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Quantitation of prenylcysteines by a selective cleavage reaction

(posttranslational processing/isoprenylation/farnesylcysteine/geranylgeranylcysteine)

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ABSTRACT **The allylic thioether bond of the prenylcysteines of prenylated proteins has been shown to be cleaved by 2-naphthol under alkaline conditions to yield substituted naphthopyrans. These products are readily resolved from interfering materials by HPLC and have a strongly absorbing chromophore. Thus, this reaction is suitable for quantitative analysis of prenyl substituents of proteins, and we have examined a number of tissues for their content of prenylcysteines. These amino acids are present in mammalian tissues at a concentration of 0.36–1.4 nmol/mg of protein, with a ratio of geranylgeranylcysteine to farnesylcysteine in the range of 4 to 10. Prenylcysteines were also found in the cytosolic fraction of two mouse tissues at about one-third the concentration of the whole organ. The level of these modified amino acids was found to be significantly less in a yeast, a fungus, a brown alga, a higher plant, and an insect. Again, geranylgeranylcysteine is predominant. Prenylcysteines were absent from *Escherichia coli* but present in an archaebacterium. The prenylcysteine content of mammalian tissue is about 1% of that of cholesterol and about equal to that of ubiquinones and dolichols. Calculations indicate that about 0.5% of all proteins are prenylated.**

Prenylated proteins contain isoprenoid-modified cysteines in the carboxyl-terminal region (1–3). This posttranslational modification has usually been identified by metabolic labeling of proteins of cells in culture with radioisotopic mevalonate. Metabolic labeling is limiting because many organisms—e.g., yeasts and plants—do not incorporate this precursor readily. In addition, this technique may be impractical, since large doses of isotope would be required for studies with animals. The specific isoprenoid involved with this amino acid (4, 5) has been established by protein hydrolysis and isolation of the prenylcysteine (6) or by cleavage of the prenylcysteine with iodomethane (7) or Raney nickel (4, 5) followed by isolation of the cleavage product. Recently, fast atom bombardment mass spectrometry has been reported as a convenient method for qualitative analysis of prenylated peptides (8). Quantitative analysis with these techniques by direct determination of mass would be difficult if not impossible. Consequently, an alternative method for identifying prenyl modifications would be useful. In prenylated proteins the allylic thioether bond of the prenylcysteines is unique and provides an avenue for selective reactions for identification of prenyl groups. A good nucleophile should react preferentially with and cleave the isoprenoid from cysteine. Prenyl groups linked through oxygen or nitrogen would be expected to be unreactive. For example, a model study using radioactive farnesyl pyrophosphate and our naphthoxide conditions showed about 0.1% of a radioactive product. If the reagent possessed a strongly absorbing chromophoric group, then the derivative would provide a means for detection of the product. We have allowed prenylated proteins to react with 2-naphthol and have found products that tentatively are prenyl-substituted naphthopyrans rather than the naphthyl ethers that were anticipated (9, 19). The naphthopyrans all have absorbance maxima at 246 and 360 nm (10, 11) that facilitate their detection by HPLC and fulfill the purpose set forth. We have applied a procedure based on this reaction to a variety of tissues and we now report the results.

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MATERIALS AND METHODS

Materials. Solvents were Omnisolv (EM Corp., Chestnut Hill, MA) or the highest quality available. Other chemicals were from Sigma or Aldrich. The potassium salt of 2-naphthol was prepared by mixing molar equivalents of 2-naphthol with KOH in ethanol. After removal of most solvent the product was crystallized twice from a toluene/ethanol mixture. Chromatography was with a Rainin (Woburn, MA) HPXL solvent delivery system in conjunction with a Knauer (Berlin) model 87 variable wavelength detector. The reversed-phase column was a 0.46 × 15 cm Rainin C_{18} Microsorb column. Normal-phase chromatography was with a Rainin Microsorb silica column of the same dimensions.

Preparation of Samples. Tissue samples were delipidated by extraction with ethanol and then diethyl ether. With CHO (Chinese hamster ovary) cells about 1% of geranylgeranylcysteine-positive material was extracted by this process. Plant tissues and mycelial mats of *Phycomyces* were homogenized in a blender or Brinkmann Polytron homogenizer. After removal of debris by centrifugation at 1000 × *g* for several minutes, the homogenate was filtered through cheesecloth, and protein was precipitated by trichloroacetic acid. Cytosolic fractions of liver and kidney were prepared by homogenizing the tissue in 5 vol of 0.05 M KH_2PO_4, pH 7, containing 0.25 M sucrose. The homogenate was centrifuged at 27,000 × *g* for 15 min and then 145,000 × *g* for 80 min. The protein in the resulting clear intermediate layer was precipitated with trichloroacetic acid. These precipitated proteins were washed successively with ethanol and diethyl ether. Protein was determined by a modified Lowry procedure (12). CHO cells were grown and labeled with [^{3}H]mevalonate as before (13). Bulk CHO cells were kindly furnished by Genentech.

Conditions for Naphthol Cleavage of Prenylated Proteins. Delipidated CHO cells were used to develop a method for naphthol-dependent cleavage of prenylated proteins, because this material permitted us to evaluate the procedure by recovery of both isotope and mass. The conditions established to be optimal for production of the naphthopyrans utilized a mixture of potassium naphthoxide and naphthol in a ratio of 4:1 as the reagent. Initially, 5 mg of this mixture was added dry to each mg of dry, solvent-extracted, cellular material, typically 10 mg of which was used. Then 50 μl of dimethylformamide was added as solvent for the reaction and the tube was gassed with argon, capped securely, and heated at 100°C for 8–15 hr. The reaction products were extracted into hexane and analyzed by HPLC with a gradient of 10% water in acetonitrile to pure acetonitrile. Later, we found that the yields were doubled if the mixture of naphthol and its

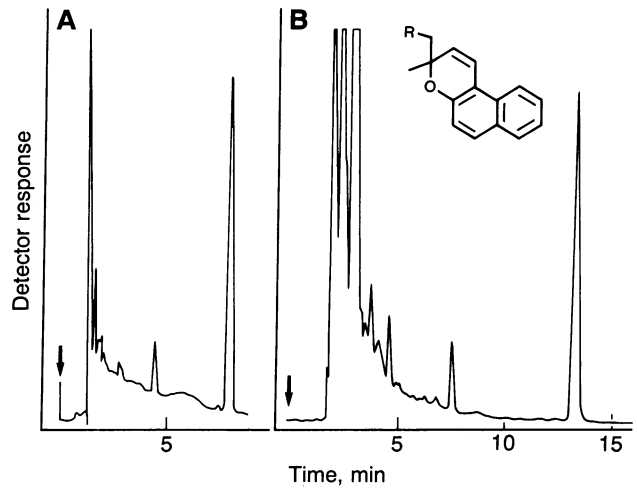

FIG. 1. (*A*) HPLC of naphthol cleavage products from 9.5 mg of CHO protein. The column was eluted with acetonitrile at a flow of 1.5 ml/min. Detection was at 360 nm with a full-scale deflection of 0.01 *A* unit. The arrow indicates sample injection. In this chromatogram the farnesylcysteine derivative is eluted at 4.3 min and the geranylgeranylcysteine derivative at 7.6 min. (*B*) HPLC of naphthol cleavage products from 2.4 mg of CHO protein. The column was eluted with a gradient of 95% acetonitrile/water to acetonitrile over 15 min. Detection was at 246 nm with a full-scale deflection of 0.04 *A* unit. The arrow indicates sample injection. In this chromatogram the farnesylcysteine derivative is eluted at 7.7 min and the geranylgeranylcysteine derivative at 13.2 min. (*Inset*) Structure of methylnaphthopyran. For the farnesyl derivative R is geranyl. For the geranylgeranyl derivative R is farnesyl. For the dimethylnaphthopyran R is CH_3.

potassium salt was added in dioxane (0.5 ml). Most dioxane was then removed with warming under a stream of nitrogen and the remainder, by gentle heating under reduced pressure. Dimethylformamide was then added and the sample was treated as described above.

Quantitation. Two methods were used for quantitation of the reaction. The first entailed the addition of a known amount of farnesylcysteine to one of a pair of samples. The amount of the two prenylcysteines present in the sample was calculated by comparison of the peak areas of the naphthopyrans obtained on elution from the column. These results were verified by a second method, in which the response of the detector was calibrated with synthetic dimethyl- and methylisohexenylnaphthopyran. Isotope recovery of the naphthopyrans from CHO proteins in conjunction with detector response was then used for calculation of prenylcysteine present in the tissue sample. The two methods were in excellent agreement and indicated a recovery of 22–25% when dioxane was used to add the reagents. When the reagents were added dry, the yield was half. While we have no explanation for this variation, it was consistent and there was no significant difference among five samples of CHO proteins analyzed by the two methods of addition of reagent. All samples were in duplicate and the results were within the standard deviation found for CHO proteins.

Table 1. Prenylcysteine composition in tissue culture cells

Cell line	Prenylcysteine, nmol/mg protein	GGCys/FCys*
CHO (hamster)	0.79	6
3T3 (mouse)	0.83	6
HeLa (human)	0.4	4
Neuroblastoma (human)	0.8	8
Fibrosarcoma (mouse)	0.5	5

*Molar ratio of geranylgeranylcysteine to farnesylcysteine.

Table 2. Prenylcysteine composition in mouse tissues

Tissue	Prenylcysteine, nmol/mg protein	GGCys/FCys*
Liver	0.32	6
Supernatant	0.15	4
Kidney	0.75	8
Supernatant	0.23	7
Brain	1.3	10
Lung	0.64	5

*Molar ratio of geranylgeranylcysteine to farnesylcysteine.

RESULTS

Identification of the Product. When CHO proteins, metabolically labeled with [^{3}H]mevalonate, were treated with 2-naphthol under alkaline conditions about 50% of the radioactivity was released as hexane-soluble material. Most of this product comigrated on HPLC with standard material prepared by allowing geranylgeranylcysteine to react with 2-naphthol under similar conditions. Although this compound is not the naphthyl ether anticipated for a simple S_N2 displacement (9), it is likely a 2-prenyl-substituted 2-methylnaphthopyran (Fig. 1 *Inset*).‡

Sensitivity of the Procedure. The experimental procedure was developed with solvent-extracted CHO cellular material. Hexane extracts of the products obtained by treating these proteins with 2-naphthol, when chromatographed on a C_{18} reversed-phase column, gave the elution profiles shown in Fig. 1. The eluate was monitored at 246 and 360 nm, absorption maxima of the product. Fig. 1*A* represents an isocratic elution (360 nm), while Fig. 1*B* is a gradient elution (246 nm). The signal-to-noise ratio found will permit detection of less than 1 pmol of product at 246 nm. In all, 15 different samples of CHO proteins were analyzed, with and without the addition of dioxane. We found 0.79 ± 0.13 nmol of prenyl residue per mg of total cellular protein, which indicates that the method is reproducible. The molar ratio of geranylgeranyl- to farnesylcysteine was 6. The comigration of products as detected by UV absorption and radioactivity has been verified by cochromatography in both reversed-phase and normal-phase systems.

Analysis of Tissues. Samples of other tissue culture cells were examined and found to have a similar content of prenylcysteines. The data are given in Table 1. Table 2 gives the results of the analysis of selected mammalian tissues. The prenyl composition varies significantly from tissue to tissue, as does the ratio of the two different prenyl entities. Sedimentation of membranes by high-speed centrifugation did not remove all of the prenylated proteins, and the cytosolic fraction of both kidney and liver contain substantial quantities of prenylcysteines. The ratio of the two prenylcysteines is the same as is found for whole tissue.

The results from analyses of nonmammalian organisms are shown in Table 3. The prenyl composition is significantly lower and the proportion of the sesquiterpene amino acid is greater. Prenylcysteines were not found in *E. coli*. This determination was on 16 mg of protein and 5 pmol/mg would have been detected.

DISCUSSION

The nucleophilic cleavage of the allylic thiol bond by 2-naphthol provides for qualitative as well as semiquantitative determination of the cysteine-bound prenyl groups of preny-

‡The basic naphthopyran structure is consistent with IR, UV, NMR, and mass spectral data, a full account of which will be published elsewhere. Although some structures are still tentative, the assignments do not compromise the present work.

Table 3. Prenylcysteine composition in other organisms

Organism	Prenylcysteine, nmol/mg protein	GGCys/FCys*
Escherichia coli	<0.005	—
Methanobacterium thermoautotrophicum	0.04	†
Saccharomyces cerevisiae	0.05	3
a factor strain	0.05	0.7
Phycomyces	0.22	3
Peletia fastigiata brown algae	0.06	‡
Spinacia oleracea	0.06	‡
Manduca sexta		
Larvae	0.14	3
Eggs	0.14	4

*Molar ratio of geranylgeranylcysteine to farnesylcysteine.
†This sample had no detectable geranylgeranylcysteine.
‡These samples had only traces of farnesylcysteine.

lated proteins. The method has a number of distinct advantages. The naphthylpyrans that are formed are readily isolated and quantified by HPLC. The tissue to be analyzed does not need to be labeled with mevalonate, and this has made it possible to examine organisms, such as bacteria, fungi, and plants, that do not incorporate this metabolite readily. Also, for metabolic labeling, tissue culture cells are frequently treated with inhibitors of mevalonate synthesis, so the observed product distribution may be skewed by this disturbance of isoprenoid synthesis. Another aspect of this method is that the naphthopyrans produced have a UV absorption spectrum that permits verification of the identity of products. This attribute may well be useful in situations where unusual prenylations could occur. For example, insects synthesize homosesquiterpenoids as hormones and similarly modified prenylcysteines would be characterized by altered retention times as well as by absorption spectra. The extinction coefficients of the naphthopyrans are 4450 and 66,070 $M^{-1} \cdot cm^{-1}$ at 360 and 246 nm, respectively.§ These values are large enough for reasonably sensitive analysis. At 360 nm the maximum sensitivity is about 10 pmol of naphthopyran and at 246 nm the sensitivity is 10-fold greater.

Naphthol cleavage of prenylcysteines has enabled us to examine different tissues for the presence of this modification and to extend the range of organisms in which it is known to occur. Our results clearly indicate that this modification of proteins is very nearly universal. The relative level of modified cysteine is lower in plants and insects than in mammals and lower yet in an archaebacterium. Prenylcysteines were not found in *E. coli* at a sensitivity that would have detected 5 pmol of prenyl residue per mg of protein. Thus, proteins so modified may not occur in all prokaryotic organisms. The tissue distribution of prenylated proteins that we have found parallels the enzymatic activity reported for protein farnesyltransferase as reported by Manne *et al.* (14). They detected this enzyme in all extracts of mammalian tissue tested but failed to find activity in extracts of *E. coli*.

The subcellular distribution of prenylated proteins in the two tissues that were examined indicates that one-third to one-half of these proteins are in the cytosolic pool. This observation is in agreement with that of Maltese and Sheridan (15), who found significant levels of cytosolic prenylated proteins. While one function of prenylation is to direct cytosolic proteins to membranes, it is apparent that this modification does not quantitatively partition proteins into membrane compartments.

Our data permit a quantitative comparison of protein prenylation to the other end products of isoprenoid metabolism normally present. Cholesterol, the most abundant polyprenol of animals, occurs in kidney and liver in the range of 40–90 nmol/mg of protein (16, 17). The concentration of ubiquinone in liver is about 0.3 nmol/mg of protein and that of the dolichols is about half this value (17). The levels of prenylated cysteines are in the range of 0.3–1.5 nmol/mg of protein, indicating that these products of mevalonate metabolism are present at levels quantitatively similar to those of the nonsterol lipid products of this pathway.

Prenylation is a surprisingly frequent modification of proteins. Assuming an average molecular weight for proteins (single polypeptide) of 25,000, there would be 40 nmol of protein in each mg of cellular protein. Our finding of about 0.7 nmol of prenylcysteine per mg of protein indicates that about 2% of all proteins are prenylated. Cysteine is one of the less common amino acids, representing approximately 2 mol % of the amino acids of total cellular proteins. One would then anticipate 1 nmol of carboxyl-terminal cysteine in each mg of cellular protein. A search (18) of the amino acid sequences in the Protein Sequencing Library of the National Biomedical Research Foundation (March 31, 1988) indicated that cysteine occurs randomly at this terminus, demonstrating that this is a reasonable assumption. Our results thus indicate that proteins with a carboxyl-terminal cysteine are likely to be prenylated.

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§These values were obtained from synthetic 2,2-substituted naphthopyrans (Fig. 1 *Inset*, R = methyl and R = isohexenyl) and literature values for the parent 3*H*-naphtho[2,1-*b*]pyran (9), all of which have nearly identical UV spectra.