Identification of *N*-myristoylated proteins by reverse-phase high performance liquid chromatography of an azlactone derivative of *N*-myristoylglycine

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A method is presented for the identification of *N*-myristoylated proteins. *N*-Myristoyl transferases have an absolute requirement for a free *N*-terminal glycine. *N*-Myristoylglycine is released upon mild acid hydrolysis of myristoylated peptides and proteins and its derivitization to a *p*-nitrobenzylazlactone with subsequent analysis by reverse phase h.p.l.c. enabled its detection to pmol levels. This facilitated the identification of *N*-terminal myristate in nmol quantities of purified proteins. Using this method we demonstrate that the α -subunit of the GTP-binding protein G_0 is *N*-terminally myristoylated.

INTRODUCTION

Fatty acylation is a recently discovered and increasingly important post-translational modification which many cellular proteins undergo. Two principal pathways are apparent. The first involves the attachment of palmitic acid via hydroxylamine labile *O*- or thioester linkages to threonine and cysteine residues in substrate proteins which may serve to anchor these proteins in cellular membranes [1]. The second involves the attachment of myristic acid to an *N*-terminal glycine residue (after the removal of the initiating methionine) immediately following transcription of the nascent protein [2]. A number of proteins have been shown to be myristoylated, including the catalytic subunit of cyclic AMP-dependent protein kinase [3], the β -subunit of the calcium-dependent phosphatase calcineurin [4], NADH-cytochrome b_5 reductase [5], the viral and cellular forms of pp60^{src} [6], and a number of viral *gag*-proteins, including those of HTLV-I and II [7]. *N*-Myristoyl transferases have been characterized in yeast cells [8–10] and mammalian tissues [11] and, consistent with the known sequences of identified protein substrates, are shown to have an absolute requirement for an *N*-terminal glycine in order for myristoylation to occur. Metabolic labelling of cultured cells with [³H]myristate followed by one-dimensional gel electrophoresis, indicates that there are between 20 and 30 proteins that incorporate myristate into hydroxylamine stable linkages [12,13]. It is apparent from those proteins already characterized, that myristoylated proteins represent a significant subset of cellular proteins and that efforts should be directed at identifying other members of this group. Methods that have been used for this purpose include immunoprecipitation of labelled protein [7], chemical analysis of the nature of the acyl-protein linkage [14], h.p.l.c. or g.c.-m.s. identification of myristate in acid hydrolysates [15] and fast atom bombardment mass spectrometry of lipophilic peptides isolated from protein digests by reverse phase h.p.l.c. [3]. These techniques are either laborious and not readily available or they fall short of a positive identification of the amide linkage of myristate to the *N*-terminal glycine. We report the development of a technique which enables a rapid identification of the *N*-myristoylglycine released

by mild acid hydrolysis from as little as 2 nmol of purified protein. The method utilizes a modification of Erlenmeyer's azlactone synthesis to produce the *p*-nitrobenzylazlactone derivative of *N*-myristoylglycine (see Fig. 1) which can then be analysed by reverse-phase h.p.l.c. It has recently been reported that the α -subunit of the GTP-binding protein G_0 from bovine brain contains myristic acid covalently bound in hydroxylamine stable linkages [14]. Using this method we have been able to confirm that this protein is indeed *N*-terminally myristoylated.

MATERIALS AND METHODS

N-Myristoylglycine was a generous gift from Dr. V. E. Marquez (Lab. Med. Chemistry, NCI, Bethesda, MD, U.S.A.). Non-acylated and acylated peptides corresponding to the *N*-terminal nine amino acids of the catalytic subunit of bovine cardiac cyclic AMP-dependent protein kinase [3], and myristoyl and non-myristoyl peptides corresponding to the *N*-terminal 15 amino acids of pp60^{src} [17] were custom synthesized and purified by Peptide Technologies Corp. (Washington, DC, U.S.A.). $G_{0\alpha}$, purified from bovine brain essentially as described by Sternweis & Robishaw [18], was obtained from Dr. R. A. Kahn in this laboratory. Bovine cardiac cyclic AMP-dependent protein kinase (catalytic subunit), *N*-acetylglycine, dipeptides and the other proteins used in this study were obtained from Sigma. All other chemical reagents were purchased as ACS grade reagents and used as supplied.

Derivatizations and acid hydrolyses

Synthesis of azlactone derivatives was carried out in 0.5 ml Pierce Reacti-vials (Pierce Chemical Co., Rockford, IL, U.S.A.). In our standard protocol 10 μ mol of anhydrous sodium acetate in methanol was added to a protein or peptide hydrolysate, or to standard tubes together with a known amount of *N*-myristoylglycine (also in methanol). The methanol was removed by evaporation in a stream of dry N_2 and the reaction initiated by the addition of 10 μ mol of *p*-nitrobenzaldehyde in 250 μ l of acetic anhydride. Vials were

heated at 100 °C for 30 min, cooled and then the acetic anhydride evaporated using an N₂ stream. The residue was re-dissolved in a small volume of acetonitrile and vortexed vigorously prior to analysis by h.p.l.c. Protein and peptide hydrolysis was performed in the same vials by heating the appropriate sample at 100 °C in 100 µl of 0.05 M-HCl for 3–6 h. The acid was removed by evaporation as previously and the residue resuspended in 50 µl of water. The vial was vortexed and the water evaporated to remove residual acid. For the analysis of G_{0a}, the protein was precipitated from 50 µl of a 4 mg/ml solution by the addition of 50 µl of 10% (w/v) trichloroacetic acid and the precipitate collected by microcentrifugation, washed once in acetonitrile and then dried in a stream of N₂. The precipitated protein was then resuspended in 100 µl of 0.05 M-HCl and hydrolysed for 3 h at 100 °C.

H.p.l.c. methodology

Separation of the azlactone derivatives of the various acylglycines was performed on a Vydac C-18 protein/peptide reverse-phase column (250 mm × 4.6 mm; 5 µm particle size, Separations Group, Hesperia, CA, U.S.A.) equipped with a CO3-032 reverse-phase pre-column (30 mm × 1.5 mm; Brownlee Labs., Santa Clara, CA, U.S.A.). The h.p.l.c. system consisted of an Altex model 420 gradient programmer with two model 110A pumps, a Waters WISP automated sample injector and M420 data module, and a Beckman M165 variable λ u.v. detector. Separation was achieved by using a stepped gradient from 70% water/acetonitrile to 100% acetonitrile as detailed in the legend to Fig. 3.

Hydrolysis of *N*-acylglycines and dipeptides

The relative stability of amide bonds in *N*-acylpeptides and dipeptides was examined by hydrolysing 1 mM solutions of *N*-acetylglycine, *N*-myristoylglycine, glycylglycine, glycylalanine, glycylserine and glycylasparagine in 0.05 M-HCl at 100 °C. 50 µl aliquots were removed periodically and free amine groups derivatized with 20 µl of *o*-phthaldialdehyde reagent solution (Sigma) for 2 min at room temperature. Reactions were quenched with 1 ml of 0.1 M-sodium acetate buffer (pH 7.0) and analysed by u.v. spectroscopy at 340 nm using a Hewlett Packard 8450A diode array spectrophotometer.

RESULTS

Preparation of azlactone derivatives of acylglycine

Erlenmeyer first described the synthesis of *N*-azlactones (anhydrides of *N*-acylamino acids) in 1893 [16]. Benzaldehyde was condensed with *N*-acetylglycine in the presence of acetic anhydride and anhydrous sodium acetate, the reaction apparently proceeding via an initial cyclization of the *N*-acetylglycine, followed by a Perkin type condensation reaction (see Fig. 1. [19]). Using this basic mechanism we were able to prepare azlactone derivatives of *N*-acetyl- and *N*-myristoylglycine by reaction with *p*-nitrobenzaldehyde. Confirmation of the expected products was achieved using solid probe m.s. (*p*-nitrobenzylazlactone of *N*-acetylglycine, 4-(*p*-nitrobenzylidene)-2-methyloxazol-5-one, *m/z* = 232, and of *N*-myristoylglycine, 4-(*p*-nitrobenzylidene)-2-tridecanoyloxazol-5-one, *m/z* = 400). The u.v. spectrum of the products revealed the expected strong chromophore with an absorbance maximum at 350 nm, thus

Fig. 1. Erlenmeyer's azlactone reaction

Initial cyclization of the acylamino acid is followed by a Perkin type condensation with an aromatic aldehyde to generate the azlactone. For X = H; R = CH₃, Erlenmeyer's original synthesis of 4-benzylidene-2-methyloxazol-5-one. For X = NO₂; R = (CH₂)₁₂CH₃, our synthesis of 4-(*p*-nitrobenzylidene)-2-tridecanoyloxazol-5-one.

Fig. 2. Standard curve for the analysis of *N*-myristoylglycine as the 4-(*p*-nitrobenzylidene)-2-tridecanoyloxazol-5-one derivative

facilitating detection of the product to low levels and offering the opportunity to characterize the *N*-acylglycine released by the hydrolysis of *N*-acylated proteins. Reaction conditions were optimized with respect to temperature and time to give those described in Materials and methods. The *p*-nitrobenzylazlactone of *N*-myristoylglycine was separated from the other reactants using reverse-phase h.p.l.c. with u.v. detection at 350 nm (see Fig. 3). Fig. 2 shows a plot of peak area versus pmol equivalents of *N*-myristoylglycine following derivatization and shows the good linearity of the reaction even at these low levels of *N*-myristoylglycine (linear correlation coefficient = 0.995). The detection limits for the analysis of *N*-myristoylglycine in our system were

Table 1. Hydrolysis of *N*-acylglycines and dipeptides

Results are expressed as relative rates versus those obtained for Gly-Gly (= 1). Our results are compared with published figures [21] obtained using higher concentrations of acid.

Dipeptide or acyl peptide	Relative rates (Gly-Gly = 1)	
	100 °C, 0.05 M-HCl	95 °C, 2 M-HCl [21]
<i>N</i> -Myristoylglycine	0.099	—
<i>N</i> -Acetylglycine	0.305	—
Glycylglycine	1.000	1.00
Glycylalanine	0.441	0.40
Glycylserine	1.106	1.83
Glycylasparagine	0.932	1.94
Glycylleucine	—	0.34

determined to be between 5 and 10 pmol equivalents of *N*-myristoylglycine. Stability of the derivatized product was evaluated by repeated analysis of crude reaction mixes following evaporation of the acetic anhydride and reconstitution in acetonitrile. Samples were stored at room temperature in the light and assayed periodically over a 6 day period. The half-life for the product under these conditions was approximately 217 h ($n = 6$).

Determination of *N*-myristoylglycine in *N*-myristoylated peptides and proteins

Towler & Glaser have reported that *N*-myristoylglycine is released upon mild acid hydrolysis of *N*-myristoylated peptides and proteins [20]. Preferential stability of the amide bond between myristate and glycine should be favoured over that between glycine and adjacent amino acids by the 14-carbon alkyl chain, such that sufficient *N*-acylglycine will be released to enable its detection by our method. Table 1 summarizes the results of our hydrolysis of *N*-acylglycines and dipeptides and shows that the *N*-myristoylglycine bond exhibits significantly enhanced stability under our conditions. We further examined this using *N*-myristoylated peptides corresponding to the *N*-terminal nine amino acids of the catalytic subunit of cyclic AMP-dependent protein kinase and to the *N*-terminal 15 amino acids of pp60^{src}. 1 mM solutions of peptide were hydrolysed in 0.05 M-HCl at 100 °C and sampled over a 6 h period by removing 50 µl samples and derivatizing as described in Materials and methods. H.p.l.c. analysis indicated that for both peptides the *N*-myristoylglycine released increased in an approximately linear fashion over this time course. At the 6 h time point, 1.054 nmol of *N*-myristoylglycine had been released/5 nmol of starting cyclic AMP-dependent kinase peptide (21.1% on a mol/mol basis; $n = 2$) and 0.501 nmol/5 nmol of starting pp60^{src} peptide (10.0%; $n = 2$). Thus, it was evident that, whilst release of *N*-myristoylglycine would be neither quantitative nor consistent from peptide to peptide, significant enough quantities of *N*-myristoylglycine would be produced to facilitate a qualitative characterization of *N*-myristoylated proteins. To test this we hydrolysed approx. 50 µg of bovine cardiac cyclic AMP-dependent protein kinase catalytic subunit for 3 h in 0.05 M-HCl and derivatized the resulting hydrolysate. We

R =

- acetyl (1)
- decanoyl (2)
- lauroyl (3)
- myristoyl (4)
- palmitoyl (5)

Fig. 3. H.p.l.c. separation of reaction products following hydrolysis and derivatization of *N*-acyl peptides

Peptides were hydrolysed for 3 h, derivatized and analysed. The chromatogram was obtained from the injection of 2.45 nmol of acetyl-, 1.76 nmol of decanoyl-, 1.74 nmol of lauroyl-, 2.58 nmol of myristoyl- and 14.8 nmol of palmitoyl-peptide hydrolysate. The C-18 column was eluted for 4 min with 30% acetonitrile in water followed by a linear gradient to 100% acetonitrile over 5 min and then a second isocratic elution at 100% acetonitrile. The flow rate was 1.5 ml/min throughout.

were able to detect the *p*-nitrobenzylazlactone of the *N*-myristoylglycine release from the 1 nmol of starting protein.

Specificity of the method

The method is specific for *N*-acylglycine adducts since the presence of β -side chains would preclude other *N*-acylamino acids from reacting. In order to examine the possibility of interference from other *N*-acyl analogues of glycine, we hydrolysed and derivatized *N*-acetyl, *N*-decanoyl, *N*-lauroyl and *N*-palmitoyl peptide analogues

Fig. 4. H.p.l.c. analysis of the acid hydrolysate of $G_{0\alpha}$

The chromatogram was obtained from the injection of 2.5 nmol of $G_{0\alpha}$ acid hydrolysate following 3 h of hydrolysis. The position of the 4-(*p*-nitrobenzylidene)-2-tridecanoyloxazol-5-one peak is marked by the arrow.

of our myristoylated cAMP-dependent kinase peptide. Fig. 3 shows that the azlactones formed from all these *N*-acylglycines are readily separated by our h.p.l.c. method. Hydrolysis and derivatization of non-myristoyl peptides, bovine serum albumin, yeast and horse liver alcohol dehydrogenase, soybean trypsin inhibitor and twice crystallized trypsin produced no interfering peaks. Finally, we used free myristic and palmitic acids in our assays and saw no interference from possible *p*-nitrobenzyl esters of these acids.

N-myristoylation of bovine brain $G_{0\alpha}$

We decided to examine bovine $G_{0\alpha}$, which has been reported to contain hydroxylamine stable covalently linked myristic acid, to see if it is present as *N*-myristoylglycine. Fig. 4 shows the chromatogram obtained from the analysis of the derivatized hydrolysate. The *p*-nitrobenzylazlactone of *N*-myristoylglycine is clearly evident, confirming that this protein is indeed *N*-terminally myristoylated. The peak area corresponds to approx. 45 pmol of myristoylglycine/nmol of hydrolysed protein.

DISCUSSION

The discovery of fatty acylation as a post-translational modification of cellular proteins has led to a great deal of research interest in this area. As part of our work in this

field, we set out to develop methodology that would facilitate the identification of *N*-myristoylated proteins from small amounts of purified starting material. Studies on mammalian and yeast *N*-myristoyl transferases and the characterization of known myristoylated proteins have revealed an absolute requirement for an *N*-terminal glycine. Identification of *N*-myristoylglycine in hydrolysates therefore represents the most specific means of determining whether an *N*-blocked protein is myristoylated. By derivatizing the *N*-acylglycine released upon acid hydrolysis to give the *p*-nitrobenzylazlactone derivative, we have been able to detect pmol levels of this *N*-terminal group and to characterize the nature of the acyl moiety. Whilst purified *N*-myristoyl transferases exhibit high specificity for acyl chain length, it has been reported that BC₃H1 cells incorporate palmitate into amide linkages [20] and *N*-acetyl is a common *N*-terminal blocking group. Should either of these acyl groups be amide linked to an *N*-terminal glycine, this method would enable the differentiation of these from *N*-myristoylated groups. We believe this procedure greatly improves our ability to identify *N*-myristoylated proteins and are currently employing it to study several proteins where either nucleotide sequence or other biochemical data suggest that the protein may possess this interesting modification. It has also been reported that *N*-myristoylglycine can be released from the Pronase digestion of myristoylated proteins in unfixed SDS/polyacrylamide gel slices [8]. We attempted this by running a Pronase-E digestion of $G_{0\alpha}$ in an SDS/polyacrylamide gel as described and partitioning the acidified hydrolysate with ethyl acetate. Derivatization of the dried organic phase resulted in the detection of small amounts of the *p*-nitrobenzylazlactone of *N*-myristoylglycine. Optimization of this procedure will further enhance the usefulness of our method.

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