The proposal of Ho and Fung (1984), that the β subunit of transducin contains three disulfide bonds, was based on the difference between the total number of cysteines identified by amino acid analysis and the number of free thiols identified by titration with dithionitrobenzoic acid following denaturation with SDS. Their method of quantitating free thiols appears to be essentially the same as the method employed in protocol 3 (Figure 2) which, as discussed above, yielded artifactually low values for free cysteines.

[3] This probability was calculated using the formula $Z_\beta = [\sqrt{2N}(\Delta)/2\sigma] - Z_\alpha$. Given that $2N$ was equal to 16 (total of data from protocols 1 and 2), Student's t values were utilized rather than Z values. Therefore, $Z_\alpha = 1.753$ (degrees of freedom = 15, α-error = 5%). The standard deviation for these data was 6.2, and Δ was set at 14.3%. A value for $Z_\beta = 2.86$ was calculated which represents a β-error of 0.6% (Friedman et al., 1982).

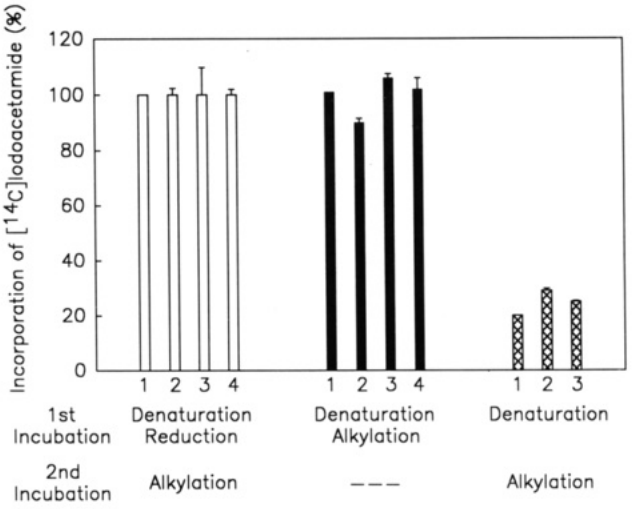

FIGURE 2: Alkylation of the β subunit with [^{14}C]iodoacetamide. Samples of G_o were denatured with 8 M urea and alkylated with [^{14}C]iodoacetamide by three separate protocols as described under Materials and Methods. Protocol 1: Samples were simultaneously denatured and reduced with DTT, and subsequently alkylated in a second incubation (open bars). Protocol 2: Samples were simultaneously denatured and alkylated under nonreducing conditions (shaded bars). Protocol 3: Samples were denatured under nonreducing conditions, and subsequently alkylated in a second incubation (cross-hatched bars). Each bar represents the average value obtained in each of four separate experiments (experiment 1, $n = 1$; experiments 2 and 3, $n = 2$; experiment 4, $n = 3$). The error bars represent the ranges for the values obtained. All values are presented as a percentage of those obtained by protocol 1, which have been normalized to 100% (values ranged from 7951 cpm to 27 031 cpm).

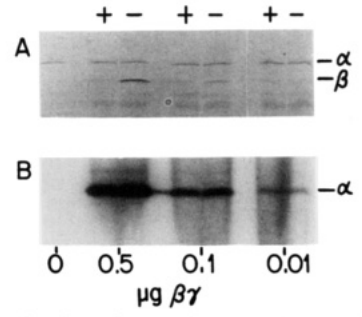

FIGURE 3: Trypsin-cleaved $\beta\gamma$ can interact with α_o to form a substrate for pertussis toxin. α_o (0.2 μg) was incubated with the indicated amounts of $\beta\gamma$ which either had (+) or had not (−) been treated with trypsin, ADP-ribosylated for 45 min at 37 °C by pertussis toxin and [^{32}P]NAD, and analyzed by SDS–PAGE, as described under Materials and Methods. Panel A shows the Coomassie Blue-stained gel, while panel B shows the autoradiogram. Densitometry of the autoradiogram confirmed that ADP-ribosylation supported by cleaved $\beta\gamma$ was 0.9 ± 0.2 of that supported by an equal amount of uncleaved $\beta\gamma$.

Cleaved $\beta\gamma$ Can Functionally Associate with α_o. The stability of the trypsin-cleaved $\beta\gamma$ complex suggested that it might still be able to associate functionally with the α subunit. Since the substrate for pertussis toxin-catalyzed ADP-ribosylation of α is the $\alpha\beta\gamma$ heterotrimer (Neer et al., 1984; Tsai et al., 1984), we used the ADP-ribosylation of α as an assay for the interaction of trypsin-cleaved $\beta\gamma$ with α. In Figure 3, various amounts of cleaved or uncleaved $\beta\gamma$ were incubated with a constant amount of α_o prior to ribosylation. Without $\beta\gamma$, α_o was not a substrate for modification by pertussis toxin (far left lane). The remaining lanes show that cleaved $\beta\gamma$ subunit supported ADP-ribosylation of the α_o subunit with the same concentration dependence as uncleaved $\beta\gamma$. The trypsin-treated samples did not contain detectable levels of uncleaved β. The possibility that ribosylation was supported

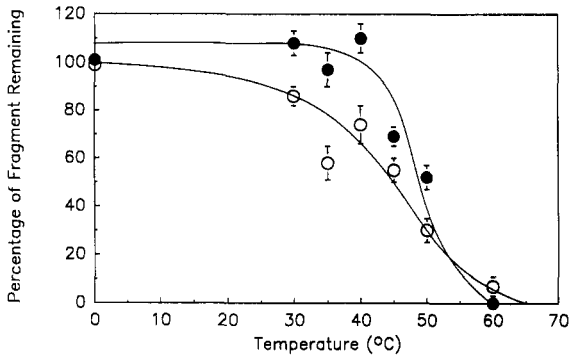

FIGURE 4: Resistance to further tryptic digestion as a measure of the thermal stability of the 14- and 27-kDa tryptic fragments. Samples were incubated at the indicated temperatures for 30 min, equilibrated at room temperature for 10 min, and treated with trypsin as described under Materials and Methods. The samples were then analyzed by SDS–PAGE, and the relative amounts of the 14-kDa (○) and 27-kDa (●) fragments were determined by densitometry of the Coomassie Blue-stained gel. Values are the average and standard error of the mean of four separate experiments (except at 35 °C, n = 2; and at 40 °C, n = 3) and are plotted as a percentage of each fragment obtained from proteolysis of the sample after incubation at 0 °C.

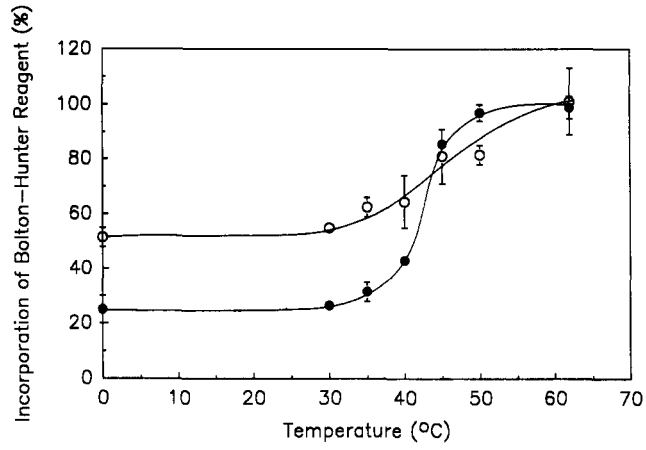

FIGURE 5: Accessibility of lysine residues in the 14- and 27-kDa tryptic fragments of β. Samples of trypsin-cleaved $\beta\gamma$ were incubated at the indicated temperatures for 30 min and at 30 °C for 5 min and then labeled with ^{125}I-labeled Bolton–Hunter reagent at 30 °C for 15 min prior to analysis by SDS–PAGE (see Materials and Methods). The radioactive bands were cut out and counted in an LKB γ counter. The incorporation after incubation at 62 °C was taken as 100%, and the values were calculated for the remaining temperatures. The specific activity of the Bolton–Hunter reagent was 340 cpm/pmol. After subtraction of background, the incorporation into the 27-kDa band was 1470 cpm, and that into the 14-kDa band was 1850 cpm at 62 °C. 1.2 pmol of cleaved $\beta\gamma$ was loaded on each lane of the SDS–PAGE gel. The 27-kDa fragment (●) contains four lysines of which 90% were modified, while the 14-kDa fragment (○) contains six lysines of which 77% were modified.

by residual uncleaved $\beta\gamma$ is ruled out by the identical dose response for the cleaved and uncleaved $\beta\gamma$ samples. For example, the trypsin-cleaved 0.5-μg $\beta\gamma$ sample clearly contained much less than 0.1 μg of *uncleaved* $\beta\gamma$. Nevertheless, it supported a much higher level of ribosylation than the uncleaved 0.1-μg $\beta\gamma$ sample. These results showed that trypsin-cleaved $\beta\gamma$ can bind to α_o and induce the conformational changes in α_o necessary for recognition by pertussis toxin. These results are consistent with the report of Fung and Nash (1983) that cleaved retinal $\beta\gamma$ can interact with retinal α_t.

The 14- and 27-kDa β Fragments Have Different Denaturation Patterns. The relative stabilities of the 14- and 27-kDa fragments were investigated by analyzing their susceptibility to thermal denaturation. This was first done by determining the susceptibility of the fragments to further tryptic digestion following incubation at temperatures ranging from 0 to 60 °C (Figure 4). The denaturation curve for the 27-kDa fragment shows a very sharp thermal transition, while the 14-kDa fragment exhibited a broader temperature-dependent unfolding. A drawback to this method is that it only quantitates the temperature-dependent accessibility of a single additional tryptic cleavage site.

We, therefore, also examined the accessibility of lysine residues to reaction with ^{125}I-labeled Bolton–Hunter reagent. Cleaved $\beta\gamma$ was first incubated at temperatures ranging from 0 to 62 °C, and then labeled at 30 °C with ^{125}I-labeled Bolton–Hunter reagent (Figure 5). Incubation of cleaved $\beta\gamma$ at 62 °C for 30 min prior to treatment with ^{125}I-labeled Bolton–Hunter reagent was sufficient to fully label the lysines present in each fragment. Once again, the incorporation of ^{125}I into the 27-kDa fragment showed a very sharp thermal transition, while the transition for the 14-kDa fragment was broader with a shallower slope. When the data in Figure 5 are replotted after subtracting the incorporation of ^{125}I-labeled Bolton–Hunter reagent at 0 °C, then the plot looks similar to the one in Figure 4 (replot not shown).

The γ Subunit Associates with the 14-kDa Fragment of the β Subunit. We have previously shown that the 27-kDa fragment of β_1 can be chemically cross-linked to either the α_o or the α_i subunit (Yi et al., 1991). We now show that the 14-kDa fragment can be cross-linked to γ. Treatment of $\beta\gamma$ with 1,6-bis(maleimido)hexane (BMH), a homobifunctional cross-linking reagent that reacts with cysteines, resulted in the formation of a product with an apparent molecular mass on SDS–PAGE of 46 kDa (see Figures 6B, 7, and 9). The average yield of this cross-linked product was 20 ± 8% (n = 7), determined by densitometry of Coomassie Blue stained gels. Anti-β antibody reacted with the 46-kDa band, as well as a few higher molecular weight cross-linked bands that were not characterized further (Figure 6A).

The γ subunit in the 46-kDa product was identified by cross-linking a sample of $\beta\gamma$ in which γ was modified with ^{125}I-labeled Bolton–Hunter reagent to a significantly higher specific activity than β (Figure 6C). Although β_1 and γ_2 contain roughly equivalent numbers of lysines (10 and 7, respectively), labeling conditions were employed that differentially labeled the γ subunit (see legend of Figure 6). Comparison of the autoradiogram in Figure 6C to its corresponding Coomassie Blue stained gel in Figure 6B clearly shows that the relative specific radioactivity of the cross-linked 46-kDa band is greater than that of the un-cross-linked β band. The β subunit must, therefore, be cross-linked to another radiolabeled protein contained in the sample. Relative specific activities were calculated by dividing the densitometrically determined areas of peaks from the autoradiogram by the area of their corresponding peaks from the Coomassie Blue stained gel. The average ratio of the specific activity of the 46-kDa product to the specific activity of β was 6.5 ± 3.5 (n = 3). Besides γ, there were only two other proteins in the sample (at approximately 64 and 17 kDa) that were labeled to a significant degree. The reduction in label in the 64- and 17-kDa bands upon BMH treatment was insufficient to account for the increase in label in the 46-kDa band. We conclude that the higher specific activity of cross-linked product compared to un-cross-linked β was due to the presence of the γ subunit in the 46-kDa band.

The cross-linked and un-cross-linked $\beta\gamma$ complexes cosedimented on 5–20% sucrose gradients (Figure 7) (3.5 S, n = 2, range ± 0.05).[4] A third experiment without markers

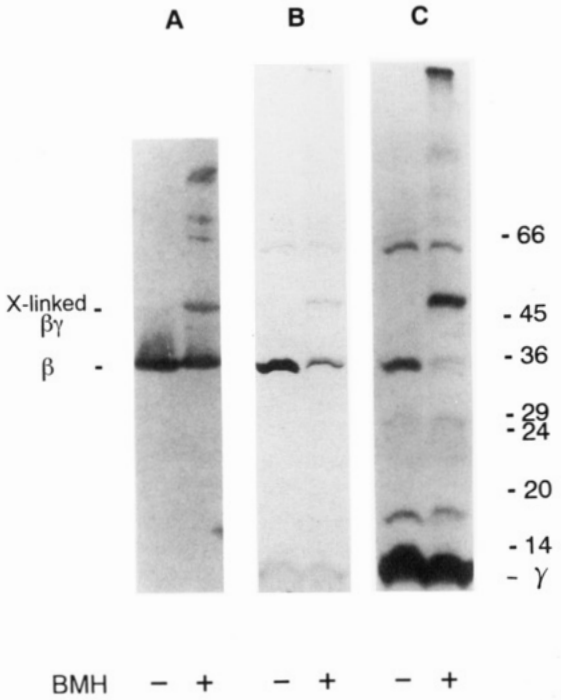

FIGURE 6: Formation of cross-linked βγ by BMH. Native βγ was incubated with 2 mM BMH for 1 h and then quenched with 12 mM DTT as described under Materials and Methods. BMH-treated (+) and untreated samples (−) were then analyzed by SDS–PAGE. Panel A: Autoradiogram of a Western blot of non-cross-linked and cross-linked samples (2.5 μg) probed with R7 rabbit antiserum against β subunit (^{125}I-anti-rabbit IgG was used for detection; see Materials and Methods). Panels B & C: Prior to cross-linking, βγ was labeled with ^{125}I-labeled Bolton–Hunter reagent (0.13 mM, 0 °C, 30 min). Panel B shows the Coomassie Blue-stained proteins (2.5 μg of βγ), and panel C shows an autoradiogram of the gel in panel B. The mobilities of specific proteins and of the molecular mass markers (kDa) are indicated. No radioactivity was observed at the position of γ when samples containing ^{125}I-labeled Bolton–Hunter reagent, but not protein, were electrophoresed. The identity of radiolabeled γ was verified by its comigration with β on sucrose gradients both in the presence and in the absence of α.

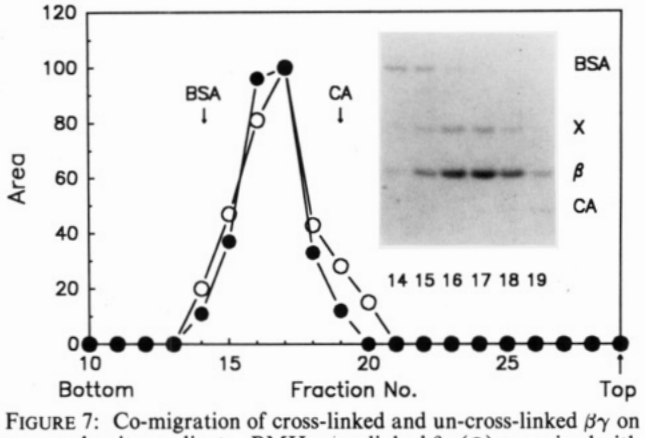

FIGURE 7: Co-migration of cross-linked and un-cross-linked βγ on sucrose density gradients. BMH-cross-linked βγ (○) was mixed with un-cross-linked βγ (●) and fractionated on 5–20% sucrose gradients as described under Materials and Methods. Fractions were analyzed by SDS–PAGE and densitometry, and the areas of individual bands are plotted as percentages of the peak fraction. The Coomassie Blue-stained gel has been inset for comparison, and the positions of bovine serum albumin (BSA), carbonic anhydrase (CA), the β subunit, and the cross-linked product (X) have been indicated.

confirmed the cosedimentation of cross-linked and un-cross-linked βγ. This finding showed that the 46-kDa product contains one β subunit cross-linked to one γ subunit, and ruled out the possibility that the 46-kDa band contained β–β or

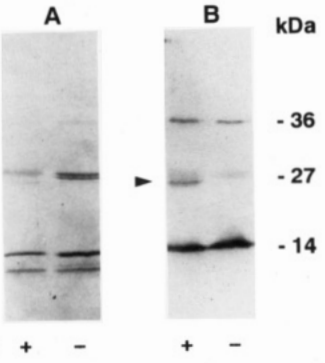

FIGURE 8: Identification of the amino-terminal fragment of β in a cross-linked tryptic cleavage product of BMH-treated βγ. βγ was cross-linked with BMH (+) and then cleaved with 0.3 pmol/μL trypsin as described under Materials and Methods. Further digestion was prevented by addition of 12.8 mM benzamidine. Non-cross-linked samples were prepared by addition of DTT (7.5 mM) to control samples before BMH (−). Panel A: Coomassie Blue-stained proteins. Panel B: Autoradiogram of Western blot probed with R7 rabbit antiserum against β subunit. The apparent molecular mass (kDa) of specific proteins is shown on the right. The position of the 25-kDa tryptic product is indicated by an arrow.

γ–γ cross-links. It did not rule out the possibility that each subunit contained intrasubunit cross-links as well.

Digestion of cross-linked βγ with trypsin produced a new immunoreactive band just below the 27-kDa fragment of β that was recognized by an antibody predominantly directed against the 14-kDa fragment of β (Figure 8B). When βγ was first digested with trypsin and then cross-linked with BMH, the same results were observed (data not shown). The appearance of a doublet at the 27-kDa position after digestion of non-cross-linked βγ has been reported previously (Winslow et al., 1986), and may be due to the presence of both the β_1 (36 kDa) and β_2 (35 kDa) subunits. When ^{125}I Bolton–Hunter-labeled and cross-linked βγ was digested with trypsin, a new radioactive band appeared on SDS–PAGE close to the 27-kDa fragment (Figure 9). This is slightly higher than the position of the 25-kDa band in Figure 8, probably due to the chemical modification. Quantitation of the labeled bands indicates that the new band contains γ and did not arise from an internal cross-link within the amino-terminal fragment of β.[5] Taken together, these results argue that the cross-linked tryptic fragment observed just below 27 kDa represents γ cross-linked to the amino-terminal fragment of β. This observation does not rule out the possibility that γ also interacts with the carboxyl-terminal 27-kDa fragment of β.

DISCUSSION

When Fong et al. (1986) deduced the amino acid sequence of β_1, they found that the sequence appeared to contain repeating units of 41–45 amino acids. These repeats, which have been called either β-transducin repeats (Keleher et al., 1992) or WD-40 repeats (Simon et al., 1991), are also found in a large number of other proteins, but there are as yet no

[4] Because brain βγ binds a substantial amount of detergent that affects both the mass and the partial specific volume ($\bar{v}$) of the particle, the *apparent* sedimentation coefficient is smaller in Lubrol PX ($\bar{v}$ = 0.96 mL/g) than in cholate ($\bar{v}$ = 0.75 mL/g) (Steele et al., 1978).

[5] Quantitation of the label in the trypsin-digested bands before and after cross-linking showed that the label in the 27-kDa region increased by 980 cpm from background while the label in the 14-kDa fragment decreased by only 270 cpm (1580 cpm in the un-cross-linked sample, 1310 cpm in the cross-linked sample). The unidentified band at 66 kDa showed a slight increase of 75 cpm. The only source of label sufficient to account for the extra 710 cpm (980 cpm − 270 cpm) in the cross-linked product in the 27-kDa region is the γ subunit.

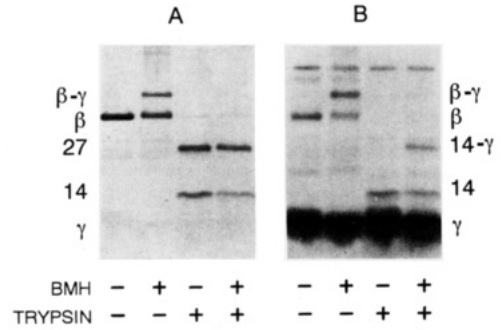

FIGURE 9: Identification of γ in a cross-linked tryptic cleavage product of BMH treated βγ. ^{125}I-Bolton–Hunter labeled βγ was cross-linked with BMH (+) and then cleaved with 0.3 pmol/μL trypsin as described under Materials and Methods. Further digestion was prevented by addition of 12.8 mM benzamidine. Non-cross-linked samples were prepared by addition of DTT (7.5 mM) to control samples before BMH (–). Panel A: Coomassie Blue-stained proteins. Panel B: Autoradiogram of tryptic products.

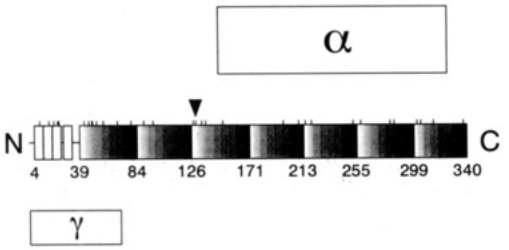

FIGURE 10: Model of G protein subunit interactions. The β subunit is represented as a linear sequence with its amino (N) and carboxyl (C) terminal ends indicated. The α and γ subunits have been placed relative to the β subunit in order to indicate the known regions of contact between these subunits. Two types of repetitive sequences have been identified within the primary sequence of β. These are putative α-helical heptad repeats (open bars) of the type observed in coiled-coil structures (Lupas et al., 1991), and WD-40 repeats (shaded bars) (Fong et al., 1986). The locations of 32 potential tryptic cleavage sites in β are indicated by vertical bars. The site at which the β subunit of native βγ is cleaved by trypsin is indicated with an arrow.

known common functions to correlate with the apparent similarities in amino acid sequence (van der Voorn & Ploegh, 1992). Further analysis of the structure of the β subunit shows that the first repeat (amino acids 1–43) fits poorly with the consensus sequence that has emerged from analysis of these repeating units in several proteins (Fong et al., 1986, 1987; Dalrymple et al., 1989). This amino-terminal region contains a stretch of four heptad repeats that have also been predicted to form an amphipathic helix of the type commonly found in coiled-coil structures (Lupas et al., 1991). The repetitive sequence of β is shown schematically in Figure 10. The tryptic cleavage site is located at the beginning of the third repeating unit. Consequently, the C-terminal 27-kDa fragment is composed entirely of five repeating units.

It is puzzling that only Arg129 of β_1 is accessible to tryptic digestion, despite the presence in other repeating units of potential tryptic cleavage sites at positions similar to Arg129. Is this arginine in a boundary region between two tightly folded structural domains, or does β have a single structure stabilized by repetitive interactions as suggested by its sequence? Our inability to separate the 14- and 27-kDa tryptic fragments of β (even though we have shown that they are not held together by disulfide bonds) fails to support a two-domain model, and suggests that tryptic cleavage has very little effect on βγ structure or function. If cleavage had caused a significant structural change, such as partial unfolding, then we would have expected the fragments to sediment more slowly than native βγ, even if they remained associated with each other. Cleaved βγ, however, had the same sedimentation coefficient as native βγ. Cleaved βγ was also able to associate with α and to support pertussis toxin-catalyzed ADP-ribosylation of α with the same efficiency as uncleaved βγ. These results all favor a model in which β folds into a compact structure stabilized by repetitive interactions.

As modeled in Figure 10, the overall structures of the 14- and 27-kDa fragments of β are predicted, on the basis of their sequences, to be quite different. The latter is composed entirely of five repeating units, while the former has two repeating units, a stretch of putative α helix that may interact with γ (Fong et al., 1986; Bubis & Khorana, 1990; Lupas et al., 1991). The overall similarity of the repeating units ranges from 59% for the most similar pair to 29% for the most different. If the similarity of these sequences is sufficient to define repetitive structures, then the 27-kDa fragments would be expected to have a very sharp thermal transition between native and denatured forms. The narrow temperature transition range for the 27-kDa carboxyl-terminal fragment is consistent with repetitive secondary and tertiary structures. The broader thermal denaturation range of the 14-kDa fragment is consistent with the more heterogeneous secondary structure predicted by its amino acid sequences. We can only speculate on the contribution of γ to the shape of the denaturation curves. A tight association with γ might be expected to stabilize the 14-kDa region against denaturation and to produce a sharp transition from native to denatured structure. However, the opposite was observed, so we conclude either that γ does not interact with the majority of the 14-kDa region or that it denatures readily by itself. If so, then association with γ may be a contributing factor to the heterogeneous nature of the denaturation of the 14-kDa fragment.

Cross-linking studies using BMH indicate that the γ subunit from bovine brain associates with the 14-kDa fragment of β. Similarly, Bubis and Khorana (1990) have found that cupric phenanthroline catalyzes the formation of a disulfide bond between Cys36/37 of retinal transducin γ_1 and Cys25 in the predicted amphipathic helix of retinal β_1. The β subunit of transducin βγ and the predominant form of β in brain are identical, but their associated γ subunits are quite different. In addition, the types of cross-links formed in our studies and those of Bubis and Khorana (1990) are significantly different. Therefore, we cannot be sure that BMH has cross-linked brain γ to the amphipathic helix in β. Another possibility is that BMH cross-linked γ to one of the two repeats in the amino-terminal region. Recent studies of Pronin and Gautam (1992) suggest that at least some of the repeating units are important for defining the specificity of β and γ interactions.

Our data show that βγ forms a stable structure that is not dissociated by cleavage at Arg129 and is able to interact with α_o even though it is nicked. The stability of β is likely to come from strong noncovalent interactions among its repeating units and perhaps also with γ. Defining the relative role of γ in determining the conformation of β, the minimum number of repeating units needed for βγ function and the detailed interactions among the units will provide new insight into the structural basis of βγ function.

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