# Functional heterogeneity of transducin α subunits

Thomas A. Neubert[1], James B. Hurley*

*Department of Biochemistry and Howard Hughes Medical Institute, University of Washington School of Medicine, Box 357370, Seattle, WA 98195, USA*

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**Abstract The N-terminal glycine of transducin α subunits is acylated by lauroyl (C12:0), myristoyl (C14:0), (*cis*-Δ[5])-tetradecaenoyl (C14:1) or (*cis*,*cis*-Δ[5],Δ[8])-tetradecadienoyl (C14:2) fatty acyl groups. We examined functional heterogeneity of transducin by sequentially eluting it from bleached outer segments using increasing concentrations of GTP then identifying the N-terminal acyl groups on the eluted α subunits. C14:2 acylated transducin eluted at low GTP concentrations followed by C12:0, C14:1 and C14:0 transducin at higher GTP concentrations. This suggests functional heterogeneity in the different forms of transducin α subunits.**



*Key words:* Transducin; Phototransduction; Myristoylation; G-protein; Mass spectrometry

## 1. Introduction

Transducin (T) is a heterotrimeric GTP-binding protein involved in the vertebrate phototransduction cascade [1]. During the initial stage of visual excitation, light-activated rhodopsin binds to transducin and catalyzes the exchange of GDP for GTP. The GTP-bound form of $T_\alpha$ then dissociates from $T_{\beta\gamma}$ and activates a cyclic GMP phosphodiesterase (PDE). Hydrolysis of cyclic GMP by PDE leads to closure of cyclic GMP-gated ion channels in the photoreceptor plasma membrane causing hyperpolarization and ultimately perception of light. After $T_\alpha$ hydrolyzes its bound GTP, it releases $PDE_\gamma$ and reassociates with $T_{\beta\gamma}$ in preparation for another cycle of phototransduction.

Transducin exists in both rod- and cone-specific forms [2]. We and others have shown that the N-terminal glycine residue of bovine rod transducin α ($T_{\alpha r}$) is heterogeneously acylated with C12:0, C14:2, C14:1, or C14:0 (myristoyl) fatty acyl groups [3,4]. Similar heterogeneous acylation also occurs at the N-terminus of other rod photoreceptor proteins [5–7]. The question remains as to whether heterogeneous acylation influences the function of transducin. We investigated this by taking advantage of the fact that transducin binds to bleached rod outer segment (ROS) membranes and can be selectively released from the membranes by GTP [8]. We used increasing concentrations of GTP to sequentially elute transducin from bleached ROS membranes and then used electrospray ionization mass spectrometry (ESI-MS) [9,10] to analyze the composition of the eluted transducin. We found that low GTP concentrations preferentially release C14:2 acylated $T_{\alpha r}$ from the membranes. Subsequent elutions using higher GTP concentrations release transducin modified mostly by C14:0 (myristate). These results suggest that the type of *N*-acyl group on $T_{\alpha r}$ affects the nature of the interaction between transducin and ROS membranes during GTP binding and hydrolysis.

## 2. Materials and methods

### 2.1. Preparation of rod transducin

All procedures were carried out at 4°C unless otherwise noted. ROS were isolated by sucrose density centrifugation from frozen dark-adapted bovine retinas (George A. Hormel Co., Austin, MN) as described by McDowell and Kuhn [11]. ROS from 50 retinas were bleached with room light for 10 min, then washed four times by manually homogenizing in 10 ml ROSE buffer (10 mM Tris-HCl, pH 7.5, 0.5 mM $MgCl_2$ and 1 mM dithiothreitol) with three passes through a glass/teflon homogenizer and centrifuging 30 min in a Sorvall SS-34 rotor at 19 000 rpm. Transducin was eluted in five steps by washing the bleached ROS as above but including increasing amounts of GTP (1–400 μM) in each subsequent step. Supernates containing transducin were concentrated separately to 150 μl using a Centricon 10 (Amicon). Protein concentrations were determined by the Bradford method [12] using a Bio-Rad Protein Assay kit. 10 μl samples from each wash supernatant (0.1% of total volume) and from each GTP elution concentrate (7% of total volume) were subjected to SDS-PAGE using 12% polyacrylamide gels. After electrophoresis the gels were stained with Coomassie brilliant blue R-250 (Bio-Rad).

### 2.2. Proteolysis of transducin

Samples containing 40 μg of protein eluted from ROS in each step of GTP concentration were digested with 1 μg trypsin in a total volume of 100 μl ROSE buffer for 30 min at 37°C. The reactions were stopped by adding 1 mM (final concentration) phenylmethylsulfonyl fluoride (PMSF) and cooling on ice.

### 2.3. Liquid chromatography/mass spectrometry

The trypsin-digested transducin samples were diluted 1:1 in distilled water (final volume 200 μl) and acidified by adding 1 μl trifluoroacetic acid (TFA). 10 μl of each sample (~20 pmol proteolysed $T_{\alpha r}$ based on the assumption that 80% of the GTP-eluted protein is transducin (see Fig. 1)) were applied to a 500 μm diameter packed capillary HPLC column (WPOREX C4) at a flow rate of 15 μl/min. After a 5 min wash during which time the column was disconnected from the mass spectrometer, all of the column effluent was directed to a PE/Sciex API-III triple quadrupole electrospray mass spectrometer operating at unit resolution and scanning mass to charge ratios (*m*/*z*) from 990 to 1040 at a rate of 2.55 s/scan (dwell time of 5.0 ms and a step size of 0.1 Da). A gradient of 0–60% acetonitrile in 0.05% TFA generated by an Applied Biosystems 140A pump over 15 min was used to elute the peptides. Selected ion plots were generated with the MacSpec 3.2 program (PE/Sciex). These plots depict the chromatographic elution profiles of components that produce ions of selected *m*/*z*. An *m*/*z* window of 1001/1002 was selected for $T_{\alpha r}$ N-terminal fragments acylated by C12:0, 1025/1026 for C14:2-containing fragments, 1027/1028 for C14:1 and 1029/1030 for 14:0-containing fragments [3].

*Corresponding author. Fax: (1) (206) 685-2320.
E-mail: jbhhh@u.washington.edu

[1]Present address: Fournier Pharma GmbH, Waldhofer Strasse 104, D-69123 Heidelberg, Germany.

*Abbreviations:* $T_r$, rod transducin; $T_{\alpha\beta\gamma}$, the α, β and γ subunits of transducin; PDE, cyclic GMP phosphodiesterase; ROS, rod outer segments; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; ESI-MS, electrospray ionization mass spectrometry; TFA, trifluoroacetic acid


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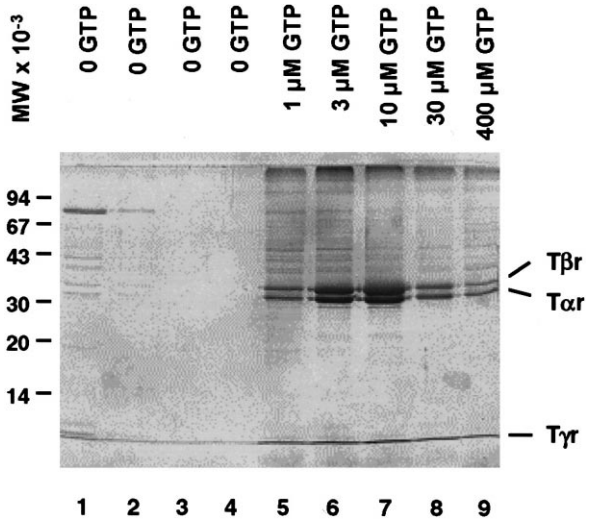


Fig. 1. Coomassie-stained SDS-PAGE of proteins eluted from bleached ROS membranes by sequential washes with increasing concentrations of GTP. Lanes 1–4 contain 0.1% and lanes 5–9 contain 7% of the total eluate (by volume) from each step. Total amount of protein in each lane are 0.88 μg (lane 1), 0.33 μg (lane 2), 0.20 μg (lane 3), 0.18 μg (lane 4), 5.5 μg (lane 5), 10.6 μg (lane 6), 12.2 μg (lane 7), 7.7 μg (lane 8) and 4.2 μg (lane 9).

## 3. Results

Fig. 1 shows an SDS-PAGE analysis of material eluted by sequential washes of bleached bovine ROS using buffer without (lanes 1–4) or with (lanes 5–9) various concentrations of GTP. The total amounts of protein eluted during each of the four washes with buffer lacking GTP were 880, 330, 200 and 180 μg. The predominant proteins eluted during these washes were the α and β subunits of PDE, which migrate between the 67 and 94 kDa markers. 79 μg total protein (consisting mostly of α, β and γ subunits of $T_r$) was eluted by 1 μM GTP, 151 μg by 3 μM GTP, 174 μg by 10 μM GTP, 110 μg by 30 μM GTP, and 60 μg by 400 μM GTP. The GTP eluates were concentrated and 40 μg (containing approximately 400 pmol $T_r$) of each concentrate was digested with trypsin. 10 μl (20 pmol) from a total of 200 μl of each digest was subjected to capillary HPLC/MS analysis to estimate the fraction of $T_{\alpha r}$ N-termini modified by each type of fatty acyl group.

Fig. 2 shows two examples of selected ion plots from these analyses. The fraction of $T_{\alpha r}$ N-termini with a given acyl group was calculated by dividing the amount of peak ions corresponding to a given acylated N-terminal by the total amount of ions corresponding to all four types of acylated N-termini. Results of a representative analysis are summarized in Fig. 3. $T_{\alpha r}$ modified with C14:2 elutes predominantly at low GTP concentrations. By comparison, forms of $T_{\alpha r}$ *N*-acylated with more saturated (C14:1 or C14:0) acyl groups remain bound to ROS membranes at low GTP concentrations and are removed only by subsequent elution with higher GTP concentrations. From the total amount of transducin eluted at all GTP concentrations and from the results summarized in Fig. 3 we calculate that 12% of all the $T_{\alpha r}$ N-termini isolated in this experiment are acylated by C12:0, 33% by C14:2, 34% by C14:1 and 21% by C14:0. This is similar to the abundance of acyl groups on N-termini of $T_{\alpha r}$ eluted directly from bleached ROS using 100 μM GTP [3]. We have performed experiments similar to the one described here five times using various concentrations of GTP and have obtained similar results each time. We did not detect differences in elution when $T_{\alpha r}$ was eluted with steps of 50 nM to 30 μM GTPγS, a non-hydrolyzable analogue of GTP. This shows that GTP hydrolysis is required for expression of the differences in elution of the different acylated forms of transducin.

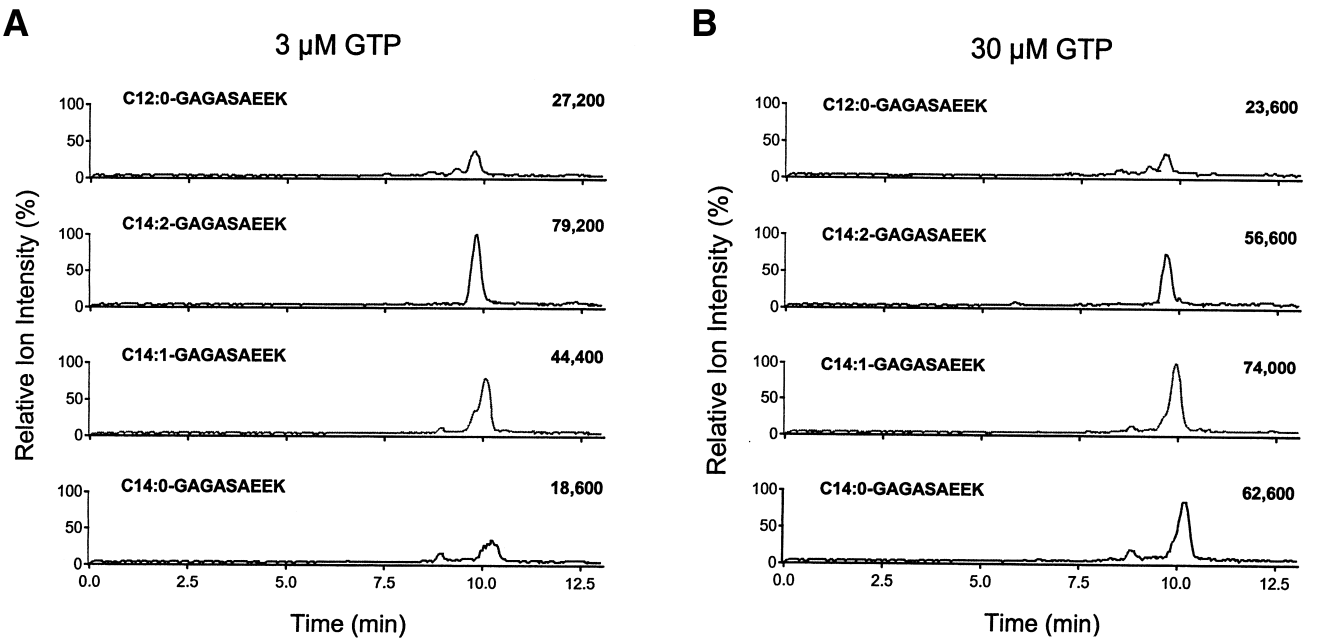


Fig. 2. A: Selected ion plots from a liquid chromatography/mass spectrometry analysis of tryptic fragments of $T_{\alpha r}$ eluted by 3 μM GTP from bleached bovine rod outer segments. B: Selected ion plots from a liquid chromatography/mass spectrometry analysis of $T_{\alpha r}$ tryptic fragments eluted by 30 μM GTP from bleached bovine rod outer segments. The selected ions have mass to charge ratios corresponding to singly charged $T_{\alpha r}$ N-terminal fragments amide-linked to C12:0, C14:2, C14:1 or C14:0 acyl groups [3]. The peak ion counts from each plot are shown in the upper right corners of each panel.

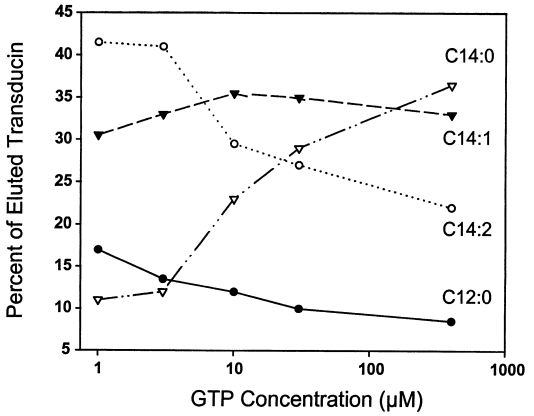


Fig. 3. Summary of relative abundances of $T_{\alpha r}$ N-terminal tryptic fragments containing C12:0, C14:2, C14:1 or C14:0 acyl groups as a function of GTP concentration used to elute the transducin during sequential washes of bleached bovine rod outer segments.

## 4. Discussion

For the analyses reported here we used ESI-MS to quantitate the types of acylated N-termini produced by tryptic digestion of transducin. For given solvent conditions and instrument settings, the increase in ion current for a given type of analyte can be linear over a wide range of concentrations [13]. Nevertheless, our conclusions are independent of absolute quantitation and instead rely only on relative abundances. Fig. 3 shows that the rank order of abundance of the four types of acylated N-termini depends on the GTP concentration used for elution. This eliminates the possibility that our results are due solely to differences in efficiency with which different forms of acylated N-termini produce ion current in the mass spectrometer. The relative abundances of N-termini determined by ESI-MS are consistent with absorbance measurements at 280 nm we detected during reverse phase HPLC analyses in which N-termini linked to C14:0 and C14:1 were each resolved by elution time from a mix of N-termini linked to C12:0 and C14:2.

Our results show that $T_{\alpha r}$ modified by C14:2 is more likely than C14:0-modified $T_{\alpha r}$ to dissociate from bleached ROS membranes at low concentrations of GTP. In the experiment shown in Fig. 3, a total of 460 μg transducin eluted from ROS membranes during the five GTP elution steps. With this much transducin in the initial extraction (0.6 μM in 10 ml), 1 or 3 μM GTP would allow only a few cycles of GTP hydrolysis before all the eluted $T_{\alpha r}$ ends up bound to GDP. One possible explanation for our results then is that C14:2-modified GDP-$T_{\alpha r}$ is less likely than C14:0-modified GDP-$T_{\alpha r}$ to reassociate with $T_{\beta\gamma}$ and/or with bleached ROS membranes. This idea has also been suggested based on the ability of acylated peptides to inhibit interaction between $T_{\alpha r}$ and $T_{\beta\gamma}$ [4]. Weaker interactions of C14:2-modified $T_{\alpha r}$ would cause it to be overrepresented in the population of $T_{\alpha r}$ molecules eluted at low GTP concentrations. In contrast, at non-limiting GTP concentrations, ongoing GTP exchange would regenerate $T_{\alpha r}$-GTP so that most of the eluted $T_{\alpha r}$ would be in the GTP bound form. Under these conditions the composition of the eluted $T_{\alpha r}$ would more closely reflect the overall composition of $T_{\alpha r}$ in the initial ROS homogenate.

Crystallography studies have suggested that the N-terminus of $T_\alpha$ and the C-terminus of $T_\gamma$ may lie on a surface of $T_\alpha$ that interacts with ROS membranes and/or rhodopsin [14]. Our results suggest that *N*-acylation by C14:0 may promote these interactions more effectively than C14:2. Alternatively, C14:2-modified $T_{\alpha r}$ may elute first because another step in the cycle, such as GTP/GDP exchange or GTP hydrolysis, is influenced by the *N*-acyl group. Heterogeneous *N*-acylation might also influence GDP/GTP exchange if the solvent accessibility of the acyl group $T_{\alpha r}$ depends on the type of bound nucleotide. Such changes have been detected upon binding of $Ca^{2+}$ to recoverin [15] and they may also occur in ADP-ribosylation factor 1, a small GTP binding protein [16]. Discrimination between these possibilities will require a separate analysis using purified forms of $T_\alpha$ modified by each individual type of fatty acid.

The nature of the N-terminal acyl group affects the ability of recoverin to inhibit rhodopsin kinase activity in vitro [17]. As we have shown here heterogeneous acylation may also have subtle effects on the activities of other phototransduction proteins such as transducin. Such subtle effects in combination might become physiologically significant, for example, by expanding the range of light intensities over which photoreceptors are able to generate detectable responses.

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