A simplified assay for the enzyme responsible for the attachment of myristic acid to the *N*-terminal glycine residue of proteins, myristoyl-CoA:glycylpeptide *N*-myristoyltransferase

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A greatly simplified assay for myristoyl-CoA:glycylpeptide *N*-myristoyltransferase (NMT) activity is described. The assay is based on the differential solubility of the acyl-peptides produced as a consequence of the NMT activity and yields results comparable with those obtained with the original assay described by Towler & Glaser [(1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 2812–2816], which requires h.p.l.c. to determine the production of the acyl-peptides. The use of the revised assay in the preliminary steps of the purification of rat brain NMT is described, and its use in determining the fatty acid-specificity of the enzyme is illustrated. The results are shown to be comparable with those obtained with the h.p.l.c.-based assay.

INTRODUCTION

The covalent modification of proteins by the post-translational addition of lipid is a recently described process [for reviews see Magee & Schlesinger (1982), Schmidt (1983), Sefton & Buss (1987) and Towler *et al.* (1988)] that appears to be a widespread phenomenon occurring in a diverse range of organisms. Interest in this modification of cell proteins has been heightened by the finding that it is important for the function of the protein products p60^{src} and p21^{ras} of two oncogenes. Indeed, mutants of these proteins that are unable to undergo this modification are unable to express their full transforming potential (Willumsen *et al.*, 1984; Pellman *et al.*, 1985; Kamps *et al.*, 1985). The importance of the attachment of fatty acid to protein function has also been shown in the studies on the retroviral gag polyproteins and their respective gag-onc fusion proteins. Thus mouse leukaemia virus p65^{gag} precursor requires myristoylation for the formation of retroviral particles (Rein *et al.*, 1986; Jorgensen *et al.*, 1988), and the Mason–Pfizer monkey virus gag-derived polyprotein requires myristoylation for the production of mature infectious viral particles (Rhee & Hunter, 1987).

Given the importance of the attached fatty acid for the correct functioning of p21^{ras}, p60^{src} and the viral proteins described above, there is a clear case for the purification and characterization of the enzymes responsible for the attachment of fatty acids to cell proteins. Two assays for the myristoylating enzyme have been described (Towler & Glaser, 1986; Glover *et al.*, 1988), both of which depend on the use of synthetic peptides to act as substrates for NMT and require the separation of the reaction products by h.p.l.c. Using the former assay we have been able to show that the rat brain is a rich source of an NMT activity, capable of attaching myristic acid specifically to the *N*-terminal glycine residue of synthetic

peptides (McIlhinney & McGlone, 1989). However, the need for h.p.l.c. makes the assay cumbersome and ill-suited for monitoring the purification of the enzyme. Therefore we have developed the assay further by making use of the differential solubility of the acylated peptides to obviate the necessity for the h.p.l.c. step. In the present paper we describe the assay, illustrate its use and compare the data with the results obtained with the conventional h.p.l.c.-based assay.

MATERIALS AND METHODS

Materials

[³H]Myristic acid (22.2 Ci/mmol), [³H]palmitic acid (30 Ci/mmol) and [³H]glycine were purchased from Amersham International. Myristoyl chloride and palmitoyl chloride were obtained from Aldrich Chemical Co. Myristic acid, palmitic acid, CoA (lithium salt), fatty acyl-CoA synthetase and ATP were all purchased from Sigma Chemical Co. All other chemicals were obtained from FSA Ltd., and were of the highest quality available. Reverse-phase f.p.l.c. columns (PEP RPC HR 5/5) were obtained from LKB Pharmacia. H.p.l.c.-grade acetonitrile and trifluoroacetic acid were purchased from Rathburn Chemicals. [³H]Stearic acid was produced by Amersham International as a customer service, by reduction of vaccenic acid with ³H₂ gas. This was subsequently purified by h.p.l.c. to give a single peak of radioactivity that co-migrated with an authentic stearic acid standard on two different chromatographic systems. The specific radioactivity was determined to be 30 Ci/mmol. The synthetic peptides used as substrates were derived from the *N*-terminal sequences of cyclic AMP-dependent protein kinase (GNAAAARR, cAMPDK peptide; Towler & Glaser, 1986) and p60^{src} (GSSKSKPKDPSQRRRY; Glover *et al.*, 1988). The

Abbreviations used: NMT, myristoyl-CoA:glycylpeptide *N*-myristoyltransferase; cAMPDK peptide, peptide representing the *N*-terminal sequence of cyclic AMP-dependent protein kinase.

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former was synthesized in the laboratory of Dr. M. Waterfield, Ludwig Institute for Cancer Research, London Middlesex Hospital University College Branch, and the latter by Cambridge Biotechnology Co. Both were exhaustively purified to homogeneity by reverse-phase h.p.l.c., and their purity was checked by amino acid analysis. Myristoyl-GNAAAARR and myristoylglycine were synthesized by reaction of the peptide with myristic anhydride or of [^3H]glycine with myristoyl chloride as described by Towler & Glaser (1986).

Tissue preparation

Male Wistar rats were stunned, decapitated, and the brains were removed and quickly placed in ice-cold 10 mM-Tris/HCl buffer, pH 7.4, containing 0.32 M-sucrose. All subsequent procedures were performed at 4 °C. The brains were washed free of Tris/sucrose and homogenized in a Dounce homogenizer, with 5 vol. of cold extraction buffer [20 mM-Tris/HCl buffer, pH 7.4, containing 1 mM-EDTA, 1 mM-dithiothreitol, pepstatin (8 µg/ml), leupeptin (20 µg/ml) and aprotinin (20 µg/ml)]. For $(\text{NH}_4)_2\text{SO}_4$ fractionation homogenates were centrifuged at 100 000 g_{av} for 1 h at 4 °C and $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant to give 35 % saturation. After standing on ice for 30 min, the precipitated proteins were removed by centrifugation at 30 000 g for 30 min in a Servall SS-34 rotor. $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant to give a final concentration of 65 % saturation and after a further 30 min on ice the centrifugation step repeated. The protein pellets were dissolved in a minimum volume of extraction buffer without the peptide inhibitors, and dialysed overnight at 4 °C against 2 litres of this buffer. Samples of the different fractions were either assayed fresh or stored at -70 °C until needed. Storage of samples at -70 °C was found to result in a low but perceptible loss in activity over a period of 2 weeks, and for this reason samples were never frozen and thawed more than once, and all comparative assays were performed on samples prepared at the same time.

Chromatography

Reverse-phase chromatography was performed on a PEP RPC HR 5/5 column with the Pharmacia LKB f.p.l.c. apparatus. The eluents were double-distilled de-ionized water containing 0.1 % (v/v) trifluoroacetic acid and acetonitrile containing 0.1 % (v/v) trifluoroacetic acid. For routine separation of the acylated peptides a gradient from 30 % (v/v) acetonitrile to 61.2 % (v/v) acetonitrile was run over 16 min, followed by a column wash with 100 % acetonitrile of 5 ml and a 5 ml re-equilibration of the column with 30 % acetonitrile. These conditions had earlier been shown to resolve the acylated peptides from other reaction products (McIlhinney & McGlone, 1989). Routinely 1 min fractions were collected and subjected to scintillation counting of radioactivity. Where conditions other than these were used they are described in the appropriate Figure legend.

N-Myristoylation assay

The assay was performed in microcentrifuge tubes (1.5 ml) with the use of a myristoyl-CoA-generating system essentially as described by Towler & Glaser (1986). Portions (50 µl) of this, containing 2.5 µCi of [^3H]myristate, were added to the reaction vials, and appropriate amounts of brain homogenates or fractions

thereof were added to give a standard volume of 100 µl. Fractions normally contained 100 µg of protein, and where necessary the volume of the added enzyme fraction was adjusted to 100 µl by the addition of extraction buffer. The reaction was initiated by the addition of the appropriate peptide (10 µl; 45 µg), continued for 20 min at 30 °C and terminated by the addition of 11 µl of 0.1 M-NaOH. Control incubations were performed by carrying out the reaction in the absence of the substrate peptide. After 30 min at room temperature the incubation mixture was acidified by the addition of 30 µl of 1 M-HCl and extracted three times with n-heptane. Proteins were precipitated by the addition of 140 µl of methanol containing 10 % (w/v) trichloroacetic acid to the remaining lower phase and standing on ice for 30 min. The precipitated proteins were removed by centrifugation (10 000 g for 15 min) and the radioactivity remaining in samples of the supernatant was determined. Subtraction of the radioactivity remaining in the supernatant of the incubations without substrate from that found in the reaction mixtures that contained the peptide substrate yielded the specific incorporation of fatty acid into the acyl-peptide, and this was confirmed by reverse-phase f.p.l.c. as described above.

Endogenous substrate incubations

The brain cytosol contained an active acyl-CoA synthetase that was capable of supporting maximum levels of NMT activity (McIlhinney & McGlone, 1989). Endogenous substrates were therefore studied by incubating 400 µg of brain cytosol with 10 µCi of [^3H]myristic or [^3H]palmitic acid, 5 mM-ATP and 1 mM-CoA (lithium salt) in a final volume of 400 µl of extraction buffer. For control incubations the ATP and CoA were omitted. The incubation was performed for 1 h at 30 °C, following which 200 µl of the reaction was treated with 1 ml of cold acetone to precipitate the proteins. The proteins, precipitated by standing on ice for 30 min, were recovered by centrifugation at 10 000 g in a micro-centrifuge for 15 min at 4 °C. They were then dissolved in SDS sample buffer and analysed by SDS/polyacrylamide-gel electrophoresis.

SDS/polyacrylamide-gel electrophoresis

Proteins were separated by electrophoresis on SDS/10 %-polyacrylamide gels and the gels subjected to fluorography as previously described (McIlhinney *et al.*, 1985).

Protein assays

Protein was determined by using the Bio-Rad dye-binding assay, with bovine serum albumin as the standard.

RESULTS

After the incubation for the NMT assay, the reaction vial contains three possible radioactive compounds, myristic acid, myristoyl-CoA and acyl-peptide. It is to separate these three products that h.p.l.c. is performed in the Towler & Glaser (1986) assay. However, we reasoned that it should be possible to separate the acyl-peptide from the other compounds by means of differential partition in organic solvents. The difficulty lay in the hydrophilic nature of the myristoyl-CoA present in the

Fig. 1. Rate of hydrolysis of myristoyl-CoA

Myristoyl-CoA was prepared as described in the Materials and methods section. Portions were then made alkaline by the addition of 0.1 M-NaOH, and incubated for the times shown, after which they were acidified with 1 M-HCl and extracted three times with hexane. The radioactivity remaining in the lower phase, which contained the unhydrolysed acyl-CoA, was then determined. The results are expressed as the percentage of the radioactivity present in the original unhydrolysed acyl-CoA, and all points represent the means of duplicate determinations.

Fig. 2. SDS/polyacrylamide-gel electrophoresis of the endogenously fatty acid-labelled proteins in rat brain cytosol

The rat brain cytosol was incubated with either [3 H]palmitic acid (tracks a and b) or [3 H]myristic acid (tracks c and d) in the presence (tracks a and c) or in the absence (tracks b and d) of ATP and CoA as described in the Materials and methods section. After the incubation the acylated proteins were analysed by electrophoresis on a SDS/10% polyacrylamide gel and the gel was processed for fluorography. The molecular-mass markers were α_2 -macroglobulin (180 kDa), β -galactosidase (116 kDa), fructose-6-phosphate kinase (84 kDa), pyruvate kinase (58 kDa), fumarase (48.5 kDa), lactate dehydrogenase (36.5 kDa) and triose-phosphate isomerase (26.6 kDa).

reaction, since this was known to remain in the aqueous phase after hexane extraction. Indeed, this property forms the basis of an assay for fatty acyl-CoA synthesis (Hosaka *et al.*, 1981; Towler & Glaser, 1986). One way to overcome this would be to convert the acyl-CoA into free fatty acid and then perform the extraction, which would then leave only the radioactive acyl-peptide in the aqueous phase. To establish the optimal conditions for the hydrolysis myristoyl-CoA was generated by using an acyl-CoA-generating system as described in the Materials and methods section. Portions of the reaction mixture were then subjected to alkaline hydrolysis by the addition of 0.1 vol. of 0.1 M-NaOH for the time periods illustrated in Fig. 1. After the hydrolysis the reaction mixture was acidified by the addition of 0.33 vol. of 1.0 M-HCl and extracted five times with hexane. The amount of radioactivity remaining in the aqueous phase was a measure of the remaining fatty acyl-CoA. The results shown in Fig. 1 indicate that the hydrolysis was complete within 15 min, and in all further experiments we have used a 30 min hydrolysis time to ensure the total conversion of fatty acyl-CoAs into free fatty acids.

However, when we applied this methodology to the assay of NMT we found that, despite the complete conversion of myristoyl-CoA into free myristic acid, large amounts of radioactivity remained in the aqueous phase after the hexane extraction. On investigation this turned out to be due to the presence of endogenous substrates for the fatty acyl-CoA, which were present in the rat brain cytosol used as a source of the enzyme. These could be readily demonstrated by SDS/polyacrylamide-gel electrophoresis of an incubation of the brain cytosol with a source of radioactive fatty acid, ATP and CoA (Fig. 2). It may be noted that these endogenous acceptors of fatty acid are more heavily labelled with

Table 1. Preliminary steps in the purification of rat brain NMT

The tissues and tissue fractions were prepared as described in the Materials and methods section. The assay was performed with 100 μ g of protein and 40 μ g of the *src*-derived peptide/assay, and the values shown were calculated from the radioactivity remaining in the aqueous phase after the hexane extractions. They represent the means of duplicate determinations, and are corrected for radioactivity found in the control incubations, from which peptide was omitted, run in parallel. This represented 10% of the radioactivity found in the aqueous phase in the presence of the peptide substrate. After this the radioactivity present as acyl-peptide was determined by reverse-phase h.p.l.c. and the results calculated from these are shown in parentheses in the Table.

Sample	NMT specific activity (pmol/min per mg)	Total NMT activity (pmol/min)
Homogenate	0.48 (0.5)	653.8
Cytosol	4.3 (4.9)	2152.1
0–35% -satn.-(NH ₄) ₂ SO ₄ fraction	1.2 (1.2)	73.8
35–65% -satn.-(NH ₄) ₂ SO ₄ fraction	10.0 (11.1)	1871.0
65% + -satn.-(NH ₄) ₂ SO ₄ fraction	0.39 (0.33)	31.4

Fig. 3. Time course for the incorporation of myristic acid into the *src*-derived peptide (● and ○) and the cAMPDK peptide (■ and □), with the 35–65%-saturation-(NH₄)₂SO₄ fraction from rat brain

The NMT assay was performed as described in the Materials and methods section with both peptides at 45 µg/assay tube. At the times shown the amount of radioactive fatty acid remaining in the aqueous layer after hexane extraction (● and ■; corrected for the control values obtained with peptide omitted) was determined. Then samples of this were analysed for the presence of acyl-peptide by reverse-phase h.p.l.c. The amount of radioactive peptide was determined from the known elution position of synthetic acylated peptide standards, and is plotted as the ○ and □ symbols.

palmitate than with myristate, with the exception of one band that appears to be specifically labelled by myristate. The stimulatory effect of ATP and CoA on protein acylation is apparent on comparison of tracks a and c with tracks b and d in Fig. 2. The presence of these proteins meant that it was necessary to remove them, and this was achieved by precipitation with methanol containing trichloroacetic acid followed by centrifugation. After this treatment the background radioactivity in the control tubes from which peptide was omitted fell to 10% or less of those in the tubes containing peptide substrate. The final optimized assay procedure is described in the Materials and methods section.

To determine whether the simplified assay method yields results comparable with those obtained in the original assay, it was used to monitor the preliminary steps in the purification of rat brain NMT. The results obtained were compared with those derived from the conventional assay. The conditions used for the assay were those previously shown to be optimal for rat brain NMT (McIlhinney & McGlone, 1989): a 20 min incubation period, 100 µg of test protein and a substrate concentration of 40 µg/assay tube. The results, obtained with the *src*-derived peptide, showed that the two assays

Table 2. Specificity of NMT from rat brain for fatty acid chain length

With the use of the 35–65%-saturation-(NH₄)₂SO₄ fraction of rat brain NMT (100 µg/assay), the two peptide substrates (40 µg/assay) and a 20 min incubation, the ability of the enzyme to use the three fatty acids listed as substrates was examined. All points represent the means of duplicate determinations, and have been corrected for the radioactivity found in the controls from which peptide was omitted. The values in parentheses show the results obtained by using reverse-phase h.p.l.c. to determine the acyl-peptide yield.

Substrate	Fatty acid	Radio-activity incorporated (d.p.m.)	Incorporation (% of myristate value)
<i>src</i> -derived peptide	Myristate	142 548	100 (117)
	Palmitate	17 462	14.4 (12.0)
	Stearate	1 987	1.6 (1.7)
cAMPDK peptide	Myristate	69 599	100 (90)
	Palmitate	5 840	8.3 (5.2)
	Stearate	0	0 (0)

yielded very similar results (Table 1), with both showing the quantitative recovery of the enzyme after a 35–65%-saturation-(NH₄)₂SO₄ fractionation of the brain cytosol, a similar increase in the specific activity of the enzyme, and an apparent increase in enzyme yield after removal of the brain membranes. This last-mentioned observation probably reflects the removal of inhibitors of the enzyme by the centrifugation.

Further evidence that the two assays yield similar results is shown in Fig. 3, in which the time course of incorporation of myristate into both the *src*-derived peptide and the cAMPDK-derived peptide are shown for the 35–65%-saturation-(NH₄)₂SO₄ enzyme fraction. As can be seen the incorporation rate is linear for both peptides over the 20 min time period shown at a protein concentration of 100 µg/assay tube, and the *src*-derived peptide is a better substrate for the enzyme than the cAMPDK peptide.

Having optimized these linear incorporation conditions for the 35–65%-saturation-(NH₄)₂SO₄ fraction we have used this NMT preparation to determine the fatty acid-specificity of rat brain NMT, using myristic acid, palmitic acid and stearic acid. Each of these is converted into the appropriate acyl-CoA by the acyl-CoA synthetase used in these experiments with similar efficiencies. With either peptide substrate myristic acid was clearly the preferred fatty acid with a rapid decrease in the incorporation of fatty acid with increasing chain length (Table 2). Again the incorporation of fatty acid was greatest with the *src*-derived peptide, and with the use of this in three separate experiments the relative rate of incorporation of palmitate was $15.1 \pm 0.6\%$ and that of stearate was $2.3 \pm 0.5\%$ of that of myristic acid. The results obtained with the assay described here were in good agreement with those obtained with the column assay, as indicated by the values in parentheses in Table 2.

DISCUSSION

In this paper a simplified version of the NMT assay devised by Towler & Glaser (1986) is described. The assay, which obviates the need for a time-consuming chromatographic step in the identification of the acyl-peptide product, is simple to perform, has proved extremely robust over some months of use, and yields results that are very comparable with those obtained with the conventional assay. The other assay described for measuring NMT activity is based on the use of iodinated *src*-derived peptide and measures the acylation of this in the presence of non-radioactive fatty acyl-CoAs (Glover *et al.*, 1988). This assay system is not suitable for modification as described here, but we have also shown that the *src*-derived peptide is a better substrate for the rat brain NMT than the cAMPDK peptide.

The assay was used to monitor the preliminary steps in the purification of rat brain NMT. An $(\text{NH}_4)_2\text{SO}_4$ fraction was obtained that contains most of the enzyme, and is enriched in specific activity some 10-fold over the original homogenate. The observation that the homogenate particulate fraction appeared to contain an inhibitory activity has been noted by us previously, and we have been able to restore this activity to the cytosol by adding back purified, washed rat synaptosomal membranes (McIlhinney & McGlone, 1989). The cause of this inhibition is not known, but it could be due to the presence of peptidases or CoA-transferases/acyl-CoA hydrolases in the membrane fraction. Similar inhibitory activities were described in reports of the purification of yeast NMT and rat liver NMT (Towler *et al.*, 1987a, 1988).

The fatty acid-specificity of NMT described here, with myristic acid being the preferred substrate, is in good agreement with the data obtained for the partially purified yeast enzyme (Towler *et al.* 1987b) and the rat brain enzyme (Glover *et al.*, 1988). Thus Towler *et al.* (1987b), using the cAMPDK-peptide and ^{14}C -labelled fatty acids, found that palmitate was barely incorporated into the peptide. Our observation that with this peptide the incorporation of palmitate was 8% that of myristate may simply reflect the use of much-higher-specific-radioactivity ^3H -labelled fatty acid, which would increase the sensitivity of the assay, or alternatively the use of a different source of the enzyme. Glover *et al.* (1988), using the *src*-derived peptide and a crude cytosol extract of rat brain, reported that palmitate was incorporated into the peptide at a rate 4.9% that of myristate and that there appeared to be no incorporation of stearate. The values reported here with the *src*-peptide are somewhat greater for palmitate (15%) and stearate (2.3%). However, we have been using a partially purified enzyme with an enhanced specific activity, which could explain these

minor differences. All the reports have noted that an increase in fatty acid chain length has profound effects upon the incorporation of the fatty acid into the peptide.

In conclusion, a simple assay for the NMT enzyme has been described, which yields results comparable with those with the previously described assays. The assay has been performed with both of the two commonly used peptide substrates, and, since these differ widely in length and amino acid sequence, it is probable that it could be used with many other potential peptide substrates. The availability of a rapid method for the determination of the activity of this novel enzyme should greatly facilitate its purification as well as allowing the activities of the enzyme to be easily measured in a wide range of biological systems and experimental situations.

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