Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 6116–6120, June 1997
Biochemistry

$G_{s\alpha}$ contains an unidentified covalent modification that increases its affinity for adenylyl cyclase

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Contributed by Alfred G. Gilman, April 9, 1997

ABSTRACT **Many G protein α subunits are dually acylated with myristate and palmitate or are palmitoylated on more than one cysteine residue near their N termini. The G_α protein that activates adenylyl cyclase, α_s, is not myristoylated but can be reversibly palmitoylated. It appears that α_s contains another, as-yet-unidentified covalent modification that decreases its apparent dissociation constant for adenylyl cyclase from 50 nM to <0.5 nM. This modification is at or near the N terminus of the protein and is hydrophobic. Palmitoylation of native α_s does not account for its high affinity for adenylyl cyclase.**

Heterotrimeric G proteins play a pivotal role in cellular signaling, functioning as molecular switches that couple a diverse array of extracellularly oriented receptors to a variety of intracellular effectors or signal generators. The guanine nucleotide-binding α subunits of G proteins are covalently modified with lipid at or near their N termini (1–3). Thus, members of the α_i subfamily contain amide-linked myristate on N-terminal glycine residues, and all α subunits (except α_t) contain palmitate in thioester linkage on one or more nearby cysteine residues. Crystal structures of G protein oligomers demonstrate that the N terminus of α is in close proximity to the C terminus of the γ subunit, which is prenylated (4, 5). Thus, the hydrophobic modifications of G protein subunits are gathered together at a point that is assumed to represent one site of interaction of the oligomer with the inner face of the plasma membrane.

Myristoylation is a stable protein modification with substantial functional consequences. Prevention of myristoylation by mutation of Gly^2 to Ala in α_i proteins causes accumulation in the cytosol of protein expressed *in vivo* (6, 7). Biochemical characterization of α_{i1} and/or α_o synthesized in bacteria in the presence or absence of myristoyl CoA:protein *N*-myristoyltransferase demonstrates that myristoylated α_i proteins have a higher affinity for both the G protein $\beta\gamma$ subunit complex and at least one effector, adenylyl cyclase, compared with their nonmyristoylated counterparts (8, 9).

Palmitoylation is a dynamic protein modification. In the case of α_s (palmitoylated on Cys^3; not myristoylated), activation of cognate receptors (e.g., β-adrenergic receptors) facilitates incorporation of [^{3}H]palmitate into the protein, apparently by stimulating depalmitoylation and thus turnover of the lipid (10, 11). Similar regulation of palmitoylation of other G protein α subunits is often assumed *but has not yet been reported*. The functional consequences of palmitoylation of α subunits are unclear, and there are inconsistencies in the literature. We (10) and Degtyarev *et al.* (12) failed to find α_s in cytosolic cellular fractions after expression of the $Cys^3 \rightarrow$ Ala mutant of the protein or following activation of the native protein by means of the β-adrenergic receptor. By contrast, others have noted both substantial distribution of Ala^3-α_s to the cytosol and appearance of the wild-type protein in soluble fractions after activation of receptors (13–15). Although the reasons for these discrepancies have been sought, they have not been found. We and others found that expression of the $Cys^3 \rightarrow$ Ala mutant of α_o caused the partial appearance of the protein in the cytosol (10, 16, 17), while modification of both palmitoylated cysteine residues of α_q had no effect on cellular distribution (18). Patterns are difficult to perceive.

Comparison of the functional properties of recombinant α_s synthesized in bacteria with those of the protein purified from liver or brain has revealed major differences (19). In particular, the apparent affinity of recombinant α_s for its effector, adenylyl cyclase, is substantially lower. We have attempted to evaluate the role of palmitoylation or another, unknown, modification of α_s in this phenomenon.

METHODS AND MATERIALS

Plasmids. A cDNA encoding bovine $\alpha_{s\text{-short}}$ was used to generate hexahistidine-modified (C terminus) α_s (α_s-CH_6), using GCGCTTAAGCTTTTAGTGATGGTGGTG-GTGATGTCCTCCGAGCAGCTCATAC as the mutagenic oligonucleotide. A cDNA encoding an $\alpha_{s\text{-long}}$ subunit that has the alternatively spliced sequence contained in exon 3 partially replaced by a hexahistidine tag (α_s-H_6) was kindly provided by Maurine Linder (Washington University).

Protein Purification. Wild-type and His_6-tagged α_s were purified as described after expression in *Escherichia coli* (20). His_6-tagged α_s proteins were also expressed in Sf9 cells by infection with recombinant baculoviruses for 40–48 hr. After extraction of Sf9 cell membranes with buffer A [20 mM Tris·HCl, pH 8.0/1 mM GDP/10 mM 2-mercaptoethanol/0.5% polyoxyethylene 10-lauryl ether ($C_{12}E_{10}$) containing protease inhibitors (21)], NaCl was added (200 mM) and the solution was applied to a column containing Ni^{2+}-NTA (Qiagen, Chatsworth, CA; NTA is nitrilotriacetate). The column was washed sequentially with buffer A containing 500 mM NaCl and buffer A containing 200 mM NaCl prior to elution with buffer A containing 150 mM imidazole. The eluate was concentrated, diluted 10-fold, and applied to $\beta\gamma$-agarose (22). Bound protein was eluted with a solution of 20 mM NaHepes (pH 8.0), 1 mM EDTA, 300 mM NaCl, 3 mM dithiothreitol, 30 μM $AlCl_3$, 20 mM $MgCl_2$, 10 mM NaF, and 0.5% sodium cholate (at room temperature). Protein was concentrated and buffer was exchanged into 20 mM NaHepes, pH 8.0/1 mM EDTA/3 mM dithiothreitol/0.3% sodium cholate. Wild-type α_s expressed in Sf9 cells was purified on a β_2-$His_6\gamma_2$-Ni^{2+}-NTA column as described (23), except that the membranes were extracted with 0.5% $C_{12}E_{10}$ and 30% (vol/vol) ethylene glycol was added to the extract. The eluate was further purified on $\beta\gamma$-agarose as described above; binding was achieved by che-

Abbreviations: G proteins, heterotrimeric guanine nucleotide-binding regulatory proteins; $C_{12}E_{10}$, polyoxyethylene 10-lauryl ether; NTA, nitrilotriacetate; GTP[γS], guanosine 5′-[γ-thio]triphosphate.

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lation of Mg^{2+}. Native α_s was purified from rabbit liver as described (24). The G protein $\beta_1\gamma_2$ subunit complex was purified after expression in Sf9 cells (23).

Purified α_s was activated with guanosine 5′-[γ-thio]triphosphate (GTP[γS]) (19, 25), followed by gel filtration into 50 mM NaHepes, pH 8.0/1 mM EDTA/2 mM $MgCl_2$/100 mM NaCl. The concentration of activated α_s was determined by inclusion of radiolabeled GTP[γS] during activation.

Adenylyl Cyclase and GTPase Activities. Membranes were prepared from Sf9 cells infected with baculovirus encoding type V adenylyl cyclase (26). These membranes (5 μg) were incubated for 3 min at 30°C with activated α_s prior to assay of adenylyl cyclase activity for 10 min at 30°C (27). Steady-state GTPase activities of α_s proteins were measured as described (28).

Mass Spectrometry. Molecular masses of certain proteins of interest were determined by electrospray ionization mass spectrometry after protein purification by reverse-phase high-pressure liquid chromatography. We are grateful to Clive Slaughter (University of Texas Southwestern Medical Center) for these determinations.

Miscellaneous Procedures. Metabolic radiolabeling with [^{3}H]palmitate, immunoblotting, and fluorography were performed as described previously (10).

RESULTS

Activation of Adenylyl Cyclase. When expressed in *E. coli* and purified, α_s (native sequence, untagged) is capable of activating membrane-bound type V adenylyl cyclase dramatically, but with an apparent affinity (EC_{50}) of only 50 nM (Fig. 1 and ref. 19). By contrast, the protein purified from rabbit liver activates adenylyl cyclase to the same maximal extent but with an EC_{50} of roughly 0.1 nM (Fig. 1 and ref. 19). A similar discrepancy was observed with detergent-solubilized adenylyl cyclase (19). This suggests the existence of a covalent modification of native α_s that facilitates interaction of the protein with the membrane (or detergent micelles) or that enhances the affinity of the requisite interaction between α_s and adenylyl cyclase. It is also possible that the conformation of recombinant α_s differs from that of the native molecule.

We have attempted to express α_s in other heterologous systems that would permit the putative covalent modification, analysis by mutagenesis, and procurement of quantities of protein sufficient for more extensive characterization. To facilitate purification, hexahistidine tags were inserted in one of three locations: the N terminus (H_6-α_s), the site in exon 3 where splice variants of α_s are produced (α_s-H_6), or the C terminus (α_s-CH_6). The latter protein was expressed in *E. coli, Saccharomyces cerevisiae,* and Sf9 cells (α_s-CH_6^{Sf9}) and purified by a combination of Ni^{2+}-NTA and βγ subunit-affinity chromatography. The EC_{50} values for activation of adenylyl cyclase by these three proteins were approximately 50 nM, 3 nM, and 3 nM, while the yields for the yeast and Sf9 cell preparations were both low—1 μg and 50 μg per liter of culture, respectively. Wild-type α_s purified from Sf9 cells was modestly more potent than the corresponding preparation of α_s-CH_6^{Sf9} ($EC_{50} \approx 1$ nM; Fig. 1), but the yield was poor because of less efficient purification (5 μg/liter).

Interaction with βγ. The affinity of G protein α subunits for the βγ subunit complex can be estimated by virtue of the capacity of βγ to slow dissociation of GDP from α and thus inhibit its steady-state GTPase activity. Such measurements are shown in Fig. 2. To be accurate, the assay must be performed at concentrations of α_s below the K_d for its interaction with βγ. However, GTPase activity cannot be assessed reliably at α_s concentrations much below 0.4 nM, limiting the range of the measurement. Addition of βγ to native α_s resulted in a steep inhibition curve with complete suppression of GTPase activity at roughly equimolar concentrations of α_s and βγ. The experiment is thus a titration, and the half-maximally effective concentration of βγ, ≈0.1 nM, is an overestimate of the K_d for the interaction between α_s and βγ. By contrast, the EC_{50} for inhibition of the GTPase activity of *E. coli*-derived α_s by βγ was 0.5 nM, reflecting a substantially reduced affinity of βγ for the recombinant protein.

Location of the Putative Modification. Limited tryptic proteolysis of GTP[γS]-bound α subunits removes a roughly 3-kDa peptide from the N terminus, leaving the rest of the protein largely intact. However, immunoblotting with an antibody that recognizes the C terminus of α_s indicated that a few amino acid residues are also removed at this site. Exclusive N-terminal cleavage was achieved with the endoprotease LysC. We have shown previously that such limited proteolysis of *E. coli*-derived α_s alters neither its capacity to activate adenylyl

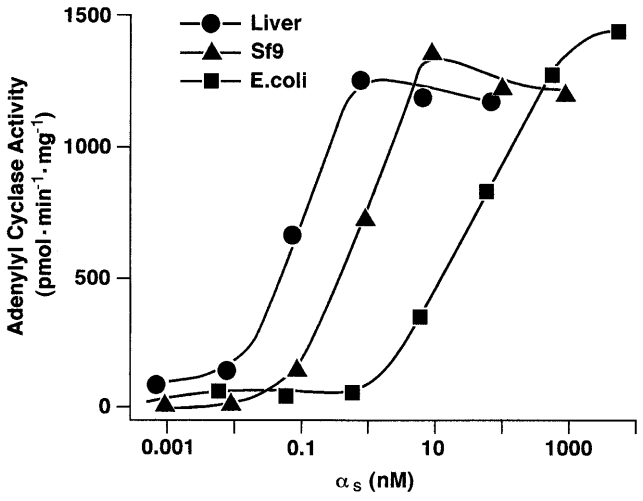

FIG. 1. Activation of adenylyl cyclase. α_s was purified from rabbit liver membranes (●) or after expression in Sf9 cells (▲) or *E. coli* (■). The proteins were activated with GTP[γS] and reconstituted with membranes (5 μg) from Sf9 cells expressing type V adenylyl cyclase. Adenylyl cyclase activity was assayed as described. Values shown are the average of triplicate determinations and are representative of at least two experiments.

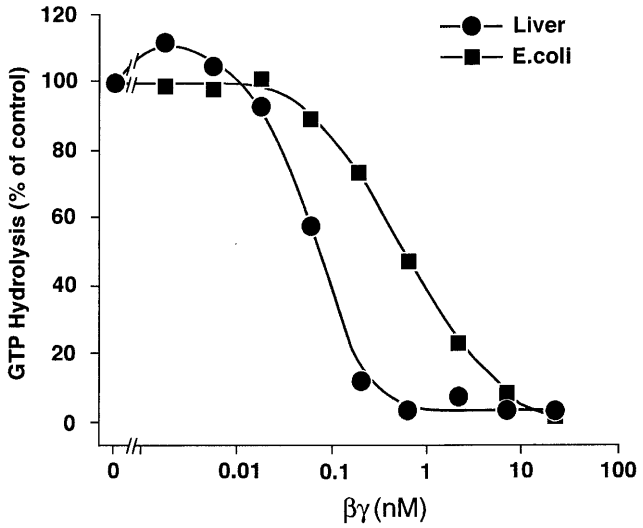

FIG. 2. Inhibitory effect of $\beta_1\gamma_2$ on steady-state GTP hydrolysis by α_s. Purified α_s from rabbit liver (●, 0.39 nM) or recombinant α_s synthesized in *E. coli* (■, 0.5 nM) was mixed with the indicated concentrations of $\beta_1\gamma_2$ and then assayed for GTPase activity (20 min, 30°C). Rates of GTP hydrolysis in the absence of $\beta_1\gamma_2$ were 0.12 and 0.25 pmol/pmol of α_s per min for the rabbit liver and recombinant protein, respectively.

cyclase nor its apparent affinity for the enzyme (26) (Fig. 3*A*). However, proteolysis of native α_s and α_s-CH_6^{Sf9} dramatically impaired, but did not eliminate, their stimulatory effect on adenylyl cyclase when tested at a fixed, submaximal concentration, presumably reflecting a decrease in the affinity of N-terminally truncated α_s for adenylyl cyclase (Fig. 3*A*). (Limited quantities of material were available, particularly of the native enzyme, precluding testing over a broad range of concentrations.) The loss of activity of the native and Sf9-derived α_s paralleled the loss of the N terminus (Fig. 3*B*). When full-length protein was no longer detectable (30 min), the three preparations of α_s (*E. coli*- and Sf9 cell-derived and the native enzyme) stimulated adenylyl cyclase to similar extents.

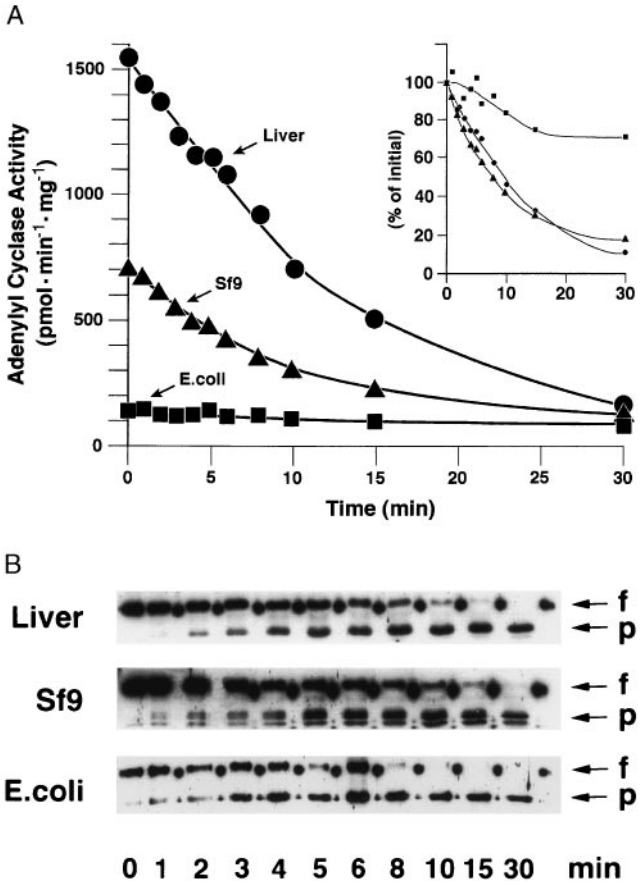

FIG. 3. Activation of adenylyl cyclase by N-terminally cleaved α_s. α_s was purified from rabbit liver (●) or after expression in Sf9 cells (▲) or *E. coli* (■), activated with GTP[γS], and digested with 0.01 μg of LysC in 150 μl of 20 mM NaHepes, pH 8.0/1 mM EDTA/2 mM $MgCl_2$/1 mM dithiothreitol/0.05% $C_{12}E_{10}$ containing 100 μg/ml bovine serum albumin at 30°C. Aliquots were withdrawn at the indicated times, N^{α}-(*p*-tosyl)lysine chloromethyl ketone (TLCK; 0.8 mg/ml) and aprotinin (0.4 mg/ml) were added, and protein was diluted and mixed with membranes from Sf9 cells expressing type V adenylyl cyclase (*A*). The proteins in duplicate aliquots were resolved electrophoretically, blotted on nitrocellulose, and visualized using an antibody specific for α_s (*B*). (*A*) Adenylyl cyclase activities of the reconstituted mixtures. The final concentrations of α_s during the assay were 8 nM for the rabbit liver protein and 56 nM and 67 nM for the proteins expressed in Sf9 cells and *E. coli*, respectively. The data shown are the average of duplicates and are representative of at least two such experiments. In the *Inset*, activity is expressed as a percentage of the value observed prior to exposure of α_s to LysC. (*B*) The immunoblot was probed with antiserum 584 (29). Arrows show the position of migration of full-length α_s protein (f) or the N-terminally cleaved product that accumulates during the incubation with LysC (p). Nondigested α_s protein was loaded onto the gel in every other slot (appears as dots).

Hydrophobic Nature of the Modification. Solutions of the nonionic detergent Triton X-114 are homogeneous at 0°C but separate into aqueous and detergent-rich phases above 20°C. Proteins that are solubilized in the cold solution partition into these two phases according to their hydrophobicity (30). The technique is sensitive, in that myristoylated and nonmyristoylated α_{i1} are completely resolved (Fig. 4). Native α_s from rabbit liver (containing both short and long splice variants) partitioned exclusively into the detergent-rich phase, α_s-CH_6^{Sf9} was found predominantly in the aqueous phase, and *E. coli*-derived α_s was found only in the aqueous phase. After treatment with LysC, all three preparations of α_s partitioned into the aqueous phase exclusively. Since proteolytic removal of the N terminus of native α_s also altered its interactions with adenylyl cyclase, it seems likely that a hydrophobic modification near or at the N terminus of α_s is responsible for the different behaviors of the native and recombinant proteins.

Mutagenesis of Potential Sites of Modification. Mutation of Gly^2 to Ala or of Cys^3 to Ser in Sf9 cell-derived α_s resulted in production of proteins that could not be distinguished from *E. coli*-derived α_s, whereas mutation of Leu^4 or Asn^6 to Ala did not change the interaction of α_s-CH_6^{Sf9} with adenylyl cyclase (data not shown). The same mutations had no effect on the behavior of the proteins after synthesis in *E. coli* (data not shown).

Cellular Localization. Sf9 cells were infected with recombinant baculoviruses encoding α_s, α_s carrying hexahistidine tags at the N or C terminus or at the site of alternative splicing in exon 3, or α_s bearing $Gly^2 \rightarrow$ Ala or $Cys^3 \rightarrow$ Ser mutations. Immunoblotting was used to determine if the α_s proteins were soluble or membrane bound. Only the detergent-soluble (1% sodium cholate) membrane-bound fraction was examined. When the proteins were expressed by themselves, 20–40% of each protein was particulate. When the proteins were coexpressed with βγ, the fraction of each protein that was membrane bound increased, such that the range of values was 50–80%. There was no clear capacity of any of the tags or mutations tested to cause predominant distribution of α_s to the cytosol (data not shown).

Effect of Palmitoylation. It is difficult to assess the stoichiometry of palmitoylation *in vivo*, and it is possible that most

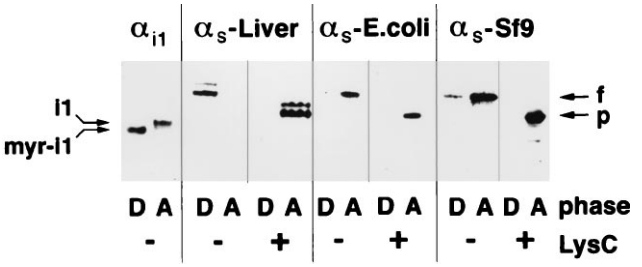

FIG. 4. Phase partitioning of G_α proteins in Triton X-114. The proteins utilized were a mixture of myristoylated and nonmyristoylated α_{i1} and α_s purified from liver or synthesized (untagged) in *E. coli* or Sf9 cells. Note that myristoylated and nonmyristoylated α_{i1} can be resolved by sodium dodecylsulfate/polyacrylamide gel electrophoresis. Approximately 1 μg of purified protein in 20 mM Tris·HCl, pH 7.5/150 mM NaCl was activated with GTP[γS], and a portion of each sample was then digested with 0.1 μg of LysC for 15 min at room temperature where indicated. Samples were diluted to 100 μl with 20 mM Tris·HCl, pH 7.5/150 mM NaCl and supplemented with 25 μl of 10% Triton X-114. The mixtures were then separated into detergent-rich (D) and aqueous (A) phases by incubation for 1 min at 30°C, followed by centrifugation at room temperature for 0.5 min at 13,000 × *g*. The separated phases were extracted two more times each with 10% Triton X-114 or buffer, adjusted to contain equal total volumes, and analyzed by sodium dodecylsulfate/polyacrylamide gel electrophoresis and immunoblotting, using α_s- or α_i-specific antisera. The positions of full-length α_s (f), the proteolyzed α_s fragment (p), nonmyristoylated α_{i1} (i1), and myristoylated α_{i1} (myr-i1) are marked.

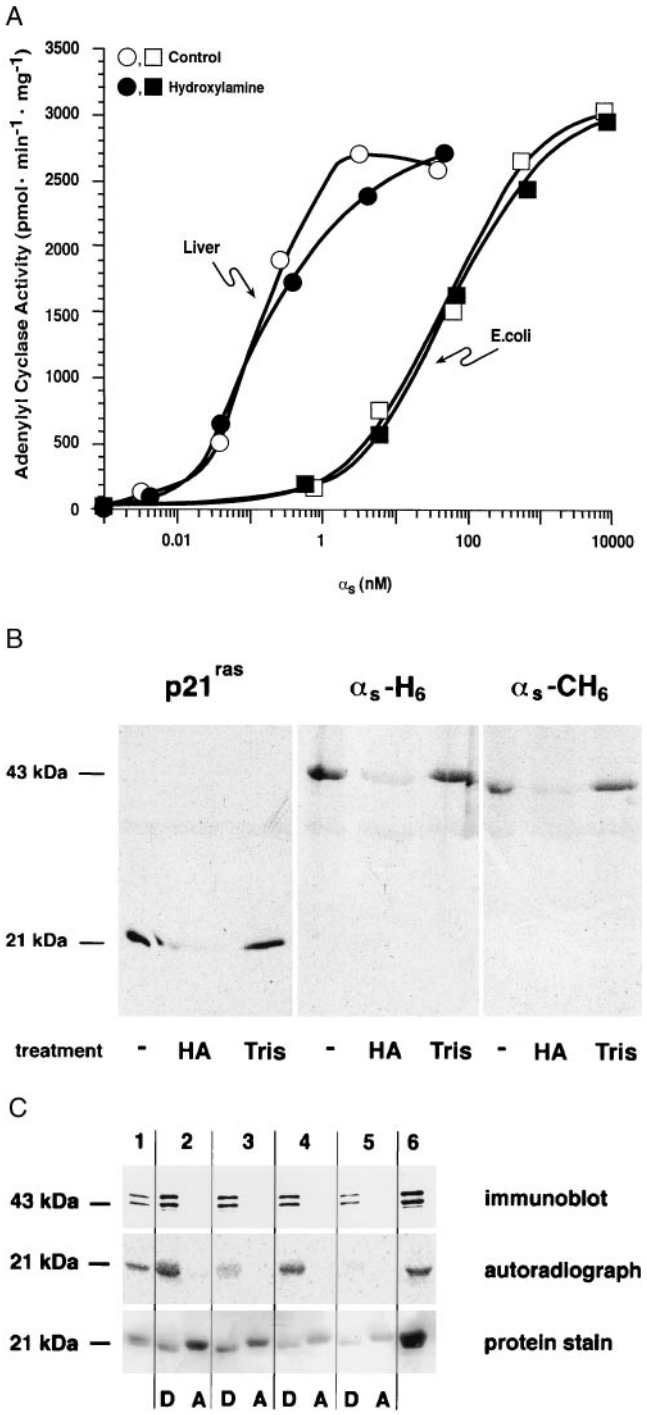

FIG. 5. Treatment of native and recombinant α_s with hydroxylamine. (*A*) α_s was purified from rabbit liver (○, ●) or after expression in *E. coli* (□, ■). Proteins were activated with GTP[γS] and then incubated with 1 M hydroxylamine at pH 7.0 (●, ■) or 1 M Tris·HCl, pH 7.0 (○, □), for 30 min at room temperature. Proteins were gel filtered prior to reconstitution at the indicated concentrations with membranes (10 μg) from Sf9 cells expressing type V adenylyl cyclase and assay of α_s-stimulated adenylyl cyclase activity. (*B*) His_6-tagged (N terminus) $p21^{ras}$, α_s-H_6, and α_s-CH_6 were synthesized in Sf9 cells and labeled *in vivo* with [^{3}H]palmitic acid. Proteins were enriched by Ni^{2+}-NTA chromatography and treated with 1 M hydroxylamine (HA) or 1 M Tris·HCl (Tris) as described for *A*. Samples were then subjected to sodium dodecylsulfate/polyacrylamide gel electrophoresis and fluorography. An untreated sample is shown in the first lane for each protein. (*C*) Purified rabbit liver α_s was mixed in 20 mM Tris·HCl, pH 7.0/150 mM NaCl with $p21^{ras}$ that had been metabolically labeled with [^{3}H]palmitic acid; this starting material is shown in lanes 1, 2, and 6. Aliquots of this mixture were treated with palmitoyl-protein thioesterase (lane 3), 1 M Tris·HCl, pH 7.0 (lane 4), or 1 M hydroxylamine, pH 7.0 (lane 5) and then subjected to Triton X-114 phase partitioning as described in the legend of Fig. 4. Proteins were resolved by electrophoresis, transferred to nitrocellulose, and detected by immunoblotting (with an α_s-specific antibody), autoradiography, or protein staining (with Ponceau S).

covalently bound palmitate is lost during purification, either enzymatically or by reaction with dithiothreitol, if present. We tested the capacity of a purified palmitoyl-protein thioesterase (31) to remove radiolabeled palmitate from α_s *in vitro*, with variable results. As an alternative, we treated native and recombinant α_s with 1 M hydroxylamine at pH 7.0. Such treatment did not affect the capacity of α_s to activate adenylyl cyclase, nor did it alter the apparent affinity of either liver or *E. coli*-derived α_s for adenylyl cyclase (Fig. 5*A*). This treatment was effective, however, in removing radiolabel from $p21^{ras}$, α_s-H_6^{Sf9}, or α_s-CH_6^{Sf9} that had been synthesized *in vivo* in the presence of [^{3}H]palmitate (Fig. 5*B*) (whereas treatment with 1 M Tris·HCl at the same pH was without effect). Of interest, treatment of rabbit liver α_s with hydroxylamine did not alter the protein's behavior in Triton X-114 partitioning experiments; the treated protein was still found exclusively in the detergent-rich phase (Fig. 5*C*).

Mass Spectroscopy. Attempts were made to determine the molecular masses of various α subunits by electrospray mass spectroscopy after removal of detergent by reverse-phase chromatography. Ions with correct masses were observed for *E. coli*-derived α_{i1} and *E. coli*-derived α_{i1} bearing an internal hexahistidine tag (data not shown). When the latter protein was myristoylated in bacteria by coexpression with *N*-myristoyltransferase, the increment in mass observed was consistent with myristoylation. When this tagged protein was synthesized in Sf9 cells, a similar larger species was observed. Problematic, however, was our failure to obtain data with native α_{i1} from Sf9 cells, *E. coli*-derived myristoylated α_{i1} with or without palmitate incorporated *in vitro* (32), or native α_i or α_o purified from brain. Failures appear ascribable to the presence of lipid modifications, the absence of the internal hexahistidine tag, and/or the possibility of additional covalent modifications that might occur in some cellular settings. We detected the anticipated signals with *E. coli*-derived α_s and with various mutant and tagged mutant constructs of α_s. However, no consistent differences were detected between *E. coli*- and Sf9 cell-derived α_s; interpretable data were not obtained with native α_s.

DISCUSSION

The findings presented herein are provocative to us but are incomplete because of technical limitations. The evidence indicates that α_s, when synthesized in mammalian cells, is covalently modified in a manner that increases its apparent affinity for adenylyl cyclase by roughly 100-fold. Further, the putative modification probably resides near the N terminus, since cleavage of approximately 30 amino acid residues at this site causes the mammalian protein to resemble its bacterially synthesized counterpart. The modification is likely hydrophobic, since it imparts to α_s the capacity to distribute into the detergent-rich phase in Triton X-114 partitioning experiments. Finally, palmitoylation of α_s does not account for the phenomena described (although α_s can be palmitoylated at Cys^3), since removal of palmitate with hydroxylamine does not lower the apparent affinity of native α_s for adenylyl cyclase or alter its distribution in Triton X-114 partitioning experiments. Conventional purification of oligomeric G_s from rabbit liver or other sources takes several days, and the protein is dissolved in solutions containing dithiothreitol. We doubt that the

stoichiometry of palmitoylation of α_s would remain high for the duration of such procedures.

Attempts to complete this project by identification of the putative modification have been frustrating. Expression of α_s in heterologous systems capable of production of large amounts of the modified protein has not been achieved. Synthesis in yeast is inefficient and, further, the protein resembles that produced in bacteria. The yield of α_s from baculovirus-infected Sf9 cells is also disappointing. Furthermore, these cells also appear to modify α_s inefficiently (or incorrectly). The apparent affinity of Sf9 cell-derived α_s for adenylyl cyclase was notably variable from one preparation to another, but was never as high as that for the rabbit liver protein. Of interest, the variable affinity of Sf9 cell-derived α_s for adenylyl cyclase appeared to correlate with the proportion of the protein that distributed into the detergent-rich phase in Triton X-114 partitioning experiments. In most experiments, the bulk of the Sf9 cell-derived α_s was found in the aqueous phase, and a relatively low stoichiometry of modification would account for the observed intermediate apparent affinities of this protein for adenylyl cyclase.

E. coli-derived α_s also differs from the native protein in having a lower affinity for the G protein $\beta\gamma$ subunit complex. Iiri *et al.* (33) have ascribed this difference to *S*-acylation of Cys^3 of α_s with palmitate. We do not refute this observation but note that it was made with α_s synthesized in Sf9 cells, and thus with a protein that we presume to be largely lacking the covalent modification hypothesized herein. Either or both the unknown adduct and/or palmitate could influence the affinity of α_s for $\beta\gamma$, particularly since both are located near the N terminus—an important site of interaction between α and $\beta\gamma$.

There is little basis for more detailed speculation on the nature and site of the modification of α_s, except to note the poor affinities of mutant α_s proteins (Gly^2 or Cys^3) synthesized in Sf9 cells. The cysteine residues that can be palmitoylated in α_q are important themselves for interaction of this protein with its effector, phospholipase C-β1 (18). However, mutation at this site of *E. coli*-derived α_s had no effect. The effect of mutation at Gly^2 is particularly interesting in view of myristoylation of members of the α_i subfamily at this site. If this is a site of modification of α_s, it would by necessity occur on the α-amino group of the protein and would likely be metabolically and chemically stable, capable of surviving long-term purification procedures. Unambiguous identification of the modification may require improvements in sample preparation for mass spectrometry and/or development of an expression system for synthesis of larger quantities of protein that more closely resembles native α_s. Perhaps almost all G_α proteins are dually modified with lipids at their N termini: most α_i family members with myristate and palmitate, α_s with X and palmitate, α_q and α_{12} family members with multiple palmitates. The exception is transducin, an α subunit unique in its facile elution from photoreceptor disk membranes by GTP; it is modified only with C_{14} (and C_{12}) fatty acids at Gly^2 (34, 35).

We thank Pamela Sternweis for superb technical assistance. This work was supported by a fellowship from the Deutsche Forschungsgemeinschaft (to C.K.) and National Institutes of Health Grant GM34497 and the Raymond and Ellen Willie Distinguished Chair in Molecular Neuropharmacology (to A.G.G.).

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