Identification of the NH_2-terminal blocking group of calcineurin B as myristic acid

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The NH_2-terminal blocking group of the Ca^{2+}-binding B-subunit of calcineurin (protein phosphatase-2B) has been identified as myristic acid by fast atom bombardment mass spectrometry and gas chromatography. The sequence, myristyl-Gly–Asn–Glu–Ala-, is very similar to that of the catalytic subunit of cyclic AMP-dependent protein kinase, the only other protein known to contain this fatty acid. This finding, and the elution of all myristyl peptides at 57% acetonitrile on reverse phase HPLC, may facilitate the identification of other proteins with this blocking group.

Protein phosphatase *Ca^{2+}* *Calmodulin* *Mass spectrometry* *Fatty acids* *High-performance liquid chromatography*

1. INTRODUCTION

Calcineurin is the name given to a calmodulin-binding protein first identified in bovine brain [1,2]. It is composed of two subunits A and B having M_r of ~60 000 and ~15 000, respectively. Calcineurin A is the subunit that interacts with calmodulin, while calcineurin B is itself a Ca^{2+}-binding protein that, like calmodulin, binds 4 mol Ca^{2+} with affinities in the μM range [2,3].

We have reported that calcineurin is a Ca^{2+}–calmodulin-dependent protein phosphatase [4,5]. It has been suggested that calcineurin A is the catalytic subunit, and that calcineurin B confers Ca^{2+}-sensitivity to the enzyme in the absence of calmodulin. The binding of calmodulin to calcineurin A increases its activity ~10-fold [4].

The determination of the primary structure of calcineurin B was undertaken in order to investigate its structural relationship to other Ca^{2+}-binding proteins such as calmodulin and troponin-C (A.A., P.C., C.B.K., in preparation). During this investigation it became clear that the NH_2-terminus of calcineurin B was blocked, and that peptides containing the blocking group were eluted from HPLC columns at very high concentrations of acetonitrile. Here, we have identified the NH_2-terminal blocking group as a long-chain saturated fatty acid.

2. MATERIALS AND METHODS

2.1. *Isolation of calcineurin B*

Calcineurin was purified from bovine brain as in [2]. The protein was dialysed against 40 mM Tris–HCl (pH 7.8)–5.0 mM dithiothreitol–30 mM NaCl–6 M urea and applied to a column of DEAE–Sephacel equilibrated in the same buffer. The column was developed with a linear salt gradient from 30–700 mM NaCl, and calcineurin B was eluted near the start of the gradient. The fractions containing calcineurin B were pooled, dialysed against ammonium bicarbonate (50 mM), freeze dried and resuspended at ~5 mg/ml. Insoluble material (residual calcineurin A) was removed by centrifugation, and calcineurin B further

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purified by HPLC using an alkyl phenyl column as in [6].

2.2. *Isolation of blocked N-terminal peptides*

Calcineurin B was cleaved with cyanogen bromide and with *S. aureus* proteinase (Miles Labs.) as in [7]. The digests were fractionated by reversed-phase HPLC using a μ-bondapak C_{18} column (Waters) with linear gradients of water/acetonitrile containing 0.1% trifluoroacetic acid [8]. The blocked N-terminal peptides eluted at 57% acetonitrile.

2.3. *Fast atom bombardment (FAB) mass spectrometry*

FAB mass spectra were recorded on a Kratos MS50 mass spectrometer fitted with a high field magnet. A standard Kratos FAB source was employed to generate a 4–6 kV xenon beam. Samples (~10 nmol) were dissolved in 1 μl of a 1:1 α-thioglycerol:diglycerol matrix, and the mixture introduced into the source on a copper probe tip. Peptides were esterified by treatment with 15 mM methanolic–HCl (50 μl) for 25 h at room temperature. This method does not result in detectable methanolysis of primary amide groups [9].

2.4. *Gas chromatography*

Peptides were hydrolysed in 6 N HCl at 110°C for 20 h in sealed glass ampoules. The hydrolysates were cooled and extracted 3 times with ether. The ethereal layer was back extracted with water to remove any HCl that had been taken up by the ether, and the organic layer dried in a stream of nitrogen. The residue was methylated with diazomethane and analysed by chromatography on a Carlo-Erba 4160 gas chromatograph fitted with an on-column injection system and a 25 m × 0.25 mm glass open tubular column coated with Sil-5 (Chrompak U.K. Ltd.).

3. RESULTS

When calcineurin B was cleaved with cyanogen bromide, and applied to an HPLC column, a peptide (CB-1) lacking a free NH_2-terminal amino acid was eluted as a sharp peak at 57% acetonitrile. The amino acid composition of CB-1 (table 1) showed that it was a decapeptide containing only two hydrophobic amino acids. This indicated that the blocking group was much more hydrophobic than the acetyl, formyl or pyrrolidone carboxylic acid groups most commonly found at the N-termini of proteins [10].

Table 1

Amino acid compositions of blocked N-terminal peptides from calcineurin B

Amino acid	CB1	SP1
Aspartic acid/asparagine	1.03 (1)	0.99 (1)
Serine	1.07 (1)	0.32 (0)
Glutamic acid/glutamine	2.24 (2)	1.02 (1)
Proline	0.98 (1)	
Glycine	0.93 (1)	1.0 (1)
Alanine	0.86 (1)	
Leucine	1.12 (1)	0.25 (0)
Tyrosine	0.97 (1)	
Lysine		0.20 (0)
Homoserine	0.85 (1)	
Total	10	3

The peptides were hydrolysed for 24 h in vacuo at 110°C in 6 N HCl containing 2 mM phenol. Serine was corrected for 10% destruction during hydrolysis

Peptide CB-1 had M_r 1271 as determined by FAB mass spectrometry in the positive ion mode [$(M + Na)^+$ = 1294, $(M + H)^+$ = 1272] and in the negative ion mode [$(M-H)^-$ = 1270]. The number of carboxylic acid groups was determined from the positive ion FAB mass spectrum of the esterified peptide. An increase in M_r of 60 was observed, which corresponds to the formation of two methyl esters plus methanolysis of the C-terminal homoserine lactone. The M_r of the amino acid component of CB-1 was 1060, indicating that the M_r of the blocking group was 211.

When calcineurin B was digested with *Staphylococcus aureus* proteinase, a peptide, SP1, lacking a free NH_2-terminal amino acid was also eluted from HPLC at 57% acetonitrile. Amino acid analysis showed it to be a tripeptide Gly,Asx,Glx (table 1). From the known specificity of *S. aureus* proteinase the C-terminal residue of SP1 must be Glu. FAB mass spectrometry established the M_r of SP1 as 528, and esterification of this peptide led to an increase in M_r to 556,

Table 2A

Assignment of the sequence ions in the FAB mass spectra of decapeptide

Type of fragment ion[a]	M_r-Values of fragment ions											M_r determining peak
	X	Gly	Asn	Glu	Ala	Ser	Tyr	Pro	Leu	Glu	Hsl	
+ Na						621	708	[b]	968	1081	1210	1294
+ NAcy								856	953	1066		1294
+ CAmin		1027	913	784	713							1294
+ CAmin		1005	891	762								1272
+ CAlk			897	768								1294
− NA						597	684	[b]	944	1057	1186	1270
− NAcy						582		832	929	1042	1171	1270
− CAmin		1003	889	760	689							1270
− CAlk		988	874	744								1270

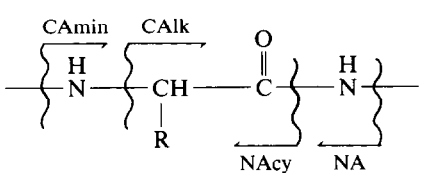
Most of the sequence ions observed in the positive ion (+) mode are cationised by Na^+. The base peaks in the positive ion FAB mass spectra of peptides that lack basic functional groups as in CB-1 often correspond to the M_r of peptides cationised by the formation of adducts with Na^+ and/or K^+, traces of which are usually present in the matrix

Table 2B

Assignment of the sequence ions in the FAB mass spectra of the decapeptide ester

Type of fragment ion[a]	M_r-Values of the fragment ions										M_r determining peak
	X	Gly	Asn	Glu-OMe	Ala,Ser	Tyr	Pro	Leu	Glu-OMe	Homo-Ser-OMe	
+ Na							[b]	982	1095	1238	1354
+ NAcy							870		1080		1354
+ CAmin		1087	973								1354
+ CAlk			957	814							1354

[a] Fragment ions are named as follows:



Bond cleavages are accompanied by a hydrogen transfer to the charged fragment except in the NAcy case. Positive and negative signs indicate cationic and anionic fragment ions, respectively

[b] Due to cyclic nature of the proline residue fragmentation of NA type does not produce fragment ions

which corresponds to the formation of two methyl esters. Since SP1 has two free carboxyl groups, the sequence of SP1 must be X–(Gly,Asn)–Glu and the M_r of the blocking group must be 211.

The assignment of sequence ions observed in the FAB mass spectrum of CB-1 and its methyl ester are given in table 2, and suggested that the sequence of CB-1 was:

X–Gly–Asn–Glu–Ala–Ser–Tyr–Pro–Leu–Glu–Hsl.

The sequence of residues 3–10 has been confirmed by automated Edman degradation of an overlapping peptide formed by *S. aureus* proteinase digestion of calcineurin B (A.A., unpublished).

Assuming that the blocking group X is linked to the NH_2-terminal glycine by the usual amide bond, then hydrolysis should yield a carboxylic acid M_r = 228. This corresponds to the M_r of a C_{14} saturated fatty acid. The mass spectra are consistent with the supposition that X is $C_{13}H_{27}CO_2^-$. To test this idea, the nature of the blocking group was examined by gas chromatography as described in section 2.4. A major peak was observed which coeluted with methyl myristate. Small amounts of methyl palmitate and traces of methyl stearate were also detected. Methyl myristate accounted for 95.4% of the fatty acid ester components and methyl palmitate for 4.6%.

4. DISCUSSION

The only protein, other than calcineurin B, known to contain an NH_2-terminal myristyl group, is the catalytic subunit of cyclic AMP-dependent protein kinase [11]. Since the two proteins that possess this unusual blocking group are a protein kinase and a protein phosphatase, it is tempting to speculate that the myristyl group is involved in their interaction with protein substrates. Alternatively, it could enable these proteins to interact with membranes, or to be translocated across membranes. A further possibility is that the myristyl group plays a role in maintaining the subunit–subunit interactions between calcineurin A and B, and between the regulatory and catalytic subunits of cyclic AMP-dependent protein kinase. These alternative ideas imply that the modification may not be confined to protein kinases and phosphatases, and raises the question of whether NH_2-terminal fatty acids are much more widespread than has hitherto been recognised. The N-terminal sequence of the catalytic subunit of cyclic AMP-dependent protein kinase is:

myristyl-Gly–Asn–Ala–Ala–Ala–Ala–Lys– [11]

Thus 3 of the first 4 residues are identical to calcineurin B suggesting that the enzyme(s) that links myristyl groups to these proteins may recognise the N-terminal sequence Gly–Asn–X–Ala– . This sequence similarity, and the finding that all myristyl peptides elute at 57% acetonitrile (A.A., unpublished), may facilitate identification of other proteins with this blocking group.

The only other example of a fatty acid linked to the α-amino group of a protein is the murein lipoprotein of the *Escherichia coli* outer membrane, where a palmityl moiety is attached to the N-terminal cysteine [12]. Two additional fatty acids are attached through a glyceryl group that forms a thioether bond with this same cysteine residue. The N-terminal sequence of this protein is quite different from that of calcineurin B and the catalytic subunit of cyclic AMP-dependent protein kinase [12].

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