# The Stoichiometry of $G\alpha_s$ Palmitoylation in Its Basal and Activated States

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ABSTRACT: Palmitoylation is the dynamic modification of proteins by the addition of palmitate to cysteine residues. The α subunits of heterotrimeric G proteins undergo palmitoylation on their amino terminus, and activation of $\alpha_s$ accelerates its palmitate turnover. In previous studies, palmitoylation was assessed by incorporation or turnover of [$^3$H]palmitate. These studies did not determine the fraction of $\alpha_s$ that is palmitoylated because the specific activity of [$^3$H]palmitoyl-CoA within cells is indeterminate. We developed an HPLC method to determine the fraction of $\alpha_s$ that was palmitoylated in the basal and activated states. COS and S49 cells were radiolabeled with [$^{35}$S]methionine, and $\alpha_s$ was immunoprecipitated from the particulate fraction. The immunoprecipitated proteins were separated by reverse phase HPLC into two peaks that were determined to contain the modified and unmodified forms of $\alpha_s$. Approximately 77% of the endogenous $\alpha_s$ in COS cells and 70% in S49 lymphoma cells were palmitoylated. The fraction of $\alpha_s$ that was modified did not change after treatment with isoproterenol, a $\beta$-adrenergic receptor agonist that causes turnover of palmitate on $\alpha_s$. These results suggest that receptor activation of $\alpha_s$ caused a rapid turnover of palmitate to maintain most of $\alpha_s$ in its palmitoylated form.

Heterotrimeric G proteins function at the cytoplasmic face of membranes to transduce signals from cell surface receptors to intracellular effectors (Hamm & Gilchrist, 1996; Neer, 1995). They consist of α, $\beta$, and $\gamma$ subunits. The α subunits are a family of homologous proteins that bind GDP and GTP and catalyze GTP hydrolysis. The membrane localization of α subunits results from their binding $\beta\gamma$ subunits and undergoing modification with the fatty acids myristate and palmitate. Myristoylation occurs on α subunits in the $\alpha_i$[1] family and strengthens their binding to $\beta\gamma$ and the membrane (Jones et al., 1990; Linder et al., 1991; Mumby et al., 1990). Palmitoylation occurs on the amino-terminus of most α subunits (Jackson et al., 1995; Linder et al., 1995; Wedegaertner et al., 1995). Palmitate posttranslationally modifies proteins by forming a thioester bond to cysteine residues (Schmidt, 1989; Schultz et al., 1988). Palmitoylation takes place at membranes and increases the affinity of the α subunits for the membrane (Degtyarev et al., 1994; McCallum et al., 1995; Mumby et al., 1994; Wedegaertner et al., 1993).

Palmitoylation is a dynamic modification—the turnover of palmitate on $\alpha_s$ is significantly faster than the turnover of the protein itself (Degtyarev et al., 1993b). Activation of $\alpha_s$ by the $\beta$-adrenergic receptor or cholera toxin accelerates palmitate turnover as demonstrated by rapid incorporation of [$^3$H]palmitate onto $\alpha_s$ in pulse studies (Degtyarev et al., 1993b) and loss of [$^3$H]palmitate from $\alpha_s$ in pulse/chase studies (Mumby et al., 1994; Wedegaertner & Bourne, 1994). All investigations on α subunit palmitoylation used metabolic labeling with [$^3$H]palmitate, a method that can detect changes over time and incorporation of small amounts of radiolabel. However, conditions that change the [$^3$H]palmitate specific activity can appear as changes in palmitoylation. For example, isoproterenol changes the intracellular pools of palmitoyl-CoA, the substrate for palmitoylation (Soling et al., 1987). The stoichiometry of α subunit palmitoylation cannot be determined by radiolabeling because the specific activity of the intracellular pool is not known. Acylation experiments in vitro show stoichiometric incorporation of palmitate on the myristoylated $\alpha_{i1}$ and less incorporation on the nonmyristoylated $\alpha_s$ and $\alpha_q$ (Duncan & Gilman, 1996).

Gas−liquid chromatography of purified protein can determine the number of fatty acid residues per molecule of protein (Bizzozero, 1995). This method was used to determine the stoichiometry of palmitoylation for a few proteins (e.g., myelin proteolipid, myelin P0 glycoprotein, rhodopsin, and band 3 protein of human red blood cells) (Bizzozero et al., 1989; O'Brien et al., 1987; Okubo et al., 1991). Reports on the chemical analysis of purified $\alpha_s$ claimed no bound fatty acids, suggesting that only a minor amount of $\alpha_s$ is palmitoylated (Buss et al., 1987). However, catalysis of palmitate thioester bonds could have occurred during the extensive purification process. Acylation can also be detected if it alters a protein's mobility on gel electrophoresis, e.g., myristoylation for $\alpha_{i1}$ (Jones et al., 1990). However, palmitoylation does not change the mobility of $\alpha_s$ or $\alpha_{i1}$ (Degtyarev et al., 1993a, 1994).

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[1] Abbreviations: G protein, guanine nucleotide-binding protein; $\alpha_s$ and $\alpha_i$, G protein α subunits associated with adenylyl cyclase stimulation and inhibition, respectively; DMEM, Dulbecco's modified Eagle's media; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TFA, trifluoroacetic acid; HPLC, high-pressure liquid chromatography.

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We determined the fraction of $\alpha_s$ that is palmitoylated in both its basal and its activated state to better understand the importance and function of this modification. We developed a technique to separate the palmitoylated and nonpalmitoylated forms of $\alpha_s$ by reverse phase HPLC. We found that in two cell lines, most of $\alpha_s$ is modified and that the amount did not change with isoproterenol treatment.

## EXPERIMENTAL PROCEDURES

*COS Cell Transfection and Metabolic Labeling*. The pCD-PS eukaryotic expression vector with the cDNAs for the rat long or short forms of $\alpha_s$ (Juhnn et al., 1992) or a mutant of the long form in which cysteine-3 was changed to alanine (Degtyarev et al., 1993a) was used for transfection. COS-7 monkey kidney cells were maintained and transfected using the DEAE-dextran method as described previously (Butrynski et al., 1992) and metabolically labeled 48 h after transfection. For labeling with [$^3$H]palmitate, cells were incubated in serum-free DMEM for 2 h and then for 30 min with 2.5 mCi of [9,10-$^3$H]palmitate (American Radiolabeled Chemicals; specific activity 60 Ci/mmol) in 5 mL of serum-free media supplemented with 1% (v/v) dimethyl sulfoxide per 75 cm$^2$ flask of COS cells. For labeling with [$^{35}$S]-methionine, the cells were incubated in 250 $\mu$Ci/mL [$^{35}$S]-methionine (Met-$^{35}$S-Label; American Radiolabeled Chemicals) in serum-free DMEM without methionine for 4 h. For experiments with the endogenous $\alpha_s$, the radiolabeling medium was changed to complete DMEM without radiolabel for 15 min followed by incubation in the absence or presence of 10 $\mu$M isoproterenol (Sigma). The cells were scraped in ice-cold phosphate-buffered saline and centrifuged at 2000*g* for 10 min and stored at −70 °C.

*S49 Lymphoma Cell Culture and Metabolic Labeling*. S49 lymphoma cells were maintained in suspension culture in DMEM with 10% fetal bovine serum, 100 units/mL penicillin, and 100 $\mu$g/mL streptomycin. For radiolabeling, $4 \times 10^8$ cells were centrifuged at 200*g* for 10 min, and the cell pellet was resuspended in 80 mL of methionine-free DMEM with 1% fetal bovine serum and 125 $\mu$Ci/ mL [$^{35}$S]methionine and divided into two 75 cm$^2$ flasks. After a 6 h incubation, the cells in one flask were treated with 10 $\mu$M isoproterenol for 2 min. Cold phosphate-buffered saline was added to stop the incubation, and the cells were prepared as described for the COS cells.

*Cell Fractionation*. The COS cell pellets were homogenized by passing 25 times through a 25-gauge needle in homogenization buffer composed of 5 mM Hepes, pH 7.4, 10 $\mu$g/mL soybean trypsin inhibitor, 0.5 $\mu$g/mL leupeptin, 2 $\mu$g/mL aprotinin, 1 mM EDTA, 0.7 $\mu$g/mL pepstatin, and 10 milliunits/mL $\alpha_2$-macroglobulin (Boehringer-Mannheim). The S49 cell pellets were homogenized by 25 strokes in a Dounce homogenizer in the same buffer. The cell lysate was centrifuged at 1000*g* for 3 min in an Eppendorf 5415 microcentrifuge to pellet the nuclei and unbroken cells. The supernatant was centrifuged at 125000*g* for 1 h at 4 °C in a Beckman TLA45 rotor. The supernatant (soluble fraction) was separated and the pellet (particulate fraction) resuspended in homogenization buffer with the use of a Dounce homogenizer for the S49 cell particulate fraction.

*Cell Lysate Preparation.* COS cell pellets were solubilized in a buffer of Hepes, pH 7.4, 0.5% (w/v) SDS, and 2 mM EDTA and then heated at 90 °C for 5 min. The samples were centrifuged at 1000*g* for 3 min at room temperature. The supernatant was adjusted to the concentrations of components in the solubilization buffer for immunoprecipitation.

*Immunoprecipitation*. One milligram of protein in 1 mL of solubilization buffer consisting of 50 mM Tris-HCl, pH 7.5 (25 °C), 150 mM NaCl, 1% (w/v) Triton X-100, 0.2% (w/v) SDS, and 1 mM EDTA and 10 $\mu$g of the affinity-purified antibody (RM) specific for the carboxy-terminal decapeptide of $\alpha_s$ (Simonds et al., 1989) was incubated overnight at 4 °C. The immunoprecipitates were recovered by incubating 2 h with protein A−Sepharose CL-4B (Pharmacia LKB) and washed 2 times in the solubilization buffer and 1 time in that buffer without detergents. After centrifugation at 8000*g* for 10 min, either the immunoprecipitate was solubilized, separated by SDS−PAGE on 10% Tris−glycine gels (Novex), and prepared for fluorography as previously described (Jones et al., 1990) or 4−8 samples were combined, centrifuged, and then stored at −20 °C for HPLC analysis.

*HPLC Analysis*. The immunoprecipitates were solubilized by suspending the samples in 0.15 mL of 0.1% TFA in Milli-Q-purified water and centrifuging at 8000*g* for 3 min. The supernatant was collected and the pellet resuspended in 0.1 mL of 0.1% TFA and recentrifuged. The supernatant was collected and combined with the first supernatant and recentrifuged for 3 min. The supernatant was injected onto a SynChropak RP-4 column (250 × 4.6 mm id.; Synchrom, Inc.), equilibrated with water/45% $CH_3CN$/0.1% TFA at 30 °C. The column was eluted in a linear gradient from 45% to 70% $CH_3CN$ in 55 min at a flow rate of 1 mL/min. Fractions of 0.5 mL were collected, and the radioactivity was determined by liquid scintillation spectrometry. For SDS−PAGE analysis, 5 $\mu$g of bovine serum albumin was added to 0.3 mL aliquots of each fraction, and then frozen on a dry ice/methanol bath. The solvent was removed by vacuum centrifugation, and the residue was solubilized in SDS−PAGE sample buffer. The samples were then heated for 3 min at 90 °C, and the solubilized proteins were separated by SDS−PAGE on 10% polyacrylamide gels. The relative size of the peaks was approximated by graphing the counts per minute for each fraction and cutting out the two peaks and weighing them.

*Hydroxylamine Treatment.* The particulate fractions of $^{35}$S-labeled COS cells were treated with 1 M hydroxylamine, pH 7.4, in homogenization buffer or in buffer alone for 30 min at room temperature. The samples were centrifuged at 125000*g* for 30 min, and the pellet was prepared for immunoprecipitation and HPLC analysis.

*Miscellaneous.* The effect of TFA on the stability of the palmitoylation modification was tested by treating immunoprecipitates of [$^3$H]palmitate-labeled proteins in 0.1% TFA as for HPLC analysis. The samples were analyzed by SDS−PAGE and fluorography. Densitometry of the fluorographs was determined with on LKB Ultroscan laser densitometer. Protein concentration was determined by the Bio-Rad protein assay kit. Immunoblotting was performed using 1 $\mu$g/mL of the affinity-purified RM antibody and the Enhanced Chemiluminescence kit (Amersham) for detection.

## RESULTS

*HPLC Separation of* $\alpha_s$. We tested conditions for separating the palmitoylated and nonpalmitoylated $\alpha_s$ by using

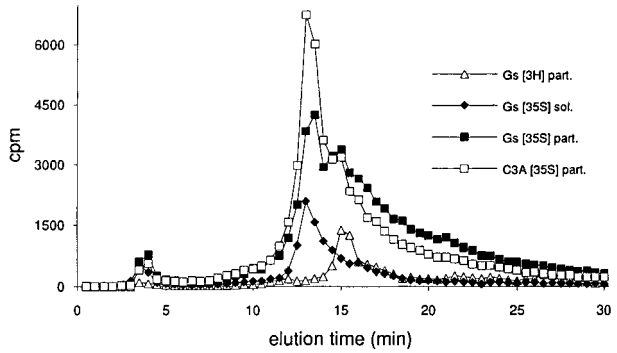


FIGURE 1: HPLC separation of transfected $\alpha_s$. COS cells transfected with the plasmid for the long form of $\alpha_s$ or the C3A mutant of $\alpha_s$ were radiolabeled with [$^3$H]palmitate or [$^{35}$S]methionine, homogenized, and separated into particulate and soluble fractions by centrifugation. $\alpha_s$ was immunoprecipitated, released from the protein A−sepharose, and applied to a reverse phase HPLC column. The proteins were eluted with an acetonitrile gradient and fractions collected every 30 s. The radioactivity in the aliquots was determined by scintillation counting.

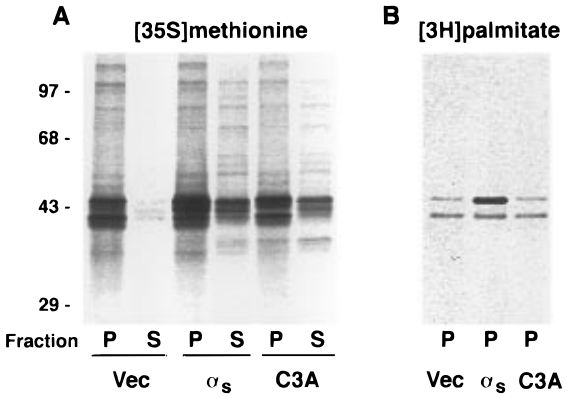


FIGURE 2: Immunoprecipitation of $\alpha_s$ and the C3A mutant. COS cells were transfected with vector alone or with the cDNAs for the long form of $\alpha_s$ or the C3A mutant. The cells were radiolabeled with [$^{35}$S]methionine or [$^3$H]palmitate and separated into particulate and soluble fractions by centrifugation. $\alpha_s$ was immunoprecipitated from 250 $\mu$g of protein in the particulate and soluble fractions of [$^{35}$S]methionine-labeled cells (A) and 900 $\mu$g of protein in the particulate fraction of [$^3$H]palmitate-labeled cells (B) followed by gel electrophoresis and fluorography. The film was exposed at −70 °C for 4 days for panel A and for 27 days for panel B. Migration of the molecular mass markers (in kilodaltons) is shown on the left.

immunoprecipitates from COS cells overexpressing the wild-type $\alpha_s$ and radiolabeled with [$^3$H]palmitate or [$^{35}$S]methionine. The endogenous and transfected $\alpha_s$ were immunoprecipitated and then applied to an HPLC reverse phase column that separated samples on the basis of hydrophobicity (Figure 1). The [$^3$H]palmitate-labeled $\alpha_s$ eluted with one peak at 15 min. The $^{35}$S-labeled $\alpha_s$ in the soluble fraction eluted earlier at 13 min. This pool, which occurs with overexpression, does not undergo palmitoylation (Degtyarev et al., 1993a). In contrast to the soluble pool of $\alpha_s$, the $^{35}$S-labeled $\alpha_s$ in the particulate fraction showed two peaks at 13 and 15 min. These results suggested that the 13 min peak contained the unmodified $\alpha_s$ and the 15 min peak contains the more hydrophobic, modified form. Aliquots of the immunoprecipitates were analyzed by gel electrophoresis and fluorography (Figure 2). The long and short forms of $\alpha_s$, seen as bands at 45 and 42 kDa, respectively, contain most of the radioactivity. Analysis of the fluorographs by densitometry showed that the density of these bands comprised greater

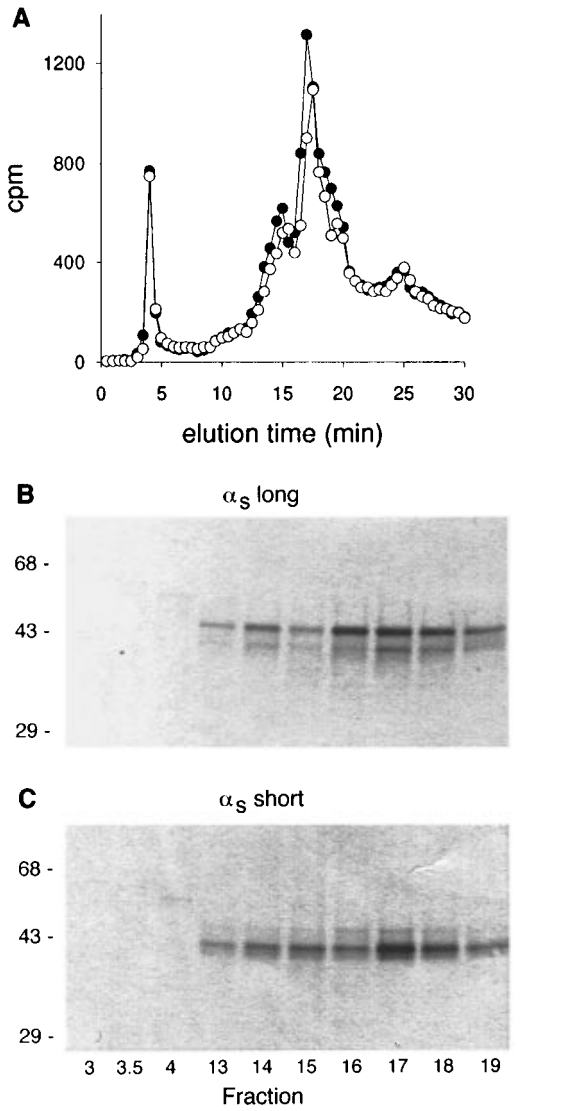


FIGURE 3: Transfection with the long and short forms of $\alpha_s$. COS cells were transfected with plasmids containing cDNAs for the long or short form of $\alpha_s$ and radiolabeled with [$^{35}$S]methionine. $\alpha_s$ was immunoprecipitated from the particulate fractions and separated by HPLC analysis as described in the Figure 1 legend. (A) Radioactivity in the aliquots of the HPLC fractions (●, long form; ○, short form). (B and C) Aliquots from fractions at elution times 3−4 min and 13−19 min from the transfection of the long form (B) and short form (C) of $\alpha_s$ were analyzed by SDS−PAGE and fluorography.

than 70% of the total density in each lane with no other band having greater than 4% of the density.

COS cells were transfected with a plasmid containing the cDNA for a mutant $\alpha_s$ that does not undergo palmitoylation because cysteine-3 was changed to alanine (Figure 2) (Degtyarev et al., 1993a). Most of the protein eluted in the 13 min peak with a small shoulder of slower eluting material at 15 min (Figure 1). The radioactivity in the 15 min fraction was probably due to the palmitoylated endogenous $\alpha_s$ because the relative amount of endogenous $\alpha_s$ compared to the overexpressed C3A $\alpha_s$ is still significant (Figure 2).

Alternative splicing of $\alpha_s$ leads to expression of long and short forms that differ by 15 amino acids (Bray et al., 1986). We transfected COS cells with plasmids containing the long and short forms of $\alpha_s$ to compare their separation by HPLC. The elution profiles from these two transfections were similar (Figure 3A). The pattern was similar to other experiments

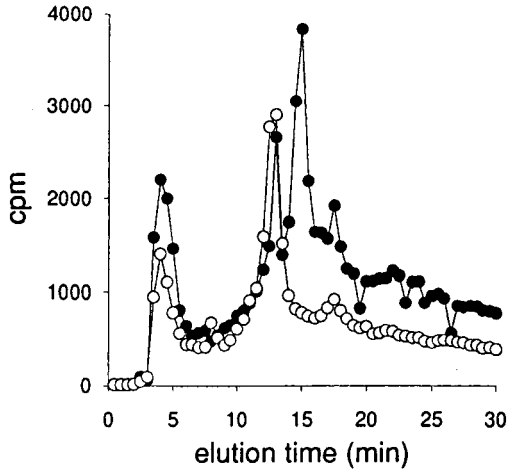


FIGURE 4: Hydroxylamine treatment of $\alpha_s$. The particulate fraction of [$^{35}$S]methionine-labeled COS cells was treated with 1 M hydroxylamine, pH 7.4, or buffer alone for 30 min at room temperature followed by centrifugation. $\alpha_s$ was immunoprecipitated and separated by HPLC as described in the Figure 1 legend. (●, Control; ○, hydroxylamine treatment.)

except that in this particular experiment the two peaks were at 15 and 17 min. Samples from elution times 13−19 min were resolved on SDS−PAGE and the radiolabeled proteins detected by fluorography (Figure 3B,C). The only radiolabeled bands seen are at 45 and 42 kDa, the molecular masses of the long and short forms, respectively. Samples from elution times of 3−4 min were also analyzed because of a peak of radioactivity at these times in all the HPLC separations. Faint $^{35}$S-labeled protein bands were detected, suggesting that free radiolabel or small radiolabeled peptides eluted in these fractions. The relative amount of the overexpressed $\alpha_s$ in the early and late peaks varied by transfection (Figure 1 versus 3A). The fraction of $\alpha_s$ eluting with the earlier peak was higher in transfections with higher levels of overexpression.

Hydroxylamine breaks thioester bonds and releases palmitate from $\alpha_s$ and other palmitoylated proteins (Degtyarev et al., 1993a; Linder et al., 1993). The particulate fractions from $^{35}$S-labeled COS cells were treated with hydroxylamine or Tris buffer, centrifuged, and then prepared for analysis by HPLC. Hydroxylamine treatment specifically removed the peak eluting at 15 min, so that the protein eluted with one peak at 13 min (Figure 4). Less total radioactivity was eluted from the particulate fraction treated with hydroxylamine probably because of a loss of $\alpha_s$ from the membrane under these conditions. These experiments indicate that the unacylated pool of $\alpha_s$ can be separated from the acylated pool as peaks eluting at 13 and 15 min, respectively.

*Palmitoylation of the Endogenous* $\alpha_s$. We determined the fraction of endogenous $\alpha_s$ that is palmitoylated by immunoprecipitating $^{35}$S-labeled $\alpha_s$ in the particulate fraction of COS and S49 lymphoma cells. For COS cells 77% and for S49 cells 70% of $\alpha_s$ eluted in the 15 min peak containing the palmitoylated protein (Figure 5). These values are the mean of two independent experiments for each cell line. We tested whether the preparation for HPLC analysis led to a loss of palmitate by comparing aliquots of a [$^3$H]palmitate-labeled sample prepared in the same manner as for HPLC to an untreated sample. Ninety percent of the [$^3$H]palmitate was retained on the protein after treatment under these conditions (mean of two independent experiments).

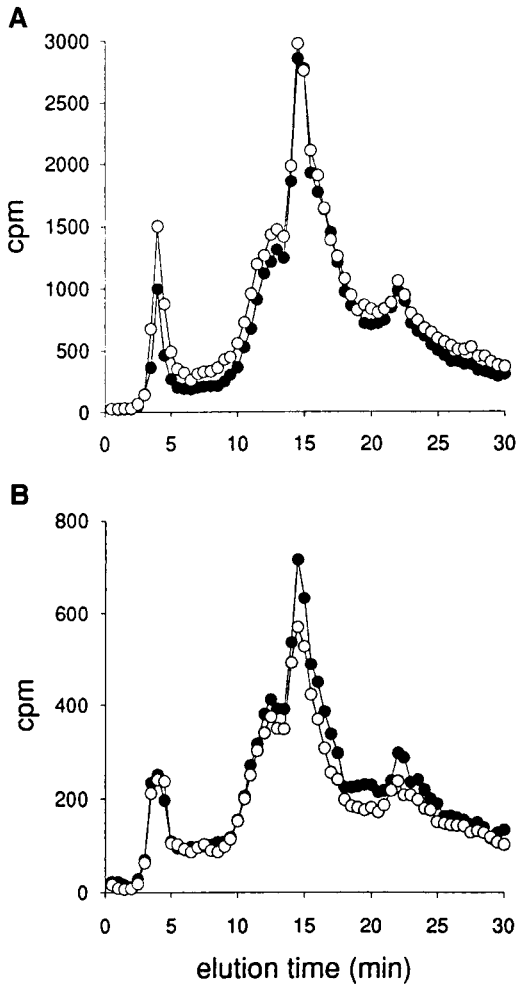


FIGURE 5: Isoproterenol treatment of COS and S49 lymphoma cells. Cells were radiolabeled with [$^{35}$S]methionine and then incubated in the presence or absence of 10 $\mu$M isoproterenol for 15 min for the COS cells (A) and 2 min for the S49 cells (B). The $\alpha_s$ was immunoprecipitated from the particulate fractions and applied to a reverse phase HPLC column as described in the Figure 1 legend. (●, Control cells; ○, isoproterenol treatment.)

*Palmitoylation after Receptor Activation.* We had shown previously that treating cells with isoproterenol increases the incorporation of [$^3$H]palmitate into $\alpha_s$ (Degtyarev et al., 1993b). We tested whether $\beta$-adrenergic receptor activation by isoproterenol would change the fraction of $\alpha_s$ that is palmitoylated. COS cells metabolically labeled with [$^{35}$S]-methionine were treated with or without isoproterenol for 15 min and then harvested. The fraction of $\alpha_s$ that eluted in the 15 min peak did not change with isoproterenol treatment (Figure 5A). The S49 lymphoma cells have a faster incorporation of [$^3$H]palmitate in response to isoproterenol (Degtyarev et al., 1993b), so the cells were exposed to isoproterenol for 2 min. The elution profiles were similar with and without isoproterenol treatment (Figure 5B). Sixty-six percent of the $\alpha_s$ in the control cells eluted in the 15 min palmitoylated peak versus 67% with isoproterenol treatment. In the COS cells, different exposure times to isoproterenol were also tested. Seventy-seven percent of the $\alpha_s$ in the control cells eluted in the 15 min peak compared to 74% with 5 min, 75% with 10 min, and 75% with 15 min of isoproterenol treatment.

The above experiments were all performed on $\alpha_s$ in the particulate fraction. We tested for the presence of $\alpha_s$ in the soluble fraction of S49 cells after isoproterenol treatment

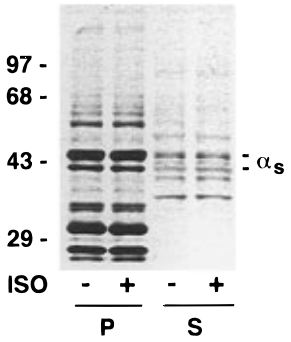


FIGURE 6: Intracellular localization of $\alpha_s$ after isoproterenol treatment. S49 cells were treated with 10 $\mu$M isoproterenol for 2 min and then harvested. The cells were homogenized and separated into particulate and soluble fractions by centrifugation. 40 $\mu$g of protein per lane was separated by gel electrophoresis, transferred to nitrocellulose paper, and immunoblotted with the RM antibody specific for $\alpha_s$. A peroxidase-labeled secondary antibody and enhanced chemiluminescence were used for detection.

because activation can change the membrane affinity of $\alpha_s$ (Levis & Bourne, 1992; Ransnas et al., 1989; Wedegaertner et al., 1996). Under these conditions with the endogenous $\alpha_s$, we found negligible amounts of $\alpha_s$ in the soluble fraction with or without activation (Figure 6). This immunoblot was overexposed to detect the small amount of $\alpha_s$ in the soluble fraction. In addition, we immunoprecipitated $\alpha_s$ from a whole cell lysate that had been solubilized in 0.5% SDS and heated for 5 min at 90 °C. In this experiment, 62% of $\alpha_s$ in the control cells and 76% of $\alpha_s$ in the isoproterenol-treated cells were found in the acylated, 15 min peak (Figure 7A). A fluorograph from the HPLC fractions shows two bands at 45 and 42 kDa with minimal contamination from other proteins (Figure 7B,C).

## DISCUSSION

Separation of the palmitoylated and nonpalmitoylated $\alpha_s$ by HPLC was a rapid, reproducible method to determine the fraction of $\alpha_s$ that was modified. This method assessed palmitoylation without the use of [$^3$H]palmitate and its problems with changes in specific activity in the cell. Using this method, we found that endogenous $\alpha_s$ was palmitoylated to near-stoichiometric levels in both COS and S49 lymphoma cells. Receptor activation of $\alpha_s$ causes palmitate turnover on $\alpha_s$, but we found that this did not change the amount of $\alpha_s$ that was palmitoylated.

These studies are the first to show that within the cell most of $\alpha_s$ is acylated. More $\alpha_s$ may be modified within the cell because a small amount of palmitate was released during preparation for HPLC and more could have been released during cell fractionation and immunoprecipitation. Some of the unmodified $\alpha_s$ may be due to newly expressed protein that has localized to membranes but not undergone palmitoylation either by a palmitoyltransferase at the plasma membrane or by autoacylation (Duncan & Gilman, 1996; Dunphy et al., 1996). Whether palmitoylation of $\alpha_s$ in vivo is an enzymatic or nonenzymatic process is yet to be determined. The relative amount of unmodified $\alpha_s$ was greater in transfected cells overexpressing $\alpha_s$ compared to the endogenous $\alpha_s$ in nontransfected cells. The nonpalmitoylated protein in transfected cells may fold aberrantly and not undergo palmitoylation. The nonpalmitoylated $\alpha_s$ may also be the result of an excess of free $\alpha$ subunit secondary

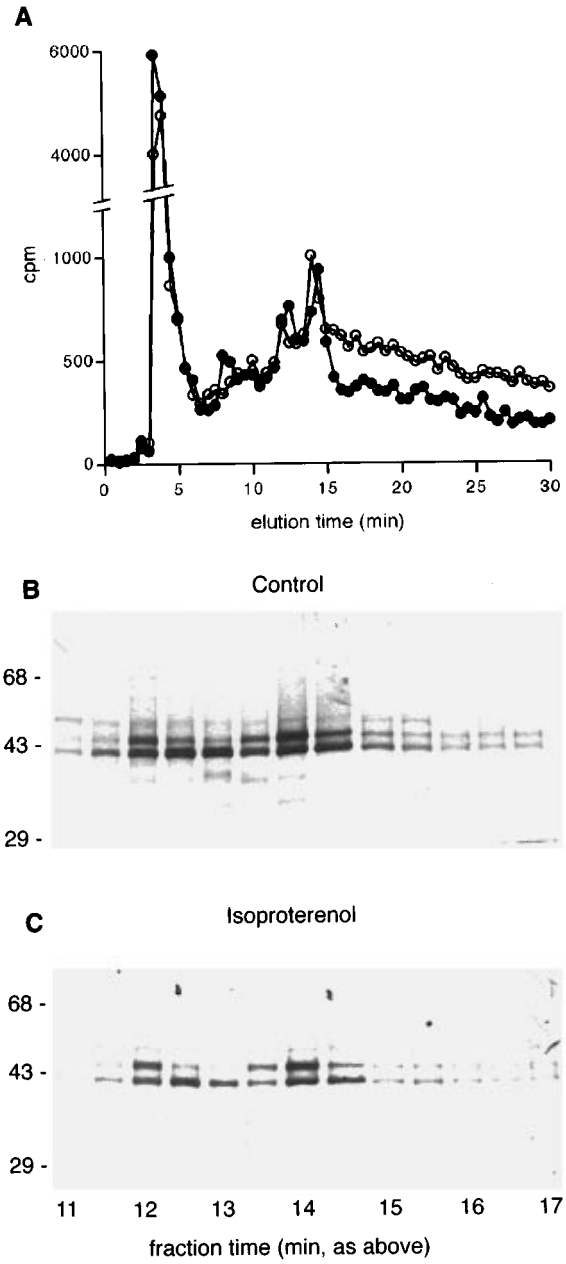


FIGURE 7: HPLC analysis of $\alpha_s$ immunoprecipitated from cell lysates. COS cells were radiolabeled with [$^{35}$S]methionine and then incubated in the presence or absence of 10 $\mu$M isoproterenol for 15 min. The cell pellet was solubilized in 0.5% SDS and heated at 90 °C for 5 min. $\alpha_s$ was immunoprecipitated from the cell lysates and applied to a reverse phase HPLC column as described in the Figure 1 legend. (A) Radioactivity in the aliquots of the HPLC fractions. (●, Control cells; ○, isoproterenol treatment.) (B and C) Aliquots from fractions at elution times of 10.5−17 min from the control cells (B) and the isoproterenol-treated cells (C) were analyzed by SDS−PAGE and fluorography.

to transfection. Free $\alpha$ subunits are a poorer substrate for palmitoylation compared to $\alpha$ subunits in the heterotrimer (Duncan & Gilman, 1996; Dunphy et al., 1996).

Quantification of the relative amounts of modified and unmodified $\alpha_s$ was only an estimate because the peaks are asymmetric and overlap. The asymmetry of the second peak may be due to heterogeneous acylation of $\alpha_s$. The G protein palmitoyltransferase activity found in membranes shows a substrate preference for palmitoyl-CoA (C16:0), but other acyl-CoAs, especially myristoyl-CoA (C14:0), stearoyl-CoA (C18:0), and oleoyl-CoA (C18:1), are also substrates (Dun-

phy et al., 1996). These acyl-CoAs are also substrates for autoacylation of α subunits (Duncan & Gilman, 1996). In platelets, α subunits incorporate arachidonate (C20:4) through a thioester bond (Hallak et al., 1994). We found in a previous study using similar [$^3$H]palmitate labeling and separation methods that the tritium incorporated into $\alpha_s$ migrated with the [$^3$H]palmitate standard on thin-layer chromatography (Degtyarev et al., 1993a). The elution of the [$^3$H]palmitate-labeled $\alpha_s$ in the peak at 15 min indicated that this peak contained $\alpha_s$ modified by palmitate but modification with acyl groups of different chain lengths is also possible.

Palmitoylation occurs on the amino terminus of $\alpha_s$ (Linder et al., 1993). Mutation of cysteine-3, the only cysteine residue in this area, prevents the modification and indicates that one palmitate is added per protein (Degtyarev et al., 1993a; Wedegaertner et al., 1993). There has been speculation that $\alpha_s$ is modified by an additional hydrophobic group because most other α subunits are modified either by myristate and palmitate or by two palmitates and because $\alpha_s$ expressed in bacteria behaves differently than $\alpha_s$ purified from tissue (Graziano et al., 1989). $\alpha_s$ was primarily detected in the two peaks at 13 and 15 min, but a small peak or shoulder at 22 min was often detected in the samples from the particulate fraction. If $\alpha_s$ undergoes an additional hydrophobic modification, it can be present in the protein eluted at 13 and 15 min and independent of palmitoylation, or the 13 min peak contains $\alpha_s$ that lacks both palmitate and the other modification.

These studies were primarily performed on the particulate fraction because we found that it contained nearly all the cellular $\alpha_s$ in both the basal and activated states (Figure 6). HPLC separation was also performed on $\alpha_s$ immunoprecipitated from cell lysates, and the results were similar to those using $\alpha_s$ from the particulate fraction. The cell lysates were treated with detergent and heat to minimize the possibility of repalmitoylation during processing (Figure 7). This treatment prevents both enzymatic and nonenzymatic palmitoylation (Duncan & Gilman, 1996; Dunphy et al., 1996). These conditions should also inhibit protein palmitoylthioesterase activity, which is abundant in the cytosol of cells (Camp & Hofmann, 1993). Translocation of $\alpha_s$ from the membranes to the cytosol upon activation has been observed by some groups (Levis & Bourne, 1992; Ransnas et al., 1989; Wedegaertner et al., 1996). This study addressed the acylation of $\alpha_s$ and not specifically the localization. Factors such as cell type, levels of protein expression, epitope-tags, fractionation, and detection methods could accentuate or minimize small changes in the membrane affinity of $\alpha_s$ induced by activation and influence whether these changes are detected as translocation.

The finding that most of the $\alpha_s$ was palmitoylated in the cell is consistent with turnover studies using [$^3$H]palmitate-labeled $\alpha_s$ that show activation of $\alpha_s$ first leads to depalmitoylation (Mumby et al., 1994; Wedegaertner & Bourne, 1994). Activation of $\alpha_s$ leads to its dissociation from $\beta\gamma$ and potentiates the removal of the palmitate by an esterase (Iiri et al., 1996). Under conditions used here, activation of $\alpha_s$ by isoproterenol treatment did not change the amount of $\alpha_s$ that was palmitoylated. Several explanations are possible. Palmitate turnover may not occur with activation—the changes in [$^3$H]palmitate incorporation are due to changes in the specific activity within the cell. Our previous work addressed this problem by showing that isoproterenol treatment does not change the [$^3$H]palmitate incorporation into other proteins. Isoproterenol effects on specific intracellular pools of [$^3$H]palmitoyl-CoA, the palmitate donor for palmitoylation, are still possible, however. Another explanation is that the $\beta$-adrenergic receptor activated only a small number of $\alpha_s$ proteins. Changes in their palmitoylation would be undetectable with this method but seen by [$^3$H]-palmitate incorporation.

A rapid turnover of palmitate can also explain our result. The current model of palmitoylation and the GTPase cycle is that G protein activation leads to dissociation of the α subunit from the $\beta\gamma$ subunits and depalmitoylation of the α subunit (Wedegaertner & Bourne, 1994). The α subunit hydrolyzes GTP, forms a heterotrimer with $\beta\gamma$, and then undergoes palmitoylation. Our result suggests that palmitoylation was rapid because a pool of depalmitoylated α subunits did not accumulate. The function of palmitate turnover is not known. Acylation localizes proteins to specific membranes and lipid domains (Cadwallader et al., 1994; Shaul et al., 1996). Depalmitoylated $\alpha_s$ could move on the membrane and contact effector proteins. Palmitoylation and a change in location would help end the interactions. A rapid turnover of palmitate would then aid in maximizing specific interactions between G proteins and receptors and minimizing nonspecific interactions. More studies are needed to understand the function of palmitoylation in G protein signaling. This HPLC separation method is another tool to study palmitoylation and to facilitate and extend this research.

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