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2-Azido-[^{32}P]NAD$^+$, a photoactivatable probe for G-protein structure: Evidence for holotransducin oligomers in which the ADP-ribosylated carboxyl terminus of α interacts with both α and γ subunits

(photocrosslinking/transducin oligomers/cysteine-347)

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ABSTRACT **A radioactive and photoactivatable derivative of NAD$^+$, 2-azido-[*adenylate*-^{32}P]NAD$^+$, has been synthesized and used with pertussis toxin to ADP-ribosylate Cys^{347} of the α subunit (α_T) of G_T, the retinal guanine nucleotide-binding protein. ADP-ribosylation of α_T followed by light activation of the azide moiety of 2-azido-[*adenylate*-^{32}P]ADP-ribose produced four crosslinked species involving the α and γ subunits of the G_T heterotrimer: an α trimer (α–α–α), an α–α–γ crosslink, an α dimer (α–α), and an α–γ crosslink. The α trimer, α–α–γ complex, α dimer, and α–γ complexes were immunoreactive with α_T antibodies. The α–α–γ and the α–γ complexes were immunoreactive with antisera recognizing γ subunits. No evidence was found for crosslinking of α_T to β_T subunits. Hydrolysis of the thioglycosidic bond between Cys^{347} and 2-azido-[*adenylate*-^{32}P]ADP-ribose using mercuric acetate resulted in the transfer of radiolabel from Cys^{347} of α_T in the crosslinked oligomers to α monomers, indicative of intermolecular photocrosslinking, and to γ monomers, indicative of either intermolecular crosslinked complexes (between heterotrimers) or intramolecular crosslinked complexes (within the heterotrimer). These results demonstrate that G_T exists as an oligomer and that ADP-ribosylated Cys^{347}, which is four residues from the α_T carboxyl terminus, is oriented toward and in close proximity to the γ subunit.**

G_T, the retinal guanine nucleotide-binding protein (G protein), is a heterotrimer composed of α, β, and γ subunits with molecular masses of 39, 36, and 8 kDa, respectively (1). G_T couples the activation of rhodopsin by photon adsorption with the regulation of cGMP phosphodiesterase in rod and cone outer segments (2). The α subunit (α_T) of G_T is similar in sequence to the α_i and α_o subunits of G_i and G_o (3), and all are substrates for ADP-ribosylation by pertussis toxin (4, 5). The site for pertussis toxin-catalyzed ADP-ribosylation of α_T is Cys^{347}, four amino acids from the carboxyl terminus (6). ADP-ribosylation of α_T Cys^{347} by pertussis toxin stabilizes the (α_T·GDP)$\beta\gamma$ complex, which is functionally unresponsive to receptor activation, thus interrupting signal transduction.

Previously, we characterized the derivatization of holotransducin ($\alpha_T\beta\gamma$) with the sulfhydryl reagent *N*-3-[^{125}I]iodo-4-azidophenylpropionamido-*S*-(2-thiopyridyl)cysteine, which covalently derivatized Cys^{210} and Cys^{347} of α_T (7). Intermolecular transfer of the iodinated photolabel after photolysis established that the carboxyl-terminal domain of α_T is folded close to the α_T GTP-binding domain. In addition, pertussis toxin catalyzed derivatization of α_T Cys^{347} with etheno-NAD$^+$, a fluorescent NAD$^+$ analogue, followed by limited proteolysis of the α_T amino terminus demonstrated that the amino and carboxyl termini of α_T are in close proximity in the tertiary structure of G_T (8).

In this study, we report the use of a photoactivatable derivative of NAD$^+$, 2-azido-[*adenylate*-^{32}P]NAD$^+$, and pertussis toxin to derivatize Cys^{347} of α_T. Upon photolysis, the light-sensitive azide moiety was activated and used as a probe to study the association of subunits and the oligomeric structure of G_T.

MATERIALS

Phosphorylation of 2′,3′-isopropylidene-2-azidoadenosine to produce 2-azidoadenosine 5′-monophosphate was performed according to the method of Yoshikawa and Kato (9). The synthesis of 2-azido[^{32}P]adenosine 5′-monophosphate at a specific activity of 10 Ci/mmol (1 Ci = 37 GBq) was performed according to the method of Boulay *et al.* (10). Both radiolabeled and nonradiolabeled nucleotides were purified by Dowex 50W-X4 H$^+$ ion-exchange chromatography as described by Boulay *et al.* (10). 2-Azido-[*adenylate*-^{32}P]-NAD$^+$ was synthesized by a modification of the method of Hoard and Ott (11) at a specific activity of 1 Ci/mmol using $^{32}P_i$. The product, 2-azido-[*adenylate*-^{32}P]NAD$^+$, was stored in methanol at −20°C.

Enzymatic Analysis of 2-Azido-[*adenylate*-^{32}P]NAD$^+$. 2-Azido-[*adenylate*-^{32}P]NAD$^+$ was enzymatically characterized by using nucleotide pyrophosphatase and alkaline phosphatase. The assay was performed at 37°C in 50 μl containing 100 mM Tris·HCl (pH 8.0), 20 mM $MgCl_2$, 0.11 unit of nucleotide pyrophosphatase, 0.11 unit of alkaline phosphatase, and 116 pmol of 2-azido-[*adenylate*-^{32}P]NAD$^+$ (≈195,000 dpm). After 1 hr, 1 μl from the assay was analyzed by silica thin-layer chromatography with (*i*) isobutyric acid/NH_4OH/H_2O (66:1:33) and (*ii*) 1-propanol/NH_4OH/H_2O (20:12:3). Hydrolysis of 2-azido-[*adenylate*-^{32}P]NAD$^+$ (*i*, R_f 0.44; *ii*, R_f 0.34) with nucleotide pyrophosphatase was complete after 1 hr, forming 2-azido-[^{32}P]AMP that comigrated with nonradioactive 2-azido-AMP (*i*, R_f 0.50; *ii*, R_f 0.37). Subsequent treatment of 2-azido-[^{32}P]AMP with alkaline phosphatase produced $^{32}PO_4$ (*i*, R_f 0.18; *ii*, R_f 0.05) and 2-azidoadenosine.

ADP-Ribosylation of Purified Holotransducin. Prior to ADP-ribosylation, purified transducin (12, 13), stored at −20°C, was passed through a 3-ml P_6 column (0.7 × 10 cm; three-drop fractions) to remove dithiothreitol and to reduce the glycerol concentration from 50% to 10% at 4°C. Removal of dithiothreitol from the transducin preparation was essen-

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Abbreviations: G protein, guanine nucleotide-binding protein; G_T, retinal G protein; α_T, α subunit of G_T.

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tial to prevent reduction of the azide moiety of 2-azido-[*adenylate*-^{32}P]NAD$^+$ during the ADP-ribosylation procedure (14). The P_6 column was equilibrated and transducin was eluted with 5 mM Tris·HCl, pH 7.2/2.5 mM $MgCl_2$/50 mM NaCl/0.05 mM EDTA/10% (vol/vol) glycerol (P_6 buffer). Fractions were assayed for protein by the Coomassie dye-binding method of Bradford (15) with bovine serum albumin used as a standard. Transducin (400 μg or 5 nmol) in P_6 buffer was ADP-ribosylated in 400 μl for 2 hr at 30°C with the following reagents: 0.5 mM ATP, 8 μg of pertussis toxin, 20 μM 2-azido-[*adenylate*-^{32}P]NAD$^+$ (250 μCi/μmol), 50 mM 2-mercaptoethanol.

Photolysis of ADP-Ribosylated Transducin. To photocrosslink the ADP-ribosylated transducin, free 2-azido-[*adenylate*-^{32}P]NAD$^+$ was removed by gel-filtration chromatography. A P_6 column, as described above, was used to remove free radiolabel. Fractions containing ADP-ribosylated transducin were assayed for protein by the Bradford method. Pooled fractions were photolyzed at 4°C through 2-mm-thick Pyrex tubes for 5 sec at a distance of 10 cm from a 1-kW mercury lamp (16). When ADP-ribosylated transducin was photolyzed in a volume that was >100 μl, the protein was precipitated as described (17). Crosslinking was analyzed by SDS/PAGE (18).

Antisera were obtained and immunoblotting was performed as described (19). Immunoreactivity was detected by the procedure described in the Bio-Rad Immuno-Blot protein A horseradish peroxidase conjugate instruction manual (catalog no. 170-6507).

Chemical Cleavage of the Thioglycosidic Bond. Cleavage of thioglycosidic bonds has been described by Krantz and Lee (20). Mercuric acetate hydrolyzes the thioglycosidic bond between Cys^{347} of α_T and the 1′ carbon of 2-azido[*adenylate*-^{32}P]ADP-ribose. Briefly, a final concentration of 10 mM mercuric acetate/0.1% SDS/0.33% aqueous acetic acid was incubated for 15 min at 30°C with ADP-ribosylated transducin. Radiolabel transfer was analyzed by 8–16% SDS/PAGE and autoradiography.

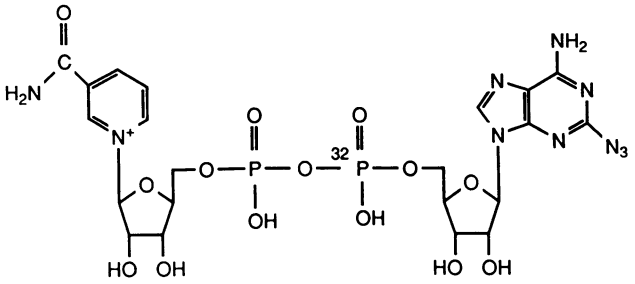

FIG. 1. Structure of 2-azido-[*adenylate*-^{32}P]NAD$^+$.

RESULTS

The important feature of 2-azido-[*adenylate*-^{32}P]NAD$^+$ (Fig. 1) is that the photoactive moiety and the radioactive atom are positioned on the same side of the phosphodiester bond. Thus, ADP-ribosylation of a G protein followed by a photolytic reaction of the azide with a neighboring polypeptide can be used to transfer the molecule to the site of azide insertion by cleavage of the thioglycosidic linkage with mercuric acetate (20) or cleavage of the phosphodiester bond with snake venom phosphodiesterase (21).

The stoichiometry of covalent modification of α_T with either [*adenylate*-^{32}P]NAD$^+$ or 2-azido-[*adenylate*-^{32}P]-NAD$^+$ was 1:1 and 30% of the transducin molecules were ADP-ribosylated (data not shown). When the 2-azido-[^{32}P]ADP-ribosylated G_T was photolyzed and the photocrosslinks were analyzed by SDS/PAGE, three primary bands in addition to the labeled 39-kDa α_T monomer were readily detected (Fig. 2, lane b). The major crosslinked polypeptides that contained [^{32}P]ADP-ribosylated α_T appeared at 47, 83, and 105 kDa. A minor photocrosslinked band at 92 kDa was

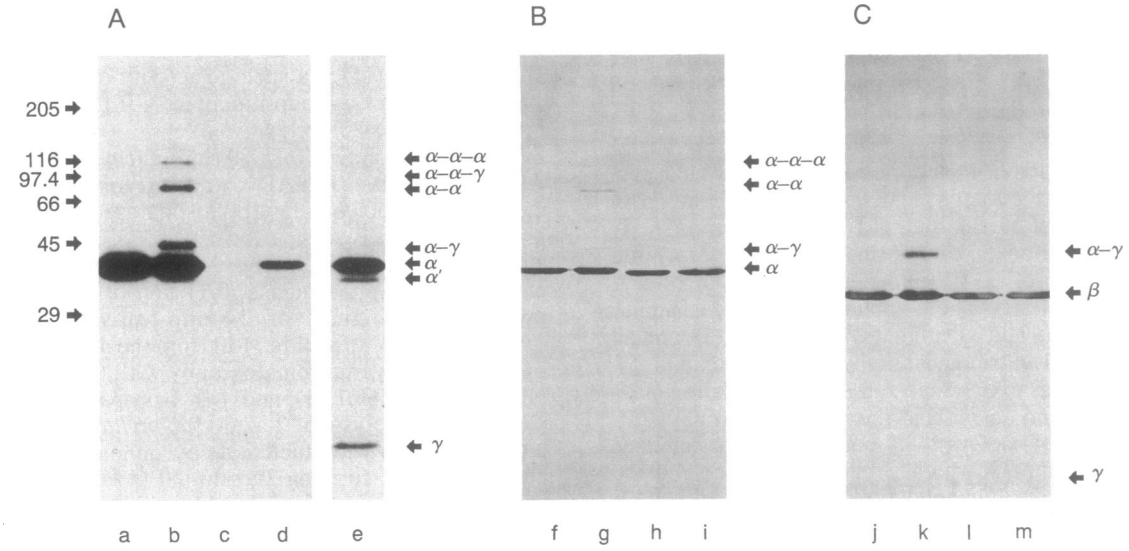

FIG. 2. (*A*) Autoradiogram of ADP-ribosylated G_T and formation of photocrosslinked oligomers by using 2-azido-[*adenylate*-^{32}P]NAD$^+$. G_T was ADP-ribosylated with 2-azido-[*adenylate*-^{32}P]NAD$^+$ (lane a) and photolyzed for 5 sec (lane b). Crosslinked products were generated (arrows) with azide activation by light. Hydrolysis of the thioglycosidic bond of ADP-ribosylated α_T by mercuric acetate removed the ^{32}P radiolabel from α_T in the absence of prior light exposure (lane c). After photolysis, the crosslinked species were reversed, thereby transferring radiolabel from α_T to either α or γ subunit (lanes d and e). The autoradiograms in *A* were obtained from the same gel. Lanes a–d were exposed for 17 hr, whereas lane e is a 75-hr exposure of lane d. (*B*) Immunoblotting of the major 39-kDa α_T bands that are ADP-ribosylated (lanes f and g) or photolabeled (lanes g and i). Lane g demonstrates that anti-α_T antiserum recognizes each of the photocrosslinked products. (*C*) Antiserum recognizing β and γ subunits recognizes the 36-kDa β subunit and the 47-kDa band, which was recognized by the anti-α_T antiserum (*B*, lane g). Subsequent treatment with mercuric acetate chased the radiolabel to the α subunit (lanes d and e) with no label in the β-subunit band. Since the photolabeled γ subunit was not transferred well to nitrocellulose, the γ subunit was difficult to detect (lanes j–m), but long autoradiographic exposures demonstrated radiolabeled γ subunit in lane e. The following protein standards (from Sigma) were used: carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa), phosphorylase B (97 kDa), β-galactosidase (116 kDa), and myosin (205 kDa).

also produced. The molecular masses (as determined by using protein standards) for the photocrosslinked species were consistent with the following crosslinks: α–γ (47 kDa), α–α (83 kDa), α–α–γ (92 kDa), and α–α–α (105 kDa). The 47- to 105-kDa crosslinked proteins required azide activation for their appearance (Fig. 2*A*, compare lanes a and b) and were highly reproducible in numerous G_T preparations.

Cleavage of the thioglycosidic bond with mercuric acetate removed >95% of the 2-azido-[^{32}P]ADP-ribose from nonphotolyzed α_T (Fig. 2*A*, lane c). In contrast, mercuric acetate treatment after photolysis demonstrated photoinsertion into α and γ subunits (lanes d and e). The majority of the label was covalently photolyzed into the α subunit; however, the destaining conditions for treatment of the gel led to some loss of the γ subunit (data not shown), making determination of the stoichiometry of label transfer from the α–γ crosslink to the γ subunit difficult. One additional ^{32}P-radiolabeled band that was seen upon reversal migrated immediately below the α subunit (lane e). This ^{32}P-radiolabeled band was not superimposable on the Coomassie-stained β subunit and is most likely an intramolecular crosslinked α subunit [referred to as α' by Ho and colleagues (22)], which has not been completely reversed with mercuric acetate.

Evidence to support the identity of the crosslinked polypeptides was provided by immunoblotting with antisera specific for either α_T or β and γ subunits (Fig. 2 *B* and *C*). Fig. 2 (*B* and *C*) shows immunoblots for the anti-α_T and anti-β and -γ antisera, respectively. It is clear that the 47-, 80-, and 105-kDa bands generated by photolysis of 2-azido-[^{32}P]ADP-ribosylated α_T Cys^{347} contain the α subunit. Only the 47-kDa crosslinked product contains the γ (Fig. 2*C*, lane k), and no evidence for the β subunit in any of the bands was observed. These results confirm that azide activation results in the insertion of the 2-azido-[^{32}P]ADP-ribose moiety into α_T by an intramolecular insertion into the α_T monomer, two types of intermolecular insertions, α–α dimers and α–α–α trimers, and an α–γ crosslink. The α–α–γ crosslink was not readily detected in this experiment by either autoradiography or immunoblotting (Fig. 2), consistent with its low abundance relative to the major crosslinked products (Fig. 2*A*, lane b).

To assess whether the α–γ crosslink was generated within the heterotrimer (i.e., $\alpha\beta\gamma$) or between heterotrimers (i.e., $\alpha\beta\gamma$–$\alpha\beta\gamma$), a series of dilution experiments were performed in which 2-azido-[^{32}P]ADP-ribosylated G_T was photocrosslinked at concentrations varying from 83 nM to 3.3 μM (Fig. 3). It was found that α–α dimers and α–α–α trimers (Fig. 3 *B* and *C*) were sensitive to dilution. Therefore, the α_T dimers and trimers must have originated from intermolecular crosslinks due to oligomeric forms of transducin heterotrimers. Formation of the α–γ crosslink was also dependent on the transducin concentration as shown in Fig. 3*A*. This result suggests that intermolecular photocrosslinking has occurred (i.e., crosslinking an α_T subunit of one heterotrimer to a γ subunit of another heterotrimer) but does not rule out the possibility that intramolecular photocrosslinking of α_T to its own γ subunit has occurred within the transducin heterotrimer. The latter possibility might occur if the ADP-ribosylated heterotrimer has reduced affinity between α and $\beta\gamma$ subunits or if the association between the α and γ subunits within the heterotrimer is dependent on association between transducin oligomers.

These findings demonstrate that oligomers of functional G_T are dynamic, sensitive to the concentration of the protein, and that crosslinking of subunits occurs primarily between heterodimers. Specificity of the oligomeric interactions was demonstrated by the highly reproducible and selective formation of α–α and α–γ crosslinks, where no α–β or α–α–β crosslinks were detected.

These findings are consistent with the following conclusions: (*i*) A significant fraction of G_T exists as dimers and/or trimers in solution. (*ii*) The γ subunit of a second G_T $\alpha\beta\gamma$ heterotrimer is positioned within 2 nm of the α_T Cys^{347}, assuming a fully extended structure for the azido-ADP-ribosyl molecule (determined by the distance of an extended molecular space-filling model from the 1′ carbon on the ribose sugar to the C-2 carbon on the adenine ring of 2-azido-ADP-ribose). (*iii*) 2-Azido-ADP-ribosyl-α_T Cys^{347} is not oriented intra- or intermolecularly toward the β subunit.

The basic new finding, therefore, is that the G_T oligomeric structure interacts in such a way that the α subunit carboxyl terminus of one heterotrimer is in close proximity with both α and γ subunits of a second G_T heterotrimer (see Fig. 4 and *Discussion*).

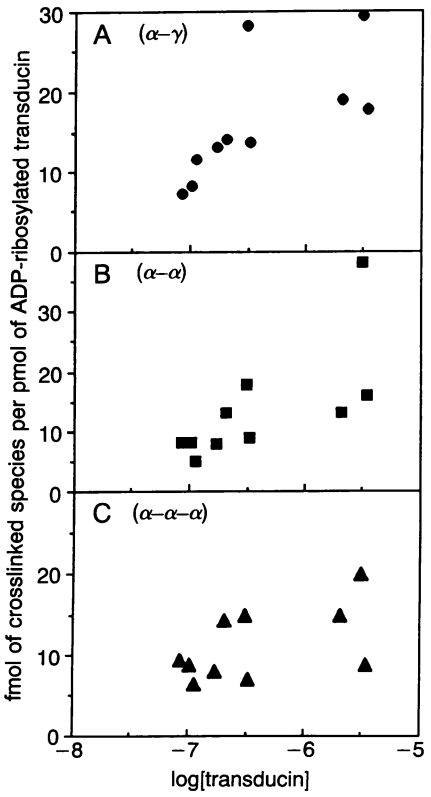

FIG. 3. Quantitation of photocrosslinking at various concentrations of ADP-ribosylated G_T. After G_T ADP-ribosylation with pertussis toxin and 2-azido-[*adenylate*-^{32}P]NAD$^+$, excess radiolabel was removed by gel filtration. After determination of G_T concentration by protein determination, pooled fractions were diluted to appropriate concentrations and photolyzed. Crosslinked species were separated by SDS/PAGE and quantitated by excising the gel band and liquid scintillation counting. The data were obtained from three independent experiments and were normalized relative to the initial amount of ADP-ribosylated G_T that had not been photolyzed. The following crosslinked species were quantitated: *A*, α–γ (●); *B*, α–α (■); *C*, α–α–α (▲).

DISCUSSION

2-Azido-[*adenylate*-^{32}P]NAD$^+$ is an NAD$^+$ analog that is radioactive and photoactivatable. It is used by pertussis toxin to ADP-ribosylate α_T Cys^{347} of the retinal G protein. The thioglycosidic linkage of the 2-azido-[^{32}P]ADP-ribosyl–Cys^{347} conjugate is readily reversible by exposure to mercuric acetate, allowing for photoinsertion and transfer of the ^{32}P radiolabel to a neighboring polypeptide. The value of this general approach to the structural analysis of proteins is that one can place a photocrosslinking reagent in a defined location in a polypeptide and transfer the label to a known position in a neighboring polypeptide. Using this approach to analyze the structure of G_T resulted in the "trapping" of

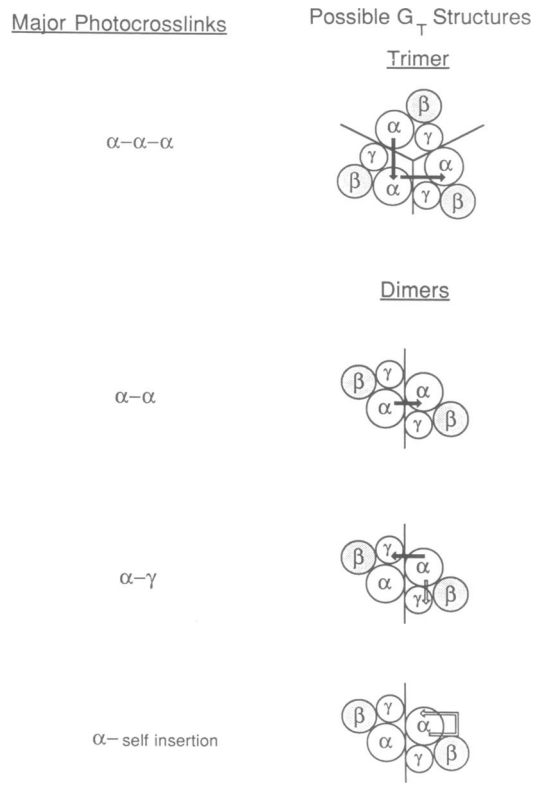

FIG. 4. Proposed model of G_T oligomers obtained from photocrosslinking analysis. The G_T oligomers could exist as trimers or dimers that are stabilized by specific α–α interactions with close association of the γ subunit to the α-subunit interaction domains. Intermolecular crosslinking of α_T is depicted by solid arrows with the point of the arrow representing the insertion site into another α_T or γ subunit. Intramolecular crosslinking of α_T to itself or to the γ subunit is depicted with an open arrow with the point of the arrow representing the insertion site.

photocrosslinks consisting of α dimers and trimers, as well as α–γ dimers. The photocrosslinked products were shown not to involve β subunits of the $\alpha\beta\gamma$ heterotrimeric G_T molecule. No photocrosslinks were observed when [*adenylate*-^{32}P]-NAD^+ was used for pertussis toxin-catalyzed ADP-ribosylation, indicating that photocrosslinks required the azide moiety of 2-azido-[*adenylate*-^{32}P]NAD^+ and were not due to photodecomposition of the adenine ring with "reverse" photoaffinity labeling (23) by activation of an amino acid residue in the binding site.

The identity of the crosslinked proteins was defined by several criteria: (*i*) The presence of radioactivity in the crosslinked proteins in which the ^{32}P radiolabel was selectively introduced at α_T Cys^{347} using pertussis toxin; thus, any crosslink product had to contain one or more α_T subunits. (*ii*) Immunoblotting identified α and γ subunits in specific photocrosslinked products. No evidence was found for crosslinking of α_T to β_T subunits. (*iii*) The molecular mass of each crosslinked product was consistent with the predicted size of the proposed oligomeric polypeptide forms. (*iv*) Transfer of the ^{32}P radiolabel was observed upon reversal of the thioglycosidic linkage and demonstrated the transfer of radiolabel only to α and γ subunits.

The oligomeric forms of G_T are dynamic and sensitive to dilution (Fig. 3). Previous studies have indicated that G_T exists and functions as an oligomer (13, 24, 25) and that the basis for the oligomers was specific α–α subunit interactions (19). The use of 2-azido-[^{32}P]NAD^+ to specifically label α_T Cys^{347} to capture specific photocrosslinked products is the most selective labeling procedure to date that "traps" oligomers of α_T subunits. Other crosslinking protocols with bifunctional reagents (22) have also produced evidence for oligomers of transducin but the exact location of the crosslinks could not be specified. The relatively low efficiency of photoactivated azide insertion into polypeptides in aqueous environments makes it difficult to quantitate the fraction of total G_T in oligomeric complexes; however, assuming a 10% insertion efficiency at 3 μM G_T at least 20–30% of the total G protein would be predicted to be in oligomeric complexes (Fig. 2).

Fig. 4 schematically diagrams the postulated arrangement of α, β, and γ polypeptides in dimer and trimer oligomers. Within the oligomeric complexes, the α_T carboxyl terminus must be oriented toward the sites of α–α subunit interaction and within 2 nm of a γ subunit of a second G_T molecule. It should be noted that the formation of transducin oligomers, through α–α association, could affect the association of α_T with its own γ subunit; that is, the association of α subunits could lead to a conformational change that brings the carboxyl terminus of α_T closer to its own γ subunit.

The orientation of the α_T carboxyl terminus near the sites of α–α subunit interaction and close proximity to an intra- or intermolecular γ_T subunit has significant implications regarding the regulation of G_T activation by bleached rhodopsin. The α-subunit carboxyl terminus is a key regulatory domain that controls both receptor activation and the intrinsic activity of the α subunit itself (26). In addition, the $\beta\gamma$ subunit complex is required for efficient receptor catalyzed G-protein activation (27) and physically interacts with the receptor polypeptide (28). The 2-nm or less proximity of the γ_T subunit to the α_T carboxyl terminus of G_T oligomers provides the intermolecular structural association of these subunits to contribute to the positive cooperative activation of G_T by bleached rhodopsin previously described (25). The orientation of the α_T carboxyl terminus toward the α–α contact domains in the oligomers would be predicted to further enhance the transfer of conformational changes induced by rhodopsin interaction with one G_T heterotrimer to the other G_T molecules in the oligomeric complex. One photolyzed rhodopsin has been shown to catalyze the exchange of 500 Gpp(NH)p for GDP in retinal rod outer segments (2, 12). Since our data indicate that transducin in solution exists as an oligomer, it may be possible that it is the oligomeric structure of transducin *in situ* that permits one photolyzed rhodopsin molecule to activate numerous transducin molecules. Similar data from reconstitution experiments of purified β-adrenergic receptor and G_S have demonstrated that one receptor molecule is capable of activating multiple G_S molecules (29). In fact, *in situ* oligomeric G-protein complexes have been postulated to be present as important intermediates for receptor/effector coupling (30, 31).

The photocrosslinking analysis of G_T subunits with 2-azido-[*adenylate*-^{32}P]NAD^+ has defined a structural orientation of the α_T carboxyl terminus within oligomeric complexes of the G protein. The concentration dependence for the formation of the oligomers suggests that the positive cooperative activation of G_T by bleached rhodopsin could be regulated in the outer segment by controlling the functional concentration of the G protein. Since oligomer formation and disruption appear to be G_T concentration dependent, regulatory mechanisms may exist that would have the potential to modulate the visual transduction pathway by controlling the amplification of cGMP phosphodiesterase activation. This could be accomplished by altering the oligomeric state of G_T molecules.

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