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Biochemistry

Protein fatty acid acylation: Enzymatic synthesis of an *N*-myristoylglycyl peptide

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ABSTRACT **Incubation of *Saccharomyces cerevisiae* strain JR153 with either [^{3}H]myristate or [^{3}H]palmitate demonstrates the synthesis of proteins that contain covalently bound fatty acids. A unique set of proteins is labeled by each fatty acid. Detailed analysis of a 20-kDa protein labeled with myristic acid demonstrates that myristate is linked to the amino-terminal glycine. We describe an enzymatic activity in yeast that will transfer myristic acid to the amino terminus of the octapeptide Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg, whose sequence was derived from a known *N*-myristoylated acyl protein, the catalytic subunit of cAMP-dependent protein kinase of bovine cardiac muscle. The acylation reaction is dependent on ATP and CoA, is enriched in a crude membrane fraction, and will use myristate but not palmitate as the acyl donor. Specificity of the glycyl peptide substrate is demonstrated by the observation that other glycyl peptides do not competitively inhibit myristoylation of Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg.**

Protein fatty-acid acylation in eukaryotes is a well-established modification of specific cellular and viral proteins (1–15). However, the function of protein acylation is only beginning to be understood. Acylation of viral glycoproteins may be important for vesicular stomatitis and sindbis virus particle formation (16). Acylation of the Rous sarcoma virus pp60^{src} is essential for association of this protein with the plasma membrane and for expression of this protein's cell-transforming potential (17–19). Pellman *et al.* (20) demonstrated that a short peptide sequence from the amino terminus of pp60^{src} can direct acylation and membrane localization when fused to heterologous proteins. However, some acyl proteins appear to be soluble (14), suggesting that protein acylation may serve functions other than facilitating protein association with membranes.

The attachment of fatty acid to proteins has been broadly divided into two categories based on the differences in acylation observed when labeling cultured cells *in vivo* with radioactive palmitate or myristate (14, 21, 22). In general, myristate becomes covalently bound to soluble and membrane proteins via amide linkage to an amino-terminal glycine (3–7, 23) early in the protein biosynthetic pathway. Palmitate, on the other hand, is linked to membrane proteins via ester or thioester linkage posttranslationally, probably in the Golgi apparatus (24, 25).

Several systems have been developed for investigating *in vitro* the acylation of proteins by ester or thioester linkage (26–29). In all cases, the enzymatic activity is membrane-associated, and little specificity for fatty-acid chain length has been demonstrated. In contrast, the enzymatic activity(s) responsible for myristoylation of proteins at NH_2-terminal glycine residues has not been identified. We report here that *Saccharomyces cerevisiae* contains proteins with NH_2-terminal myristoylglycine residues. We describe an enzymatic activity that specifically transfers myristic acid to the amino-terminal glycine residue of the synthetic peptide Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg.

MATERIALS AND METHODS

S. cerevisiae strain JR153 (ref. 30; *MATα, trp1, prb1, prc1, pep4-3*) was the kind gift of Peter Burgers of this department. The peptide Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg was synthesized by Joseph Leykam (Washington University, St. Louis, MO). The peptide Gly-Ser-Ser-Lys-Ser-Pro-Lys-Asp-Pro-Ser was a kind gift of S. Adams and S. Eubanks (Monsanto, St. Louis, MO). Peptides were >95% pure as determined by reverse-phase HPLC on a Whatman μBondapak C_{18} column. Sequences were verified by automated Edman degradation. Pronase E and *Pseudomonas* acyl CoA synthetase were purchased from Sigma. [9,10-^{3}H]Palmitic acid and [9,10-^{3}H]myristic acid were purchased from New England Nuclear.

Labeling and Extraction of Yeast Protein for Electrophoretic Analysis. Yeast was grown to an OD_{660nm} of 1–3 in a rotary shaker at 30°C in YPD medium (1% yeast extract/2% Bactopeptone/2% dextrose in distilled water). Fifteen-milliliter aliquots were labeled for 30 min with 1 mCi (1 Ci = 37 GBq) of ^{3}H-labeled fatty acid, cooled for 5 min on ice, and cells were pelleted at 4°C by centrifugation at 7600 × *g* for 10 min. Cell pellets were washed, centrifuged with 1 ml of 10 mM NaN_3 in 140 mM NaCl/10 mM phosphate, pH 7.2, then suspended in 100 μl of 5 mM Tris·HCl, pH 7.4/3 mM dithiothreitol/1% $NaDodSO_4$/1 mM phenylmethylsulfonyl fluoride. Cells were broken with one cell volume equivalent of 0.5-mm glass beads by six 30-sec spurts of vigorous mixing in a Vortex, cooling on ice between each mixing. Debris was removed by centrifugation for 30 sec at 8000 × *g* in a tabletop Eppendorf centrifuge. The supernatant was then alkylated with 125 μl of 20 mM iodoacetamide in 8 mM Tris·HCl (pH 8.0) for 1 hr at room temperature. Twenty-microliter aliquots were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis and fluorography (9).

Analysis of the Linkage of ^{3}H-Labeled Fatty Acids to Proteins. Twenty microliters of reduced and alkylated ^{3}H-labeled fatty acid-labeled yeast protein was treated for 4 hr at 23°C with 7 μl of freshly prepared 4 M hydroxylamine/20 mM glycine, pH 10, followed by electrophoresis and fluorography.

To determine the hydroxylamine-stable linkage of [^{3}H]myristic acid to the 20-kDa acyl protein in JR153, the cultures were labeled as described above except that the cells were treated for 15 min prior to addition of fatty acid with cerulenin at 2 μg/ml, a known inhibitor of yeast fatty-acid synthesis (31) that enhances the labeling of the specific acyl proteins in JR153 severalfold (data not shown). After separation of protein by $NaDodSO_4$/12% polyacrylamide gel electrophoresis, gel slices were cut from the undried gel sample lanes through the 20-kDa region, rinsed once rapidly with 0.5 ml of 10% methanol in water, then individually

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digested under toluene atmosphere for 72 hr at 37°C with 1 mg of Pronase E in 1 ml of 50 mM ammonium bicarbonate (pH 7.9) with mixing. One milligram of fresh Pronase E was added at 24 hr. Following digestion, the digest from the slice containing radioactivity was removed, the gel slice was rinsed once with 500 μl of 0.1% $NaDodSO_4$, the digest and rinse were combined and then acidified to pH 1–2 with 40 μl of 6 M HCl. The acidified solution was extracted twice with 1.5 ml of chloroform/methanol (2:1, vol/vol). The combined organic phases were backwashed once with 1 ml of chloroform/methanol/0.01 M HCl (1:10:10, vol/vol/vol) and dried under a stream of nitrogen gas. The residue was redissolved in 50% methanol/50% HPLC buffer A. Ninety-seven percent of the radioactivity present in the original protein digest was recovered after extraction. The sample was analyzed by reverse-phase HPLC on a Whatman μBondapak C_{18} column at a flow rate of 1 ml/min using as buffer A 0.1% trifluoroacetic acid/0.05% triethylamine in water, and as buffer B 0.1% trifluoroacetic acid in acetonitrile, eluting with a 1% per min acetonitrile gradient. The myristoyl-[^{3}H]glycine standard was synthesized as described (23).

Synthesis of Fatty Acyl Peptide Standards. The synthesis of acyl peptide standards was performed by reacting the radioactive symmetric myristic acid or palmitic acid anhydride with Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg in pyridine. One hundred microcuries of ^{3}H-labeled fatty acid was treated with 4 μl of the respective fatty acyl chloride and then suspended in 150 μl of pyridine containing 4.8 mg of the respective nonradioactive fatty acid. After incubation for 60 min at 23°C, 65 μl of this solution was then added to 400–500 μg of Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg. The reaction was allowed to proceed overnight with mixing. Pyridine was evaporated under vacuum, and the residue was extracted twice with 0.3 ml of petroleum ether and then redissolved in 400 μl of 50% methanol in water. The reaction products were purified and analyzed by reverse-phase HPLC as described above.

Preparation of Yeast Extract for the Assay of *N*-Myristoylglycyl Peptide Synthetase Activity. Yeast cultures were grown as described above to OD_{660nm} of 1–3. All subsequent steps were carried out at 4°C. Cells from 40 ml of culture were collected by centrifugation at 7600 × *g* for 10 min. The cell pellet was washed by centrifugation with 1 ml of 10 mM Tris·HCl (pH 7.4), then resuspended in 400 μl of extraction buffer [10 mM Tris·HCl, pH 7.4/1 mM dithiothreitol/0.1 mM EGTA/aprotinin (10 μg/ml)] by pipetting. Cells were broken with glass beads as described above. After the beads were allowed to settle, cellular debris was removed from the supernatant fluid by centrifugation at 1000 × *g* for 10 min. The supernatant was then centrifuged at 45,000 rpm for 30 min in a Beckman 75 Ti rotor. The supernatant was removed, and the crude membrane pellet was resuspended by pipetting into 400 μl of extraction buffer. The enzymatic activity was stable at −60°C for at least 1 week. Protein was determined by the method of Peterson (32).

Assay for *N*-Myristoylglycyl Peptide Synthetase Activity. ^{3}H-labeled fatty acyl CoA was synthesized enzymatically and added to the incubation as follows: per assay tube, acyl CoA synthetase reaction consisted of 0.5 μCi of [^{3}H]myristic acid; 25 μl of 2× assay buffer (20 mM Tris·HCl, pH 7.4/2 mM dithiothreitol/10 mM MgCl/0.2 mM EGTA); 5 μl of 50 mM ATP in distilled water, adjusted to pH 7.0 with NaOH; 2.5 μl of 20 mM lithium CoA in distilled water; 15 μl of *Pseudomonas* acyl CoA synthetase at 1 mU/μl in 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid, pH 7.3; 2.5 μl of distilled water. The reaction was allowed to proceed for 20 min at 30°C. Typically, 40–50% of the ^{3}H-labeled fatty acid was converted to its CoA ester by this procedure, as measured by determining the radioactivity remaining in the reaction after acidification with 6 M HCl to pH 2.0 and extraction six times with 5 vol of heptane, a modification of the method of Hosaka *et al.* (33). Fifty microliters of this reaction mixture was added to tubes containing 40 μl of extraction buffer (see above) and 10 μl of 1 mM Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg. The assay was initiated by the addition of 10 μl of yeast cell extract (50 μg of protein) followed by incubation at 30°C for 10 min. The assay was terminated by the addition of 110 μl of methanol and 10 μl of 100% trichloroacetic acid (wt/vol), followed by cooling for 10 min at 0°C. Precipitated protein was removed by centrifugation at 8000 × *g* for 3 min. (Under these conditions, 95% of synthetic [^{3}H]myristoyl peptide or [^{3}H]palmitoyl peptide remained soluble when added to an assay mixture.) Fifty microliters of the supernatant was analyzed by reverse-phase HPLC on a 3.9-mm by 30-cm Waters μBondapak C_{18} column using the same HPLC buffers described above, starting at 35% acetonitrile and eluting with a 1% per minute acetonitrile gradient. [^{3}H]Myristoyl-Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg eluted at 24 min, while [^{3}H]palmitoyl-Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg eluted at 30 min.

RESULTS

To explore the use of *S. cerevisiae* as a source of *N*-myristoylglycyl peptide synthetase, we investigated the acylation of proteins in the protease-deficient strain JR153 (30). Cellular protein from yeast was labeled with [^{3}H]myristic or [^{3}H]palmitic acid and analyzed by gel electrophoresis and fluorography. To characterize the nature of the linkage of the fatty acids to the acyl proteins, cellular protein extracts were treated with 1 M hydroxylamine. In Fig. 1, it can be seen that the two fatty acids become attached to different proteins, and that none of the protein-bound fatty acid appears to be

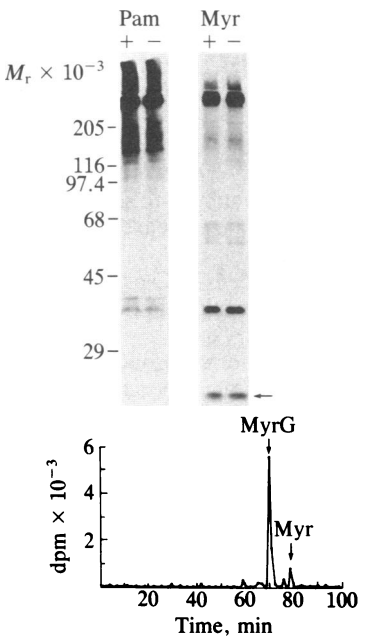

FIG. 1. Fatty acid acylation of yeast cellular proteins. (*Upper*) Yeast cultures were labeled with either [^{3}H]palmitate (Pam) or [^{3}H]myristate (Myr), and cellular proteins were analyzed by $NaDodSO_4$/10% polyacrylamide gel electrophoresis and fluorography. Samples were either treated (+) or not treated (−) for 4 hr with 1 M hydroxylamine (pH 10) before electrophoresis. Horizontal arrow indicates the [^{3}H]myristate-labeled protein that was digested with Pronase to isolate myristoylglycine, which was identified by HPLC (*Lower*). Vertical arrows indicate the elution positions of myristoylglycine (MyrG) and myristate (Myr).

hydroxylamine-labile, indicating either that fatty acid is present in amide linkage or in a very stable *O*-ester linkage (14, 34). To establish the nature of the linkage of the [^{3}H]myristate bound to the myristate-specific 20-kDa acyl protein, [^{3}H]myristate-labeled 20-kDa protein was exhaustively digested with Pronase, then analyzed by reverse-phase HPLC. Fig. 1 shows that 74% of the radioactivity comigrated with the myristoyl-[^{3}H]glycine standard. We have shown previously that a variety of synthetic myristoyl amino acids can be separated from myristoylglycine under these conditions (23). Thus, this yeast strain synthesizes at least one acyl protein with myristic acid covalently attached to its amino-terminal glycine residue, an acyl linkage also found in acyl proteins in higher eukaryotes.

To identify the enzyme activity responsible for this protein modification, we developed an *in vitro* assay that measures the transfer of [^{3}H]myristic acid to a synthetic acceptor peptide. The enzymatic product is then identified by HPLC. We chose to use an octapeptide whose sequence was derived from the amino terminus of the catalytic subunit of bovine cardiac muscle cAMP-dependent protein kinase, a protein that contains myristic acid covalently bound to its amino-terminal glycine residue (3). The sequence of the synthetic peptide Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg contains the six amino-terminal residues of cAMP-dependent protein kinase, followed by two arginine residues that replace two lysine residues in the native sequence. This latter substitution allowed ease of synthesis of the *N*-[^{3}H]acylglycyl peptide standards without undesired side-chain acylation (see *Materials and Methods*) while maintaining the basic charge characteristics of the peptide that enhanced the solubility of the acyl peptide in trichloroacetic acid/methanol buffer.

Chemically synthesized standards of [^{3}H]myristoylglycyl peptide and [^{3}H]palmitoylglycyl peptide were found to elute from a Waters 3.9 mm × 30 cm C_{18} μBondapak reverse-phase HPLC column with 59% and 65% acetonitrile, respectively. Cell lysates were prepared and fractionated into crude membrane and soluble fractions. *N*-Myristoylglycyl peptide synthetase activity was detected in both crude membrane and soluble fractions, with the specific activities of total, soluble, and membrane fractions being 1410, 1320, and 2260 dpm per μg of protein per 10-min assay, respectively. We estimate that 65% of the activity resides in the crude membrane fraction. This estimate is subject to some uncertainty because of the presence of cytosolic proteases that may hydrolyze the peptide substrate.

Pronase digestion of the HPLC-purified standard as well as the enzymatic product resulted in their conversion to myristoylglycine (Fig. 2).

To avoid the problem of cytosolic proteases, we chose to further characterize the activity associated with the membrane fraction. As can be seen in Table 1 (experiments 1 and 2), myristoylation of the peptide was dependent on ATP and CoA and was heat labile. To demonstrate the requirement for ATP, it was necessary to omit the exogenous acyl CoA synthetase, since this preparation contained significant amounts of ATP. In the absence of exogenous acyl CoA synthetase, equal *N*-myristoylglycyl peptide activity was observed, suggesting that endogenous acyl CoA synthetase converts enough of the [^{3}H]myristic acid to its CoA ester to satisfy the requirements for peptide acylation in our assay.

Fig. 3 shows time dependence, enzyme dependence, and peptide substrate dependence of the *N*-myristoylglycyl peptide synthetase. The reaction is linear with time through 10 min with 50 μg of yeast membrane protein, but it plateaus at longer times. Likewise, for 10-min assays, the reaction was linear with respect to enzyme up to ≈75 μg of yeast membrane protein. Under the condition of our assay, the enzyme exhibits an apparent K_m for Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg of ≈150 × 10^{-6} M.

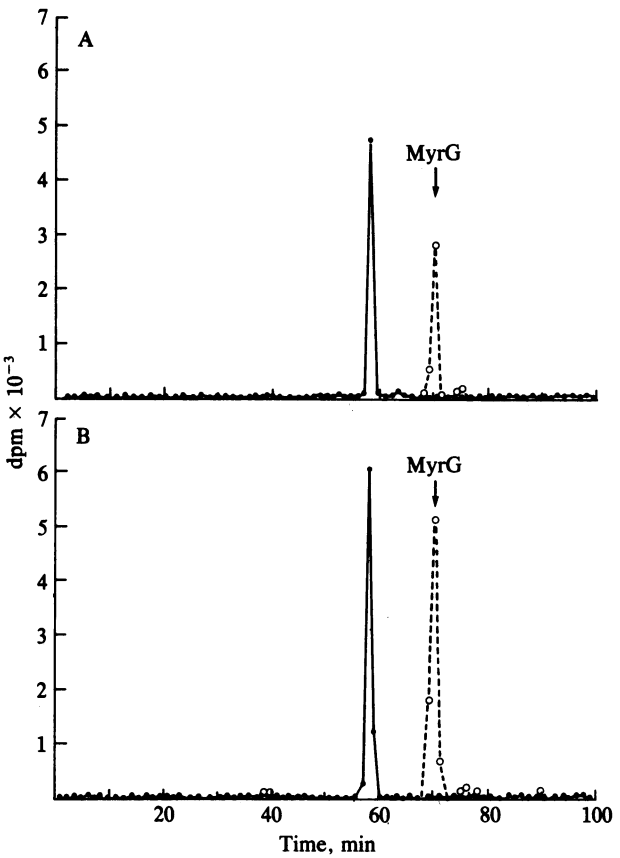

FIG. 2. Pronase digestion of myristoyl peptides. Myristoyl peptide was prepared either enzymatically or chemically and isolated by HPLC (solid lines). The isolated peptides were digested with Pronase and myristoylglycine identified by HPLC (dashed lines). (*A*) Chemically synthesized myristoyl peptide; (*B*) enzymatically synthesized myristoyl peptide. MyrG identifies the elution position of authentic myristoylglycine.

To examine the specificity of the peptide acylation reaction for the fatty-acid chain length, the transfer of [^{3}H]palmitate to Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg was investigated. As

Table 1. Characterization of *N*-myristoylglycine peptide synthetase

Experiment	Rate of myristoyl peptide synthesis, dpm × 10^{-3} per 10 min
1. Control	111
− ATP	9
− CoA	1
2. Control	83
Heated membranes (5 min; 65°C)	2
3. Control	26.7
+ 1 mM GN	28.0
+ 1 mM GPRP	25.6
+ 1 mM GSSKSPKDPS	27.4

Assays were carried out using crude membrane fractions from yeast with changes as indicated. In Exp. 1, we test the dependence of the assay on ATP and CoA, in the absence of exogenous fatty acid CoA ligase. In Exp. 2, we demonstrate that the yeast enzyme is heat labile, and in Exp. 3, that addition of other peptides containing NH_2-terminal glycine does not inhibit the reaction, which in this experiment was measured using only 18 μM peptide substrate rather than the usual 90 μM, in order to maximize possible inhibitory effects. The one-letter amino acid code is used to identify the sequence of the peptides.

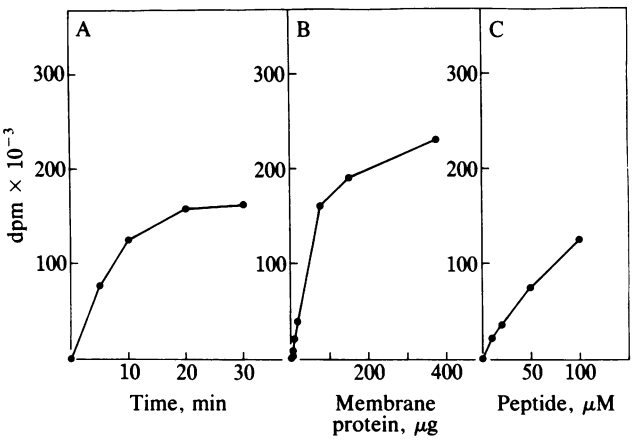

FIG. 3. Characterization of membrane-associated *N*-myristoylglycyl peptide synthetase activity. *N*-Myristoylglycyl peptide synthetase activity present on yeast membrane fraction was assayed as described in *Materials and Methods*. (*A*) Time dependence of the activity (50 μg of membrane protein). (*B*) Dependence on enzyme concentration (10-min assay period). (*C*) Dependence on Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg concentration. The apparent K_m for the peptide is 150 μM. The data in *A* and *B* are from different enzyme preparations.

can be seen in Fig. 4, synthesis of [^{3}H]palmitoylglycyl peptide could not be detected, even though 4 times as much radioactivity was used when studying peptide acylation with palmitate than with myristate. The absence of palmitoyl peptide synthesis could not be attributed to differences in the conversion of the fatty acids to their CoA esters by the acyl CoA synthetase, since 49% of the [^{3}H]palmitate and 44% of

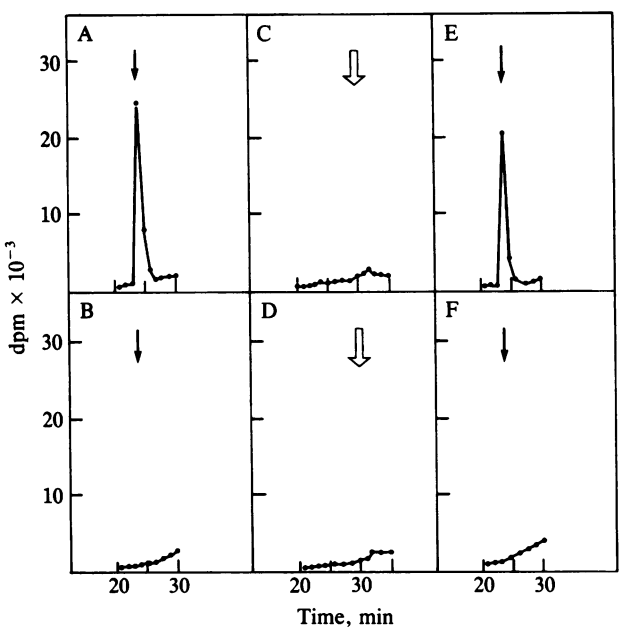

FIG. 4. Acyl donor specificity of the *N*-myristoylglycyl peptide synthetase. Assays of membrane-associated peptide acylating activity were performed as described in *Materials and Methods*. Shown are typical HPLC elution profiles. (*A*, *C*, and *E*) Assays carried out in the presence of 90 μM glycyl peptide substrate. (*B*, *D*, and *F*) Assays carried out in the absence of glycyl peptide. (*A* and *B*) Assays performed with 0.5 μCi of [^{3}H]myristate. (*C* and *D*) Assays performed with 2 μCi of [^{3}H]palmitate. (*E* and *F*) Assays performed with 0.5 μCi of [^{3}H]myristate and 2 μCi of [^{3}H]palmitate. Solid arrows, elution position of myristoyl peptide standard. Open arrows, elution position of the palmitoyl peptide standard.

the [^{3}H]myristate were converted to their respective acyl CoA esters during the preincubation with the acyl CoA synthetase. Moreover, as also seen in Fig. 4, [^{3}H]myristoylglycyl peptide synthesis could occur in the presence of [^{3}H]palmitate, indicating that there was no contaminant in the [^{3}H]palmitate preparation that was inactivating the peptide acylating activity. Therefore, the enzyme exhibits very high specificity with respect to the fatty acid transferred to the peptide. This is consistent with the fatty-acid specificity seen with the *N*-myristoylated proteins analyzed to date (3–7, 14, 23).

To begin investigation of the specificity of the *N*-myristoylglycyl peptide synthetase for the peptide substrate, we examined the ability of other glycyl peptides to competitively inhibit acylation of Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg. As can be seen in Table 1 (experiment 3), 1 mM concentrations of three different glycyl peptides had no effect on myristoylation of 18 μM peptide substrate (≈1/8th its K_m). Thus, the *N*-myristoylglycyl peptide synthetase exhibits specificity for the peptide substrate.

DISCUSSION

The presence in yeast of protein containing covalently bound palmitate has previously been demonstrated by Wen and Schlesinger (15). We have shown that, like higher eukaryotes, yeast also synthesizes proteins that contain covalently bound myristic acid present in amide linkage to an amino-terminal glycine residue.

The use of synthetic peptides to identify and characterize the enzymes responsible for covalent modifications of proteins has been demonstrated in a number of cases (35–38). We describe an enzyme activity that links myristic acid to the amino-terminal glycine of the synthetic peptide Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg. The ATP and CA dependence of the acylation reaction suggests that the enzyme uses myristoyl CoA as the acyl donor, a characteristic shared with the enzymes of ester-linked acyl protein synthesis (26–29). The activity apparently exists in both crude membrane and soluble fractions. Preliminary observations suggest that the attachment to membranes is relatively loose. However, more stringent fractionation will be required before assigning with certainty the subcellular location of the enzyme.

The *N*-myristoylglycyl peptide synthetase exhibits a high degree of specificity for the fatty-acid donor; the enzyme covalently attaches [^{3}H]myristate but not [^{3}H]palmitate to the amino terminus of the glycyl peptide substrate. This contrasts markedly with the activity described by Berger and Schmidt (26), which acylates the Semliki Forest virus E1 protein in thioester linkage; myristate, palmitate, stearate, and oleate all functioned as acyl donors in their system. This acyl donor specificity for the *N*-myristoylglycyl peptide synthetase agrees well with the specificity observed for acylation of the 20-kDa acyl protein in yeast and is consistent with *in vivo* data for *N*-myristoylated proteins in other eukaryotes, where myristate is the only fatty acid found linked to amino-terminal glycine residues (3–7, 23).

It is interesting to note that several other glycyl peptides, one of which has a sequence similar to the amino terminus of pp60^{src}, did not competitively inhibit myristoylation of Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg. Given the high degree of specificity of the activity for acyl donor and peptide acceptor, the precise signals involved in protein *N*-myristoylation may now be determined by examining the ability of other defined peptides to become acylated and/or inhibit acylation of Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg. Others have begun this analysis for myristoylation of pp60^{src} by using the techniques of oligonucleotide-directed mutagenesis (19) and gene fusion (20). Inspection of the sequences of the known myristoylated proteins reveals no obvious consensus sequence for acylation

(3–7), suggesting the possibility of more than one *N*-myristoylating activity. Use of various synthetic peptide substrates *in vitro* should allow detection of multiple *N*-myristoylglycyl peptide synthetases and be equally applicable to the investigation of the peptide sequence specificity of the enzymes present in higher eukaryotes. In preliminary experiments, we have detected this activity in extracts of HeLa cells.

Determining the subcellular location of myristoylation will be important for understanding acylprotein biogenesis. The myristoylation of proteins has been shown to occur very early in the protein biosynthetic pathway, possibly cotranslationally (21, 22, 39). Further characterization of the *N*-myristoyl peptide synthetase(s) will reveal the components involved in protein myristoylation and its regulation.

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