Functional Significance of G Protein Carboxymethylation†

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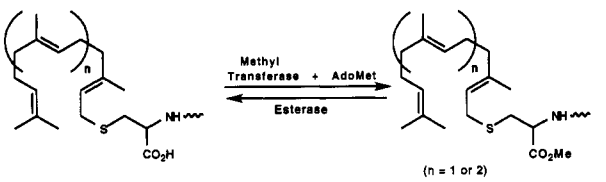
ABSTRACT: Heterotrimeric G proteins are isoprenylated and methylated on their γ subunits. Since methylation is the only reversible reaction in the isoprenylation pathway, it could be a site of control of G protein activity. A method for selectively demethylating isoprenylated and methylated G proteins is reported here using retinal transducin (T) as a model system. It was found that pig liver esterase is capable of completely hydrolyzing $T_{\beta\gamma}$, but not $T_{\alpha\beta\gamma}$, to its unmethylated form. This allows for the direct determination of the activities of methylated and unmethylated $T_{\beta\gamma}$. The activities of the $T_{\beta\gamma}$s were determined by measuring their abilities to stimulate GTP-γ-S exchange in the presence of T_α and photoactivated rhodopsin (R*). It is reported here that, in detergent, unmethylated $T_{\beta\gamma}$ was at least as active as its methylated counterpart. Therefore, methylation does not affect the intrinsic ability of $T_{\beta\gamma}$ to functionally interact with T_α and R*. However, in disk membranes an approximate 2-fold effect was observed, with the methylated $T_{\beta\gamma}$ being more efficient. Therefore, isoprenylated protein methylation may play a quantitative role in signal transduction, even though the intrinsic activities of the methylated subunits in detergent may be no different from their unmethylated counterparts. Finally, the use of pig liver esterase to demethylate isoprenylated proteins should allow for a clarification of the physiological role(s) of isoprenylated protein carboxymethylation in general.

Proteins which carboxyl-terminate with CX_3 (where C = cysteine and X = any amino acid) (Hancock et al., 1989; Farnsworth et al., 1990; Reiss et al., 1990; Lai et al., 1990; Clarke et al., 1988; Maltese, 1990; Sinensky & Lutz, 1992; Gibbs, 1991), CAC (Farnsworth et al., 1991), and CC (Khosravi-Far et al., 1991) are often isoprenylated with either farnesyl or geranylgeranyl moieties at their cysteine residues. In the case of CX_3-containing proteins, endoproteolysis occurs at the isoprenylated cysteine (Ma & Rando, 1992; Ashby et al., 1992) followed by *S*-adenosylmethionine-linked carboxymethylation (Gutierrez et al., 1989) to yield the methylesterified isoprenylated residue as the new carboxyl terminus. CAC- and CC-containing proteins are bis-geranylgeranylated at their cysteine residues (Farnsworth et al., 1991; Khosravi-Far et al., 1991). Methylation also occurs in the case of the CAC-terminating proteins (Farnsworth et al., 1991). As all known subunits of heterotrimeric and "small" G proteins are modified in these ways, it has been of substantial interest to determine what roles these modifications might play in G protein function.

Since the reactions in the isoprenylation pathway occur in sequence, it is relatively straightforward to assess the overall functional importance of the modifications by techniques such as site-specific serine for cysteine substitutions (Hancock et al., 1989) or by studying cloned proteins isolated from cells unable to carry out isoprenylation reactions (Inglese et al., 1992). Not surprisingly, isoprenylation has generally been found to be essential for isoprenylated protein function. For example, nonisoprenylated *ras* (Hancock et al., 1989), the heterotrimeric G protein retinal transducin (T)[1] (Fukada et al., 1990), and rhodopsin kinase (Inglese et al., 1992) are all biochemically inactive. It would be of particular interest to determine the functional role of carboxymethylation (Scheme 1) independently of the other reactions in the pathway because carboxymethylation is the only reversible step in the pathway (Tan & Rando, 1992), and hence the only reaction subject to dynamic control. Indeed, there is ample precedent for the functional importance of reversible protein carboxymethylation in signal transduction in bacterial chemotaxis (Kort et al., 1975) and in fungal mating (Anderegg et al., 1988). Moreover, it has been suggested that the βγ subunit of T is required to be methylated in order to interact with T_α (Ohguro et al., 1991). In this article, we describe an enzymatic method to specifically demethylate $T_{\beta\gamma}$-OCH_3 and show that unmethylated $T_{\beta\gamma}$-OH is as active as its carboxymethylated counterpart in interacting with T_α and photoactivated rhodopsin in detergent. Therefore, methylation does not affect the intrisic interaction of T_α with $T_{\beta\gamma}$. However, methylated $T_{\beta\gamma}$ interacts more efficiently with T_α when the rhodopsin is membrane-bound in disks, suggesting a quantitative role for methylation in the association of $T_{\beta\gamma}$ with membranes.

Scheme 1



MATERIALS AND METHODS

Materials

Frozen bovine retinas were obtained from Wanda Lawson Co. (Lincoln, NE). GTP, GTP-γ-S, dithiothreitol (DTT),

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[1] Abbreviations: T, retinal transducin; $T_{\beta\gamma}$-OCH_3, carboxymethylated transducin; $T_{\beta\gamma}$-OH, unmethylated transducin; R*, photoactivated rhodopsin; ROS, rod outer segments; DTT, dithiothreitol; PLE, pig liver esterase; iPLE, immobilized pig liver esterase; GTP-γ-S, guanosine 5′-(γ-thio)triphosphate.

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leupeptin, aprotinin, pepstatin, and trypsin inhibitor were from Boehringer Mannheim. GTP-γ-^{35}S (1340 Ci/mmol; 1 Ci = 37 GBq) was from NEN/Dupont. Blue Sepharose CL-6B was from Pharmacia. Guanidinium chloride, pig liver esterase, pig liver esterase–acrylic beads, and hexylagarose were from Sigma. Filtron-X scintillation fluid was from National Diagnostics.

Methods

Preparation of Transducin, $T_{\alpha\beta\gamma}$. The preparation of transducin was based on a previously published procedure (Wessling-Resnick & Johnson, 1987). All manipulations were carried out under room light. Bovine retinas (50) were suspended in 20 mM Tris-HCl, pH 7.4/1 mM $CaCl_2$ (buffer A) containing 45% sucrose. After centrifugation, the crude ROS collected at the surface were washed with buffer A. Further ROS purification was accomplished by a step gradient of 25–35% sucrose in buffer A. The ROS membranes collected from the 25%/35% interface were washed four times with isotonic buffer B (10 mM Tris-HCl, pH 7.4/100 mM NaCl/5 mM $MgCl_2$/1 mM DTT/0.1 mM EDTA/pepstatin, leupeptin, aprotinin, and trypsin inhibitor, 10 μg/mL each) and four times with hypotonic buffer C (10 mM Tris-HCl, pH 7.4/1 mM DTT/0.1 mM EDTA).

Transducin was extracted completely from the ROS membranes by two treatments with 40 μM GTP in buffer C (total volume 75 mL) for 15 min. Further purification of transducin was accomplished by hexylagarose chromatography (Fung et al., 1981). Fractions were analyzed for the ability to bind GTP-γ-^{35}S (see Assay of GTP Exchange Activity) for 30 min at 25 °C (4 nM rhodopsin, 2 μM GTP-γ-S (5000–7000 cpm/pmol), 25 μL of each column fraction in 125 μL total assay volume), and those fractions with GTP exchange activity were concentrated to a small volume.

The transducin heterotrimer was separated into its purified T_α and $T_{\beta\gamma}$ subunits by Blue Sepharose chromatography based on previously published procedures (Wessling-Resnick & Johnson, 1987; Fukada et al., 1989). A column of Blue Sepharose (260 mm × 10 mm) was equilibrated (25 mL/h) with buffer D (10 mM Tris-HCl, pH 7.4/5 mM $MgCl_2$/1 mM DTT/0.1 mM EDTA). To the above transducin sample was added $MgCl_2$ (1 M) to bring the concentration of $MgCl_2$ to 5 μM. This solution was loaded onto the Blue Sepharose column, after which the sample was washed onto the column thoroughly with buffer D (20 mL). A step gradient of buffer D with 100 mM NaCl (125 mL), then 300 mM NaCl (75 mL), followed by 1.0 M NaCl (150 mL) was employed to selectively elute the transducin subunits.

Preparation of Rhodopsin and ROS Membranes. Solubilized rhodopsin in dodecyl maltoside (Longstaff & Rando, 1985) and urea-treated ROS (Fawzi & Northup, 1990) were prepared in the dark as described previously.

Hydrolysis of Transducin Subunits. Transducin samples (either $T_{\beta\gamma}$ or $T_{\alpha\beta\gamma}$) were treated with either pig liver esterase (PLE) or pig liver esterase conjugated to acrylic beads (iPLE) and nutated at 4 °C. In a typical experiment to accomplish full hydrolysis of T_γ-OCH_3 to the corresponding acid, purified $T_{\beta\gamma}$ subunits (600 μL, 15.5 μM) were treated with iPLE (40-50 μL settled volume, 12–15 units) which had been thoroughly washed with buffer D containing 100 mM NaCl (4 times). After this reaction was nutated for the desired time period at 4 °C, the supernatant was removed after centrifugation (14 000 rpm, 5 min) and the beads were further washed with buffer D containing 100 mM NaCl (500 μL). After 1 h, a second supernatant was removed after centrifugation. Each supernatant typically contained 5–7 μM $T_{\beta\gamma}$-OH.

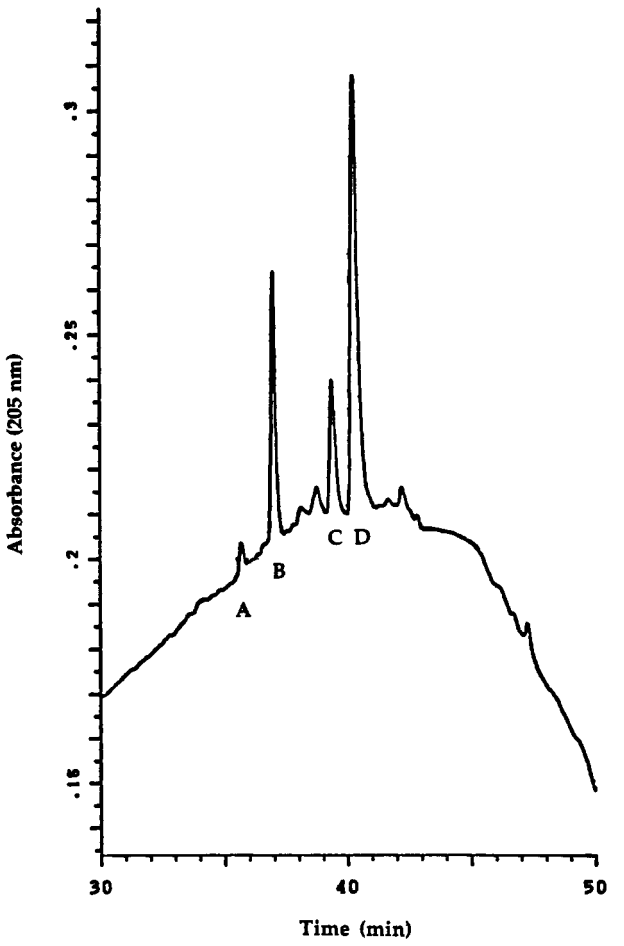

FIGURE 1: HPLC chromatogram of transducin, $T_{\alpha\beta\gamma}$, after hexylagarose purification. UV peaks are identified as (A) 36 min, T_γ degraded (minus carboxy-terminal Gly-Cys(farnesyl)-OCH_3; (B) 37 min, 16.8-kDa protein; (C) 39 min, T_γ-OH; (D) 40 min, T_γ-OCH_3.

Assay of GTP Exchange Activity. In a test tube were combined 50 μL of assay buffer (50 mM Tris-HCl, pH 7.4/500 mM NaCl/25 mM $MgCl_2$/5 mM DTT/0.5 mM EDTA), water (an amount that will bring the total assay volume to 250 μL), and T_α and/or $T_{\beta\gamma}$ subunits. In the dark, either detergent-solubilized rhodopsin or urea-treated ROS was added and the tube was incubated at the desired temperature (2 or 25 °C). The sample was bleached for 1 min under ordinary room light before addition of GTP-γ-^{35}S (2 μM final concentration, 5000–7000 cpm/pmol). Aliquots (35 μL) of each experiment were removed 1, 5, 10, 15, 20, and 30 min after GTP-γ-^{35}S addition. All assays were done in duplicate. Each aliquot was filtered through a nitrocellulose membrane (Schleicher and Schuell, BA85) and immediately washed three times with ice-cold 10 mM Tris-HCl, pH 7.4/100 mM NaCl/5 mM $MgCl_2$/0.1 mM EDTA (4 mL). Each membrane was dissolved in Filtron-X scintillation fluid (10 mL) and counted.

HPLC Analysis of T_γ. Analysis of the γ subunit of transducin was accomplished by reverse-phase HPLC using a C18 column (Dynamax 300A, Rainin) running a linear gradient at 0.75 mL/min. After 10 min at 5% AcCN in H_2O with 10 mM TFA, a 40-min gradient was run to 95% AcCN in H_2O with 10 mM TFA. At that point, the eluant was held at 95% AcCN for an additional 10 min. All transducin samples were injected with added guanidinium chloride at a final concentration of greater than 3 M. UV absorbance was monitored at 205 nm. Typically, T_γ-OCH_3 was retained on

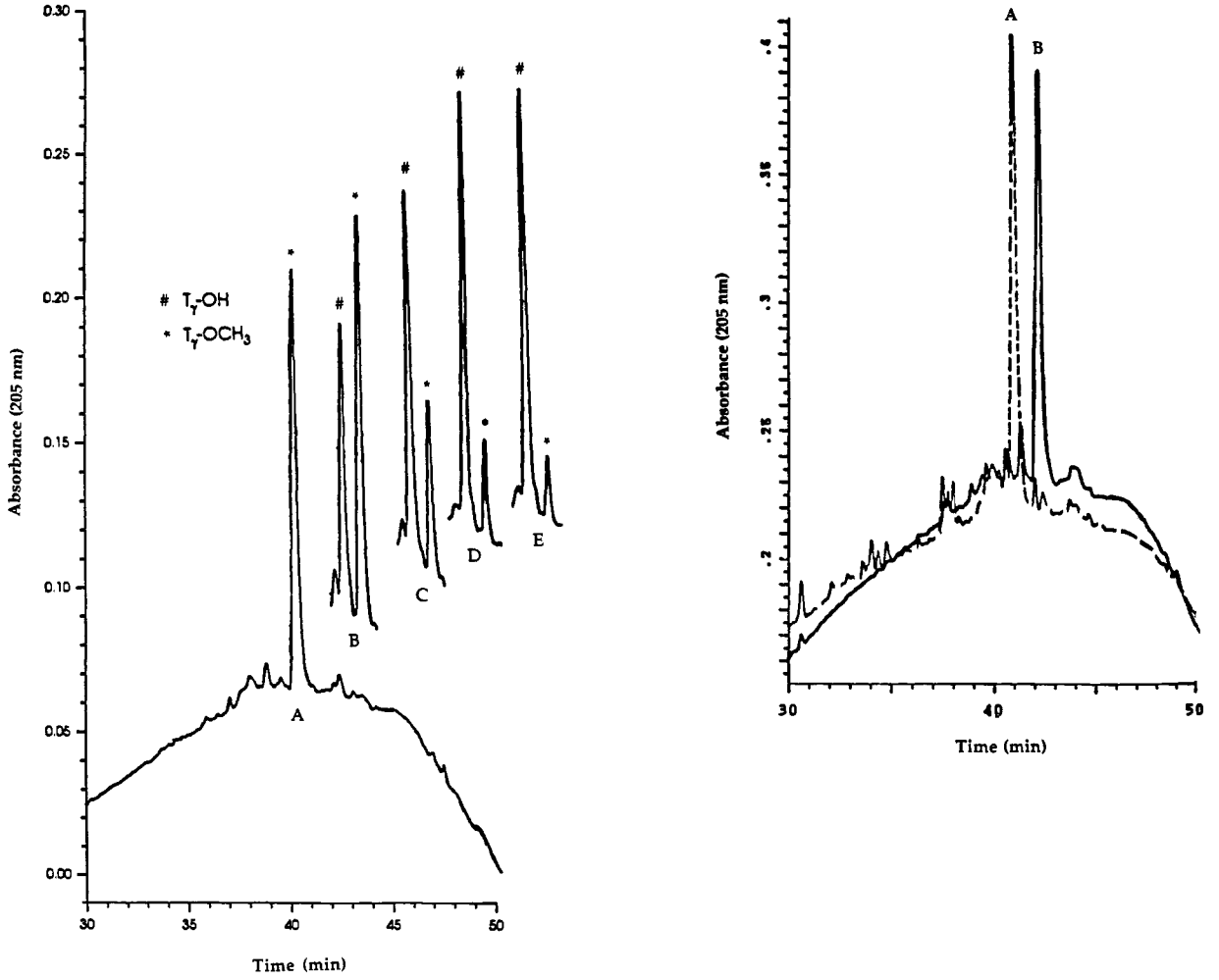

FIGURE 2: (a, left) Time course of $T_{\beta\gamma}$ hydrolysis. $T_{\beta\gamma}$ (9 nmol) in 600 μL of buffer D with 100 mM NaCl was nutated at 4 °C with iPLE (25 μL settled volume, 7.5 units). At each time point, an aliquot (100 μL) was analyzed by HPLC after centrifugation (14 000 rpm). Time points were as follows: (A) t_0, (B) 21 h, (C) 40 h, (D) 61 h, and (E) 70 h. (b, right) Complete hydrolysis of $T_{\beta\gamma}$ with iPLE. $T_{\beta\gamma}$ (7.5 nmol) in 500 μL of buffer D with 100 mM NaCl was nutated at 4 °C with iPLE (50 μL settled volume, 15 units) for 70 h. After centrifugation (14 000 rpm), an aliquot (300 μL) of the supernatant was analyzed by HPLC (dashed line, A = T_γ-OH). A control which was left untreated (70 h) was analyzed under identical conditions (solid line, B = T_γ-OCH_3).

the column approximately 1 min more than the corresponding free carboxylic acid with this protocol (see Figure 1). The exact retention times of these peaks varied within a few minutes depending on column and HPLC conditions, but a standard injection was utilized to confirm the identity of each component.

Miscellaneous Procedures. Protein concentrations were determined using a Lowry assay (Bio-Rad DC assay). Electrospray mass spectrometry was performed at Harvard Microchemistry. Laser desorption mass spectroscopy was performed on a Millipore PM^3-1000 laser desorption mass spectrometer.

RESULTS

Since transducin is the best understood of all of the heterotrimeric G proteins (Stryer, 1991; Hargrave et al., 1993), we sought to investigate the relative abilities of pure unmethylated $T_{\beta\gamma}$($T_{\beta\gamma}$-OH) and $T_{\beta\gamma}$ methyl ester ($T_{\beta\gamma}$-OCH_3) to interact with T_α and the transducin receptor, activated rhodopsin (R*) (Stryer, 1991). T_γ is farnesylated and substantially methylated when isolated (Lai et al., 1990; Fukada et al., 1990). An HPLC chromatogram of freshly prepared T_γ is shown in Figure 1. Most of the T_γ subunit is in the isoprenylated and methylated form (theoretical mass 8329.7 Da, measured mass 8331.6 Da). The position of proteolytically processed T_γ is also indicated. Electrospray mass spectrometry of this peak shows that it is cleaved between the two glycine residues adjacent to the modified cysteine residue (theoretical mass 7950.5 Da, measured mass 7953.9 Da). The other indicated peak, which at this point has not been identified, has a mass of 16.8 kDa as measured by laser desorption mass spectrometry.

Isolated $T_{\alpha\beta\gamma}$ contains approximately 5-15% unmethylated T_γ (Figure 1, theoretical mass 8315.7 Da, measured mass 8319.4 Da). In our hands, $T_{\beta\gamma}$-OCH_3 and $T_{\beta\gamma}$-OH were separable by Blue Sepharose chromatography. $T_{\beta\gamma}$-OH eluted in the void volume along with proteolytically cleaved $T_{\beta\gamma}$ and the 16.8-kDa protein. However, $T_{\beta\gamma}$-OH could not be purified away from these contaminants by Blue Sepharose chromatography. Pure $T_{\beta\gamma}$-OCH_3 was obtained from those fractions eluted with 100 mM NaCl in buffer D (see Methods). The optimal route to $T_{\beta\gamma}$-OH required controlled hydrolysis of this prepared $T_{\beta\gamma}$-OCH_3. Base-catalyzed hydrolysis proved to be unsuitable, as $T_{\beta\gamma}$-OCH_3 is unstable to the basic conditions (pH 10) required to effect hydrolysis. Therefore an enzymatic means was sought to carry out the hydrolysis of $T_{\beta\gamma}$-OCH_3. It was found that pig liver esterase (PLE) readily hydrolyzes *N*-acetyl-*S*-farnesyl-L-cysteine methyl ester (AFCM) (J. Cañada and R. R. Rando, unpublished experiments), a molecule which is identical to the carboxyl-terminal

residue of T_γ. PLE also proved capable of hydrolyzing $T_{\beta\gamma}$-OCH_3. Although soluble pig liver esterase appeared to be a more effective catalyst than its immobilized counterpart, the latter was more useful in the experiments reported. The soluble enzyme interfered with both the HPLC analysis of T_γ and the GTP binding assays. In Figure 2 a time course for the hydrolysis of $T_{\beta\gamma}$ by iPLE is shown. It is clear that after approximately 70 h at 4 °C there is virtually no methylated $T_{\beta\gamma}$ remaining. Interestingly, $T_{\alpha\beta\gamma}$ is virtually inert to hydrolysis by iPLE and no more than 10% hydrolysis was effected over 60 h under conditions identical with those described in Figure 2a. This suggests that the isoprenylated and methylated cysteine moiety of $T_{\beta\gamma}$ is inaccessible when complexed to T_α. This would be consistent with a scenario in which the carboxyl terminus of T_γ interacts with T_α.

In order to assess the relative abilities of methylated and unmethylated $T_{\beta\gamma}$ to support GTP-γ-S exchange for GDP in the presence of T_α and R*, a dose–response curve was first generated using T_α and methylated $T_{\beta\gamma}$ in detergent (Figure 3a). After numerous sets of conditions were analyzed, the greatest relative enhancement of GTP-γ-S binding for T_α with $T_{\beta\gamma}$-OCH_3 over T_α alone was observed at low temperature (2 °C) and rhodopsin concentration (2 nM). As can be seen here, T_α or $T_{\beta\gamma}$-OCH_3 alone shows a very low background GTP-γ-S binding in this assay, and progressively more binding is observed as the concentrations of methylated $T_{\beta\gamma}$ are increased. A direct comparison was made between methylated and completely hydrolyzed $T_{\beta\gamma}$ using this assay system, and the results are shown in Figure 3b. Clearly, in detergent there is no difference between methylated and unmethylated $T_{\beta\gamma}$ with respect to their abilities to interact with T_α in the presence of R*. Therefore, the intrinsic activity of $T_{\beta\gamma}$ is unaffected by its state of methylation. A rather different result was obtained when the same experiments were conducted in the absence of detergents and in the presence of freshly prepared bovine photoreceptor disk membranes containing rhodopsin (Figure 4). Here the state of $T_{\beta\gamma}$ methylation did make a difference, with the methylated $T_{\beta\gamma}$ being approximately 2-fold more active than the unmethylated form. The lower temperatures used to suppress the basal activity of T_α also led to diminished exchange activity using disk membranes. The experiments were repreated at a higher temperature (Figure 4c). Although higher background activity of T_α relative to T_α with $T_{\beta\gamma}$ was observed at the higher temperature (25 °C), these experiments still indicated an approximate 2-fold decrease in activity over background for unmethylated $T_{\beta\gamma}$. Although further experiments are being performed to uncover the underlying mechanism responsible for this effect, it is clear that disk membranes do reveal a difference between the methylated and unmethylated forms of $T_{\beta\gamma}$.

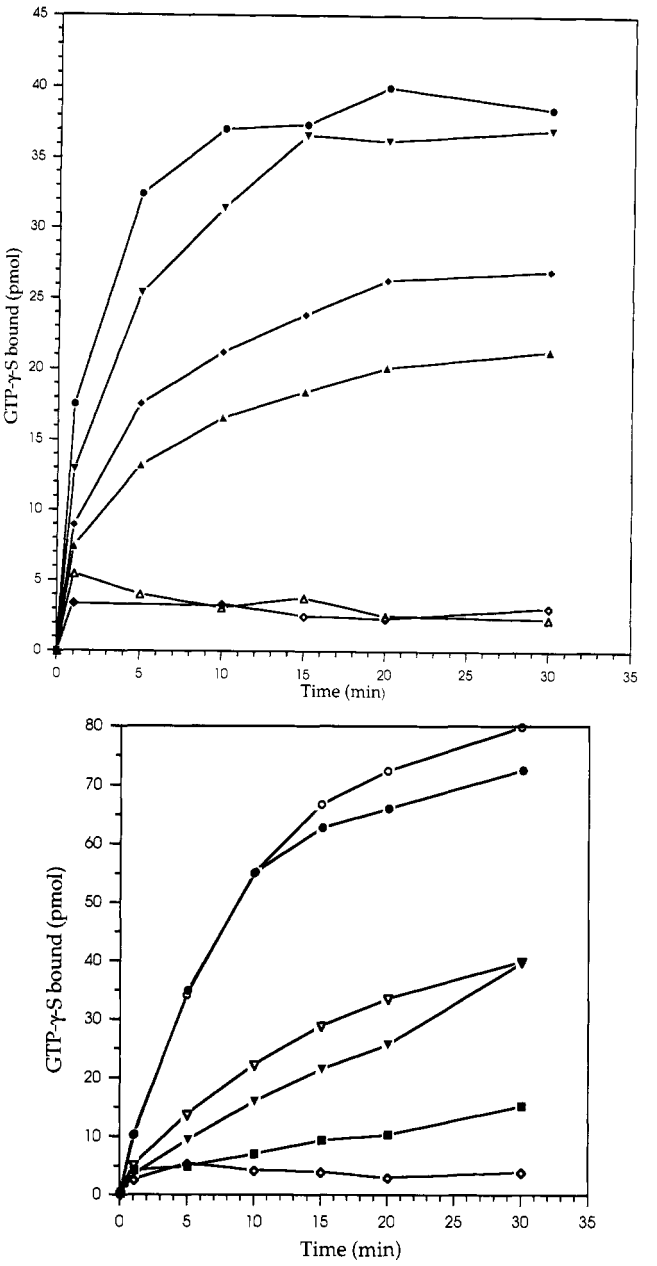

FIGURE 3: GTP-γ-S exchange assays in detergent. (a, top) Time course of GTP-γ-S exchange with 1 μM T_α only (◇), 1 μM $T_{\beta\gamma}$-OCH_3 only (△), or with both 1 μM T_α and $T_{\beta\gamma}$-OCH_3 at 0.25 μM (▲), 0.5 μM (◆), 1.0 μM (▼), or 2.5 μM (●). Each assay contained 2 μM GTP-γ-S, 2 nM detergent-solubilized rhodopsin, and 50 μL of assay buffer (total assay volume = 250 μL) and was performed in duplicate at 2 °C. (b, bottom) Time course of GTP-γ-S exchange with 1.5 μM T_α only (◇) or with both 1.5 μM T_α and $T_{\beta\gamma}$-OCH_3 at 0.30 μM (●), 0.20 μM (▼), or 0.10 μM (■) or with both 1.5 μM T_α and $T_{\beta\gamma}$-OH at 0.30 μM (○) or 0.20 μM (▽). Each assay contained 2 μM GTP-γ-S, 2 nM detergent-solubilized rhodopsin, and 50 μL of assay buffer (total assay volume = 250 μL) and was performed in duplicate at 2 °C. Duplicate points in all assays were within 5% of each other and experiments on several occasions gave equivalent results.

DISCUSSION

Methylation is the only reaction in the isoprenylation pathway that is reversible. As such, it is possible that methylation is of regulatory significance. To begin to test this possibility it is first important to determine whether or not methylation is important in the function of an isoprenylated protein. Transducin appears to be a good choice to study this issue as its mechanism of action is relatively well characterized (Stryer, 1991; Hargrave et al., 1993) and it is available in abundant amounts. Moreover, while $T_{\beta\gamma}$ is not absolutely essential for GTP exchange in T_α in the presence of R*, it markedly enhances it (Phillips et al., 1992) so that the role of the state of methylation of $T_{\beta\gamma}$ in the exchange reaction can be readily studied here.

As isolated, transducin appears to be heterogeneous in its γ subunit. A truncated subunit is found in which the isoprenylated cysteine residue is lacking. Holotransducin formed from this subunit appears to be functionally inactive. This has been interpreted to mean that isoprenylation is essential for the function of transducin (Fukada et al., 1990). Further heterogeneity in the γ subunit is found in the state of methylation of the isoprenylated cysteine residue (Fukada et

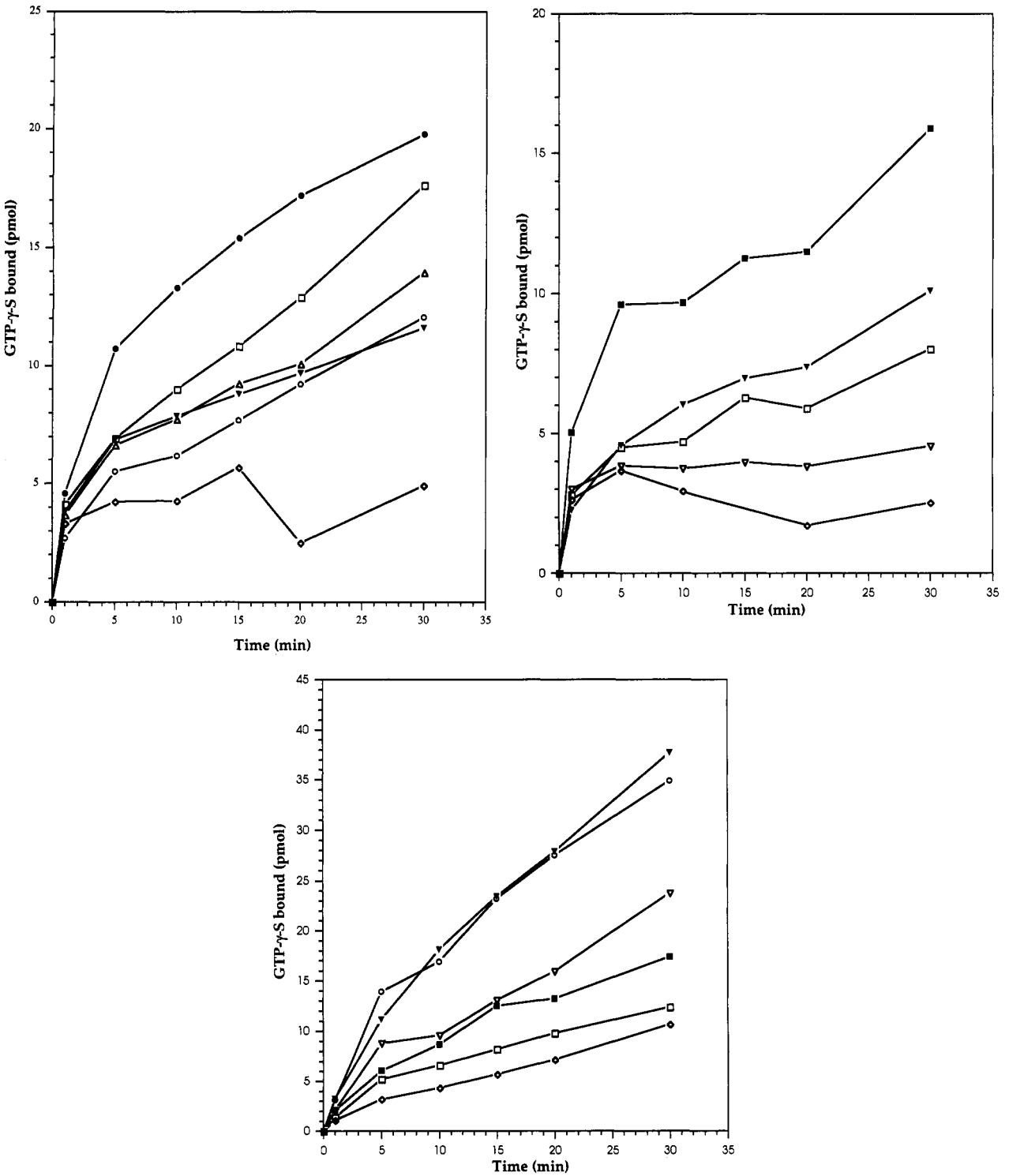

FIGURE 4: GTP-γ-S exchange assays with urea-washed ROS membranes. (a, top left) Time course of GTP-γ-S exchange with 1.5 μM T_α only (◇) or with both 1.5 μM T_α and $T_{\beta\gamma}$-OCH_3 at 0.60 μM (●) or 0.30 μM (▼) or with both 1.5 μM T_α and $T_{\beta\gamma}$-OH at 1.60 μM (□), 1.10 μM (△), or 0.60 μM (○). Each assay contained 2 μM GTP-γ-S, 15 nM urea-treated ROS, and 50 μL of assay buffer (total assay volume = 250 μL) and was performed in duplicate at 2 °C. (b, top right) Time course of GTP-γ-S exchange with 1.5 μM T_α only (◇) or with both 1.5 μM T_α and $T_{\beta\gamma}$-OCH_3 at 1.2 μM (■) or 0.70 μM (▼) or with both 1.5 μM T_α and $T_{\beta\gamma}$-OH at 1.2 μM (□) or 0.50 μM (▽). Each assay contained 2 μM GTP-γ-S, 15 nM urea-treated ROS, and 50 μL of assay buffer (total assay volume = 250 μL) and was performed in duplicate at 2 °C. (c, bottom) Time course of GTP-γ-S exchange with 1.5 μM T_α only (◇) or with both 1.5 μM T_α and $T_{\beta\gamma}$-OCH_3 at 0.30 μM (▼) or 0.11 μM (■) or with both 1.5 μM T_α and $T_{\beta\gamma}$-OH at 0.50 μM (○), 0.30 μM (▽), or 0.11 μM (□). Each assay contained 2 μM GTP-γ-S, 5 nM urea-treated ROS, and 50 μL of assay buffer (total assay volume = 250 μL) and was performed in duplicate at 25 °C. Duplicate points in all assays were within 5% of each other, and experiments on several occasions gave equivalent results.

al., 1990; Ohguro et al., 1991). Both unmethylated and methylated βγ subunits are observed, as confirmed here. When mixtures of $T_{\beta\gamma}$-OCH_3 and $T_{\beta\gamma}$-OH are treated with photoactivated disk membranes, $T_{\beta\gamma}$-OCH_3 appears to be preferentially bound to the photoactivated disks (Ohguro et al., 1991). During preparation of this paper, a large-scale method for separating $T_{\beta\gamma}$-OCH_3 and $T_{\beta\gamma}$-OH was published utilizing gel-filtration chromatography, and it was again shown that $T_{\beta\gamma}$-OCH_3 is preferentially bound to disk membranes (Fukada et al., 1994). These experiments show that $T_{\beta\gamma}$-OCH_3 and $T_{\beta\gamma}$-OH can behave differently with respect to interactions with disk membranes.

The experiments described here probe the functional consequences of $T_{\beta\gamma}$ methylation. The state of methylation of $T_{\beta\gamma}$ proved to be unimportant in governing the intrinsic interactions between $T_{\beta\gamma}$, T_α, and R*. This is shown by demonstrating that in detergent there is no substantial difference between $T_{\beta\gamma}$-OCH_3 and $T_{\beta\gamma}$-OH with respect to their abilities to enhance GTP-γ-S uptake by T_α in the presence of R*. If anything, $T_{\beta\gamma}$-OH may have been a bit more efficient than $T_{\beta\gamma}$-OCH_3 in catalyzing the exchange reaction. However, a distinct (2-fold) difference between $T_{\beta\gamma}$-OCH_3 and $T_{\beta\gamma}$-OH was observed when urea-stripped disk membranes were used as the source of R*. Here, $T_{\beta\gamma}$-OCH_3 proved to be more efficient. These differences could be of physiological importance in retinal physiology. It will be interesting to determine whether transducin is demethylated in disk membranes on a physiological time scale.

It is of interest to consider what the 2-fold effect that we have measured might be caused by. Two straightforward mechanisms involve (1) a receptor in disk membranes which preferentially binds $T_{\beta\gamma}$-OCH_3 or (2) the enhanced hydrophobic binding of $T_{\beta\gamma}$-OCH_3 to membranes via hydrophobic interactions. A recently published study bears on this issue. This report (Fukada et al., 1994) described experiments similar to some of those reported here, except that the rhodopsin was incorporated in lecithin-based liposomes. These experiments demonstrated a 35% decrease in GTP-γ-S exchange with $T_{\beta\gamma}$-OH as compared with $T_{\beta\gamma}$-OCH_3. Since this result is of a similar magnitude as that seen with disk membranes, as reported here, it suggests that this effect is driven primarily by hydrophobic membrane interactions and not by a specific disk membrane-associated receptor. Along these lines, Hancock et al. (1991) have determined that the binding of isoprenylated $p21^{K\text{-}ras(B)}$ to membranes is enhanced by methylation. These studies again suggest that methylation may enhance the membrane association of isoprenylated proteins.

It is assumed that similar results will be obtained for other heterotrimeric G proteins as well. Indeed, the methodology developed here using iPLE to demethylate isoprenylated proteins should be readily adaptable for use with other heterotrimeric $\beta\gamma$ subunits and "small" G proteins, as well as with other non-G proteins that are isoprenylated and methylated, enabling one to unequivocally answer whether or not methylation is essential in the function of these proteins. The literature on this point is quite unclear thus far, mainly because direct, unequivocal experiments have not been performed to address some of these issues. For example, while methylation of the small G protein *rac-2* has been reported to be important in its role in mediating superoxide formation in neutrophils (Philips et al., 1993), other studies report that cloned and unprocessed (nonisoprenylated) *rac-2* is as active as the processed form with respect to activating the oxidase complex (Kreck et al., 1994).

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