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A conserved deamidation site at Asn 2 in the catalytic subunit of mammalian cAMP-dependent protein kinase detected by capillary LC-MS and tandem mass spectrometry

PAUL T. JEDRZEJEWSKI,¹ ANDREAS GIROD, ANDREAS THOLEY, NORBERT KÖNIG,
SANDRA THULLNER, VOLKER KINZEL, AND DIRK BOSSEMEYER

Department of Central Spectroscopy and Department of Pathochemistry, German Cancer Research Center,
INF 280, D-69120 Heidelberg, Germany

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Abstract

The N-terminal sequence myr-Gly-Asn is conserved among the myristoylated cAPK (protein kinase A) catalytic subunit isozymes C α , C β , and C γ . By capillary LC-MS and tandem MS, we show that, in approximately one third of the C α and C β enzyme populations from cattle, pig, rabbit, and rat striated muscle, Asn 2 is deamidated to Asp 2. This deamidation accounts for the major isoelectric variants of the cAPK C-subunits formerly called C_A and C_B. Deamidation also includes characteristic isoaspartate isomeric peptides from C α and C β . Asn 2 deamidation does not occur during C-subunit preparation and is absent in recombinant myristoylated C α (rC α) from *Escherichia coli*. Deamidation appears to be the exclusive pathway for introduction of an acidic residue adjacent to the myristoylated N-terminal glycine, verified by the myristoylation negative phenotype of an rC α (Asn 2 Asp) mutant. This is the first report thus far of a naturally occurring myr-Gly-Asp sequence. Asp 2 seems to be required for the well-characterized (auto)phosphorylation of the native enzyme at Ser 10. Our results suggest that the myristoylated N terminus of cAPK is a conserved site for deamidation in vivo. Comparable myr-Gly-Asn sequences are found in several signaling proteins. This may be especially significant in view of the recent knowledge that negative charges close to myristic acid in some proteins contribute to regulating their cellular localization.

Keywords: cAMP-dependent protein kinase; deamidation; isoaspartate, isoenzymes; myristate

The cAMP-dependent protein kinase is activated by a great variety of stimuli that cause an elevation in the level of cAMP (Robison et al., 1971) and dissociation of the inactive holoenzyme into a dimer of regulatory subunits bound to four molecules of cAMP, and two monomeric catalytic subunits. The free C-subunit has the

ability to phosphorylate a large number of substrates in the cytoplasm and the cell nucleus, and thus represents a multifunctional enzyme (Walsh & Van Patten, 1994). To date, the factors that control the phosphorylation of specific substrates by this enzyme are not fully understood. The subcellular distribution of the C-subunit, governed by regulatory subunits and anchoring proteins (Scott, 1991; Hubbard & Cohen, 1993), may play an important role as well as the translocation of C-subunit between the cytoplasm and the cell nucleus (Meinkoth et al., 1990; Harootunian et al., 1993; Wen et al., 1995). Most C-isoenzymes are myristoylated at their N-terminal glycine (Carr et al., 1982), a modification responsible in many other proteins for their translocation and membrane interactions (Knoll et al., 1995; McLaughlin & Aderem, 1995). The possible role(s) of the different C-isozymes of cAPK and their myristoylation in determining substrate specificity has not yet been elucidated.

The major C-subunit isoform in mammalian striated muscle has been sequenced at the protein level (Shoji et al., 1981) and has

Reprint requests to: D. Bossemeyer, Department of Pathochemistry (0210), German Cancer Research Center, INF 280, D-69120 Heidelberg, Germany; e-mail: bossemeyer@dkfz-heidelberg.de.

¹Present address: The Barnett Institute, Northeastern University, 341 Mugar Building, 360 Huntington Avenue, Boston, Massachusetts 02115.

Abbreviations: cAPK, cAMP-dependent protein kinase; C, catalytic subunit of cAPK; T#, tryptic peptide #; NMT, protein N-myristoyltransferase; μ LC-ESI-MS, capillary liquid chromatography-electrospray-mass spectrometry; m/z , mass to charge ratio; myr, myristoylated; TFA, trifluoroacetic acid; MeCN, acetonitrile; IPTG, isopropylthiogalactoside; IEF, isoelectric focusing; DIC, diisopropylcarbodiimide; DCM, dichloromethane; DMF, *N,N*-dimethylformamide; PCMT, protein carboxylmethyltransferase.

later been termed $C\alpha$. Cloning work revealed the existence of additional mammalian gene products, $C\beta$ and $C\gamma$ (Showers & Maurer, 1986; Uhler et al., 1986b; Beebe et al., 1990), as well as the existence of alternative splice products such as $C\beta 2$ and $C\beta 3$ (Wiemann et al., 1991a; Qi et al., 1996). $C\alpha$ is ubiquitously expressed in mammalian tissues, whereas $C\beta$ is most highly expressed in brain and reproductive tissues. The abundance of $C\alpha$ relative to $C\beta$, the high degree of sequence homology between them, and the similarity in their physical properties make the biochemical detection of the latter in normal tissues more difficult (Lee et al., 1996). Biochemical studies so far have required the expression of recombinant enzyme (Gamm et al., 1996).

Yet another type of isozyme in the form of isoelectric variants is indicated in C-subunit preparations from striated muscle. Cation exchange chromatography yields two peak fractions, A and B, in an approximately 1:2 ratio, that differ by about 0.4 pH units in their isoelectric values (Peters et al., 1977; Kinzel et al., 1987; Van Patten et al., 1988; Herberg et al., 1993). Proteolysis of the bovine catalytic subunit by endoproteinase Asp-N indicated a cleavage site at the N terminus unique for the enzyme recovered in fraction A, providing initial insight into the nature of these isoelectric differences (Hotz et al., 1989). The N-terminal myristoylation prevented analysis by conventional Edman degradation. However, the sensitivity, precision, and capability of sequencing modified peptides by mass spectrometry have allowed us to study the various C-subunit isoforms in detail. We have used capillary liquid chromatography coupled to electrospray mass spectrometry and tandem mass spectrometry for the analysis of tryptic peptides of the C-subunit from heart and/or skeletal muscle of four mammalian species (pig, cattle, rabbit, and rat), as well as from bovine recombinant myristoylated rC α expressed in *Escherichia coli*. With the sensitivity and resolution of μ LC-ESI-MS, we show here that a mixture of proteins may be analyzed in a single analysis, which is difficult to accomplish by other methods. The results suggest that Asn 2 near the myristoylated N terminus of the cAMP-dependent protein kinase catalytic subunit irrespective of isozyme is a conserved site for deamidation in vivo.

Results

Chromatographic separation of the catalytic subunits into fractions A and B

Catalytic subunit preparations of different mammalian species and tissues (bovine heart, bovine skeletal muscle, porcine heart, rabbit skeletal muscle, rat skeletal muscle) were chromatographically separated on a cation exchange resin (Kinzel et al., 1987; Herberg et al., 1993) that consistently yielded two peak fractions, A and B. Figure 1a shows a representative elution pattern for the porcine heart enzyme. The A:B ratio was about 1:2 in all cases. The measured total masses from each species did not differ significantly between fractions (Fig. 1b; Table 1), confirming that the difference in chromatographic behavior was not simply caused by alterations in phosphate content (Kinzel et al., 1987; Herberg et al., 1993). The phosphate content of the porcine heart C-subunit determined colorimetrically was 1.91 ± 0.17 in fraction A and 1.98 ± 0.14 in fraction B, in agreement with the measured total masses of bovine C α and rat C α and indicative of a twofold phosphorylation (Table 1). Isoelectric values of fractions A and B differed by about 0.4 pH units (data not shown).

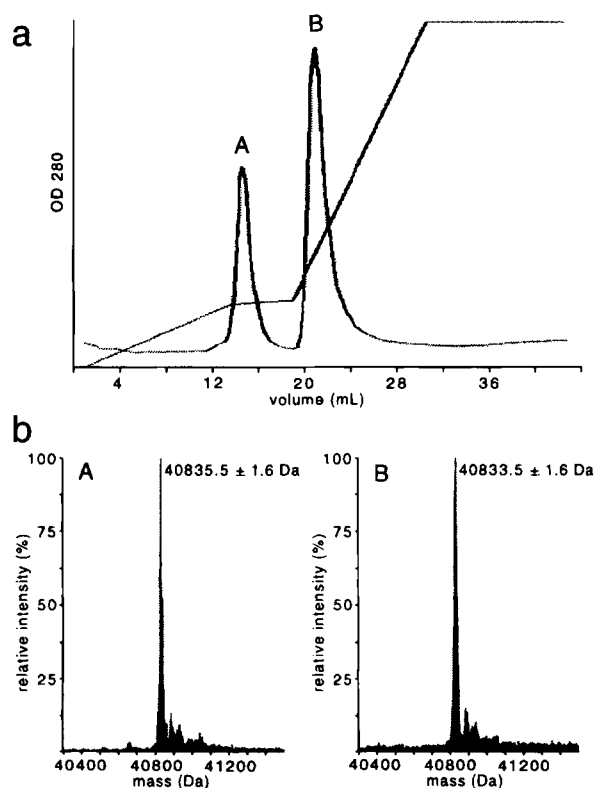


Fig. 1. FPLC Mono-S LC separation of porcine C-subunits into fractions A and B (a), using gradients from 0 to 1 M LiCl in 20 mM BisTrisPropane, pH 8.5. μ LC-ESI-MS mass analysis of fractions A and B are shown (b).

Analysis of fraction B

Tryptic digests of fraction B from different species and tissues were analyzed for myristoylated amino-terminal peptides by μ LC-ESI-MS. The search yielded the expected protonated peptide signals at m/z of 812.5 for heptapeptides from the catalytic subunit isoform C α (myrGNAAAAK). As a representative example, the mass spectrum for the N-terminal peptides from the porcine heart digest is given in Figure 2. The elution time of these peptides was

Table 1. Molecular weight of cAPK C-subunit in fractions A and B from cattle, rabbit, and pig determined by μ LC-ESI-MS^a

	Fraction A	Fraction B	Calculated for C α
Bovine ^b	40,858.4 \pm 1.6	40,855.7 \pm 1.6	40,859.1
Pig ^b	40,835.5 \pm 1.6	40,833.5 \pm 1.6	—
Rabbit ^c	40,835.2 \pm 2.0	40,833.6 \pm 2.0	—
Rat ^c	40,847.6 \pm 1.8	40,848.3 \pm 1.8	40,858.7

^aThe molecular weight calculated from nucleic acid sequence includes a myristate and two phosphoryl groups. The sequence of porcine C α is only partially known (see Fig. 6); the rabbit sequence is unknown.

^bFrom heart muscle.

^cFrom skeletal muscle.

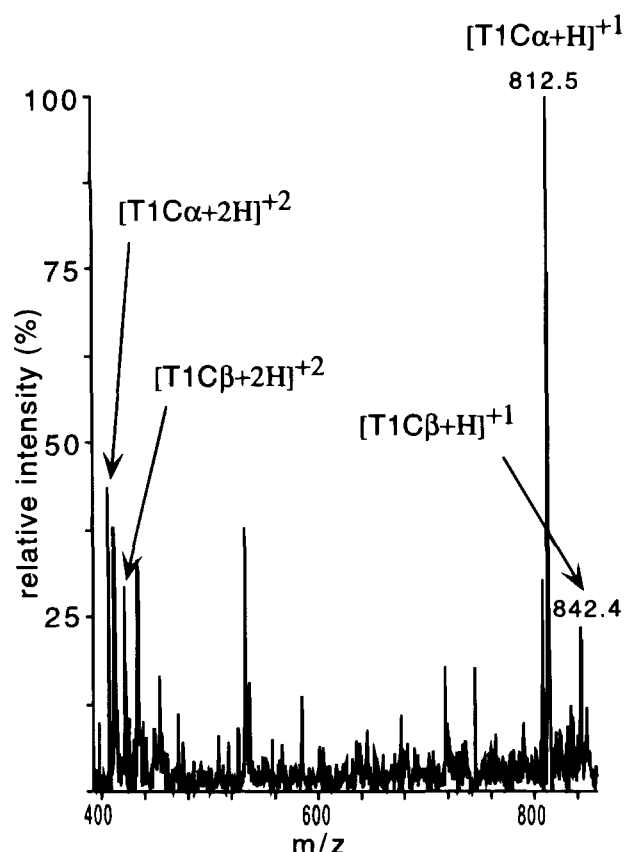


Fig. 2. Electrospray mass spectrum of the protonated N-terminal tryptic peptides from μ LC-ESI-MS analysis of total tryptic digest from porcine heart fraction B, showing masses indicative of the presence of C α and C β .

also confirmed by the myristoyl modification-specific chromatogram (data not shown), where myristoylated peptides exhibited a marker fragment ion at m/z 211.2 (Jedrzejewski & Lehmann, 1997). Selected mass chromatograms for the protonated N-terminal peptides in fraction B for all species are shown in Figures 3a, 4a, b, and c. The sequence of the bovine C α -subunit is known from both protein and cDNA sequencing (Shoji et al., 1981; Wiemann et al., 1992), and that of rat C α from cDNA sequencing (Wiemann et al., 1991b), whereas the porcine and rabbit C α sequences are known only partially (Nelson & Taylor, 1981) or are unknown, respectively. The measured masses suggest the same sequence for all N-terminal peptides in the B fractions; this was confirmed by sequencing with electrospray tandem mass spectrometry (the porcine sequence is shown in Fig. 5a). In addition to the signal at m/z of 812.5, tryptic digests of the B fractions yielded significant amounts of a second myristoylated peptide at m/z of 842.4 (Figs. 2, 3b). A difference of 30 Da coincides with a threonine residue replacing one of the alanines. For confirmation, the peptides of the porcine enzyme indicated in Figures 2 and 3b were sequenced by tandem mass spectrometry (Fig. 5b). As indicated by the difference of 101.0 Da between the Y_3'' and Y_2'' ions, Thr (with a sequence mass of 101.0) was confirmed at the fifth position. Additional evidence for Thr 5 is the loss of water (B_5-H_2O ion) characteristic of this amino acid. The Thr in position five is characteristic of isozyme C β , for which this sequence is conserved among the known cDNAs from human, cattle and mouse. Further evidence (to be discussed

later) for the presence of C β comes from the identification of C β unique peptides (Table 4). The N-terminal sequences of porcine C β (Adavani et al., 1987), as well as the sequences of rabbit C α , C β , and rat C β , have not been reported yet. A summary of the N-terminal peptides found in this study is given in Table 2. Consistent with previous studies on N-acylation (in bovine brain tissue) for both N termini, no N-terminal peptides corresponding to acylation by C14:1, C14:2, or C12:0 were detected, indicating the high degree of specificity for C14:0 acylation (Johnson et al., 1994). Together, the data show that fraction B is comprised of the catalytic subunits C α and C β , each with an amino terminus in its DNA-encoded form.

Analysis of fraction A

Analysis of the myristoylated tryptic peptides from fraction A by μ LC-ESI-MS resulted in more complex chromatograms. In the selected mass chromatograms of fraction A from all species and tissues, we found a peak corresponding to a peptide with m/z 813.4, which was preceded by a smaller peak indicating an earlier eluting peptide of identical m/z (Figs. 3c, 4d,e,f). The possible significance of earlier and later eluting forms with the same peptide mass will be examined below. In all cases, both of these myristoylated peptides were 1 Da higher in mass than the corresponding peptides in fraction B (Figs. 3a, 4a,b,c). Because these analyses were performed with an average mass precision of 0.3 Da, the difference was significant. To find the rationale behind the

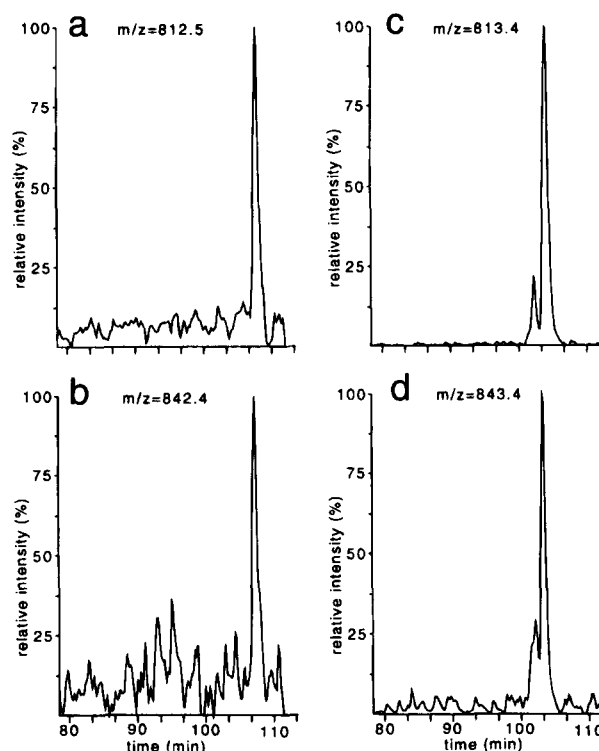


Fig. 3. Selected mass chromatograms for four protonated N-terminal peptides from μ LC-ESI-MS analysis of the porcine heart total tryptic digests, obtained from fraction B (a,b) and fraction A (c,d). These chromatograms are representative figures from a number of performed experiments.

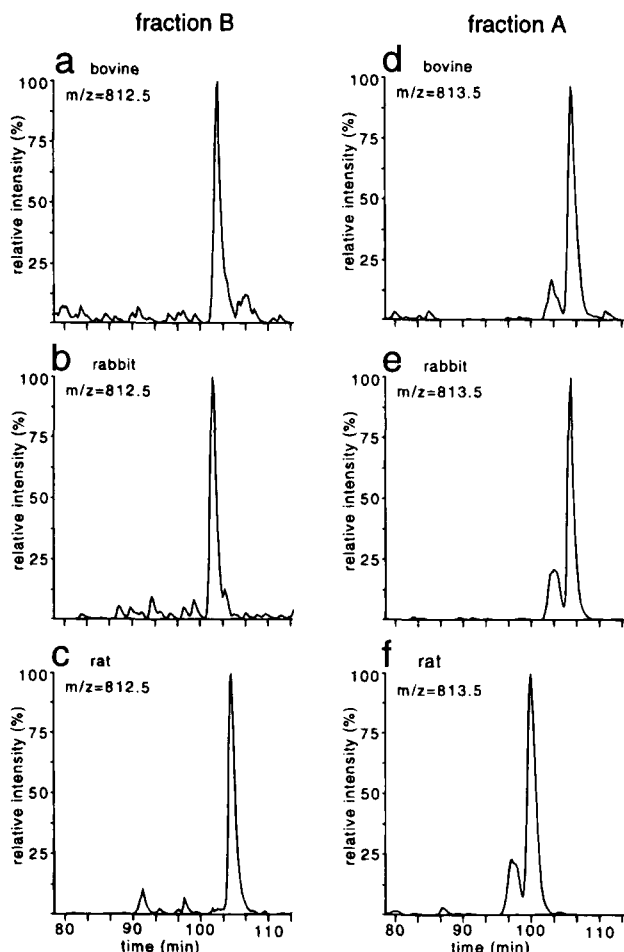


Fig. 4. Selected mass chromatograms for six protonated N-terminal peptides from μ LC-ESI-MS analysis of total tryptic digests of C-subunit from bovine heart muscle (a,d) and from rabbit (b,e) and rat (c,f) skeletal muscle, obtained from fractions B and A.

1-Da mass difference, peptides from the main peaks of fraction A (Figs. 3c, 4d,e,f) were sequenced by electrospray tandem mass spectrometry. We found that, in all species, the amino-terminal peptides of fraction A had an aspartate in position two instead of the asparagine found in fraction B. The porcine sequence, homologous to the others, is shown in Figure 6a. This is consistent with the AspN-endoproteinase cleavage site seen previously in bovine heart enzyme (Hotz et al., 1989).

In addition to the major peptide peak at m/z 813.4, a less abundant peptide with m/z 843.4 was detected in all species. In the cases of porcine heart (Fig. 3d), bovine heart, and bovine skeletal muscle, the selected mass chromatograms again indicated a small amount of an earlier eluting peptide with identical m/z 843.4. In rabbit and rat skeletal muscle, peptides with m/z 843.4 were detected at a much lower relative abundance (Table 3) and the earlier eluting peptides were not detected, probably because they were below the detection limit. The mass difference of 30 Da between m/z 813.4 and 843.4 suggested that the heavier peptides may also have a Thr residue in position five, as observed in fraction B. This was confirmed by the sequence of this peptide from porcine heart

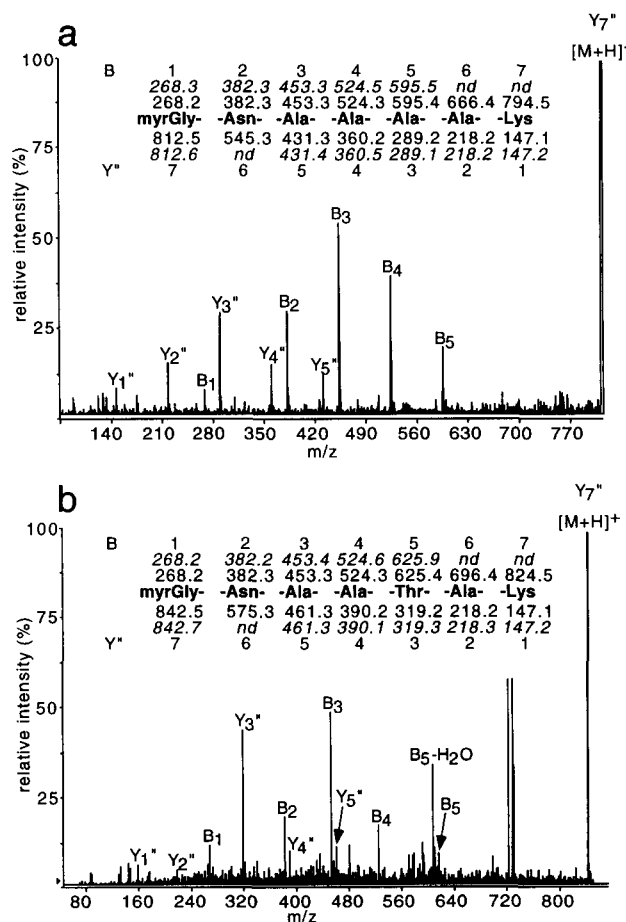


Fig. 5. Electrospray tandem mass spectra of the porcine heart N-terminal tryptic peptides from fraction B with the m/z 812.5 (a) and peptides with the m/z 842.4 (b). In the top section of each panel, the sequence of each peptide and the expected B_n (N terminus derived fragment ions) and Y_n⁺ (C terminus derived fragment ions) masses are shown. Predicted and observed (average error of ± 0.25 Da) fragment ions are shown in normal and italic fonts, respectively. For both peptides, the complete sequences were obtained from the tandem mass spectra. The location of myristoylation is conclusively shown by the fragment ion B₁. The predicted and observed m/z for B₅-H₂O ion are 607.4 and 607.5, respectively. Unlabeled fragment ions correspond to immonium or internal fragment ions. For complete nomenclature of tandem fragment ions, see Roepstorff and Fohlman (1984).

(Fig. 6b). Indeed, the heavier peptide contains the Thr 5 residue, which is characteristic of isozyme C β , but, in addition, contains an aspartate residue in position two, like the other peptides from fractions A having an m/z of 813.4. The peptides with m/z of 843.4 consistently found in all A fractions apparently represent the myristoylated amino termini of C β (Asp 2) isozymes. In accordance with N-terminal peptides in fraction B, the acylation of both major N-terminal peptides in fraction A was exclusively with C14:0; this was consistently observed in data from all species and tissues investigated.

Taken together, these data provide direct evidence that the myristoylated peptides in fraction A from all species share an aspartate residue in position two, whereas the related peptides in fraction B share an asparagine in this position, irrespective of whether they are derived from C α or C β . The identity of the second residue,

Table 2. Summary of N-terminal peptides found in C-subunits from pig and cattle heart muscle, and cattle, rat, and rabbit skeletal muscle^a

Fraction	N-terminal peptide		C isoform
B	myr-G-N-	A-A-A-A-K	C α
A	myr-G-D-	A-A-A-A-K	C α (Asp 2)
	myr-G-D _{iso} -	A-A-A-A-K	C α (isoAsp 2)
B	myr-G-N-	A-A-T-A-K	C β
A	myr-G-D-	A-A-T-A-K	C β (Asp 2)
	myr-G-D _{iso} -	A-A-T-A-K	C β (isoAsp 2)

^aN-terminal sequences for all species of C α and C β have been determined by tandem mass spectrometric sequencing, whereas the identification of isoAsp-containing peptides was established by mass analysis and chromatographic behavior.

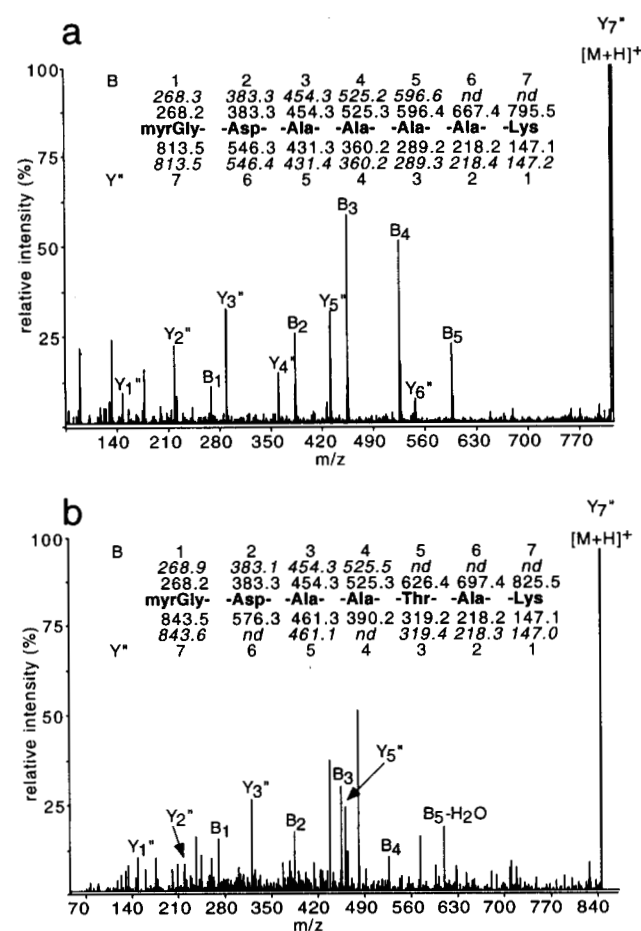


Fig. 6. Electrospray tandem mass spectra of the porcine heart N-terminal tryptic peptide with the m/z 813.4 (a) and peptide with the m/z 843.4 (b) from fraction A. In the top section of each panel, the sequence of each peptide and the expected B_n (N terminus derived fragment ions) and Y_n (C terminus derived fragment ions) masses are shown. The predicted and observed (average error of ± 0.25 Da) fragment ions are shown in normal and italic fonts, respectively. For both peptides, the complete sequences were obtained from the tandem mass spectra. Predicted and observed m/z for B_5 -H₂O ion are 608.4 and 608.6, respectively. Unlabeled fragment ions correspond to immonium or internal fragment ions.

aspartate or asparagine, determines the elution behavior of the catalytic subunit on cation exchange chromatography, and accounts for the formerly described C-subunit isoforms C α and C β (Van Patten et al., 1986, 1988; Kinzel et al., 1987; Toner-Webb et al., 1992; Yonemoto et al., 1993).

Analysis of the β -aspartate-containing peptides in fraction A

The observation of earlier and later eluting forms of peptides with the same mass, indicated in the selected mass chromatograms (Figs. 3c,d, 4d,e,f), suggests the presence of amino acid isomers. Considering deamidation as a likely mechanism for the formation of the Asp 2 found in fraction A, the two peaks may represent homologous peptides containing either isoaspartate or aspartate. To examine the elution behavior of such isomers, the synthetic myristoylated heptapeptides myrGD_{iso}AAAAK and myrGDAAAAK were chromatographed separately (Fig. 7a,b) and in a mixture (Fig. 7c) under otherwise identical conditions. As shown in Figure 7, the isoAsp-containing peptide eluted earlier, suggesting that the preceding small peaks in fraction A (Figs. 3c,d, 4d,e,f) represent the isoAsp isomers of the N-terminal peptides. Moreover, synthetic myrGDAAAAK comigrated with the major peak in porcine fraction A and synthetic myrGD_{iso}AAAAK comigrated with the corresponding preceding small peak (data not shown). Isoaspartate-containing peptides often precede their aspartate-containing isoforms when chromatographed under conditions similar to those used here (Murray & Clarke, 1984; Stephenson & Clarke, 1989; Szendrei et al., 1994). Isoaspartate is a characteristic product of the β -aspartyl shift mechanism of deamidation (Wright, 1991), indicating deamidation via this mechanism as a likely cause of the observed Asn 2/Asp 2 variability in the catalytic subunit isoenzymes. It should be noted that the ratio of Asp- to isoAsp-containing peptides for both C α and C β differed between about 10:1 for the heart enzyme and about 5:1 for skeletal muscle enzyme (Table 3). Collectively, these data indicate that tryptic digests of fraction A from the different species contain, in addition to C α (Asp 2) and C β (Asp 2), an amino-terminal peptide characteristic of C α (isoAsp 2). The minor peptide with the mass 843.4 (Fig. 3d) accordingly most likely represents C β (isoAsp 2). Summaries of the amino-terminal peptides and their relative abundance are given in Tables 2 and 3.

Verification of the presence of C β in porcine fractions A and B

Considering the relatively large amounts of C β -related N-terminal peptides detected in the routine preparations of C-subunit from heart and skeletal muscle, we wished to confirm the identity of this isoform by additional C β -specific sequence information using porcine heart samples. Some C β sequence information at the protein level has been reported for bovine brain only (Johnson et al., 1994) and brain tissue contains the highest level of C β mRNA (Uhler et al., 1986a). Partial sequence analysis of porcine C α and C β have shown a high degree of homology in the known sequence regions (Adavani et al., 1987; Buechler et al., 1989). An analysis of μ LC-ESI-MS data for C β unique peptides is shown in Figure 8 and Table 4. For this purpose, selective mass chromatograms and mass spectra (data not shown) from the μ LC-ESI-MS analysis of total digests from fractions A and B were evaluated. Of the 27 peptides unique for porcine C β , 10 and 11 peptides, respectively, were identified by the presence of multiply charged ions in fraction A

Table 3. Summary of quantification of C-subunits isoforms in various species^a

Species/tissue	T1 C α /T1 C β	T1 C α D2/T1 C β D2	T1 C α D2/T1 C α D _{iso2}	T1 C β D2/T1 C β D _{iso2}
Porcine heart muscle	3.3	3.9	8.3	9.0
Bovine heart muscle	2.7	ND ^b	11.4	9.7
Bovine skeletal muscle	9.3	2.8	5.1	5.3
Rabbit skeletal muscle	10.8	27.2	4.7	nd ^c
Rat skeletal muscle	15.2	39.8	4.7	nd ^c

^aGiven is the ratio of the more abundant form to the less abundant form. Values are derived from the integration of peak areas of the appropriated selected mass chromatograms. Values are from single measurements, except for the porcine data, which are from three replicate experiments with a mean error of 6%.

^bND, not determined.

^cnd, not detectable.

and B. The measured values corresponded to the predicted values of these C β -specific peptides within the error of the measurement (± 0.3 Da) (Table 4). For additional confirmation, peptide T22 in fraction A was sequenced by tandem mass spectrometry (data not shown). A schematic summary of C β -specific sequence information is given in Figure 8. Together, these data verify that the myristoylated peptides of m/z 843.4 and 842.4 found in fractions A and B, respectively, indeed represent the N-terminal tryptic peptides of the C β isoenzyme. The total contribution of C β was estimated by a comparison of the integrated peak areas from selected mass chromatograms for the myristoylated peptides in fractions A and B. In porcine and bovine tissues, C β contributes to about 30% of the total enzyme in fractions A and B; in rabbit and rat, C β comprises up to about 9% of the total enzyme in each fraction.

Analysis of recombinant rC α by μ LC-ESI-MS and copurification of bovine C-subunit with ³⁵S-labeled myr-rC α

To examine whether the C α -subunit is subject to spontaneous Asn 2 deamidation, the bovine recombinant C α gene was coexpressed with yeast protein-N-myristoyltransferase in *E. coli*. Expression of rC α was induced for 18 h, and the enzyme was affinity purified (Girod et al., 1996). The protein was enzymatically active,

and mass spectrometric analysis of the whole enzyme indicated myristoylation and twofold phosphorylation (data not shown). The purified enzyme was digested with trypsin and analyzed for myristoylated N-terminal peptides. Both the mass spectrum of the myristoylated N-terminal peptides (Fig. 9a) and the selected mass chromatogram (Fig. 9b) indicated myristoylated peptides containing asparagine only; deamidation products were not detected. To further exclude the possibility that C-subunit deamidation occurs during the preparation procedure, we purified A and B fractions from fresh bovine heart tissue in the presence of ³⁵S-methionine-labeled myristoylated rC α , added before homogenization of the tissue. To avoid any influence of holoenzyme formation, the cells were broken in the presence of cAMP, and C-subunits were purified by use of an affinity column [PKI(5-24) agarose, Olsen & Uhler 1989; Girod et al., 1996], followed by chromatography on a MonoS column into fractions A and B. IEF (Fig. 10) showed that radiolabeled myr-rC α coeluted exclusively with fraction B (lanes 2

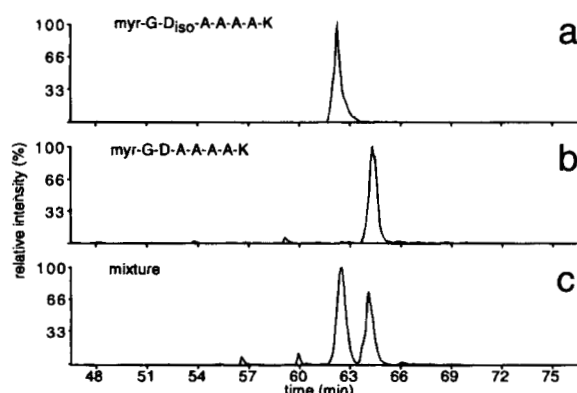


Fig. 7. Elution profile from μ LC-ESI-MS analysis of synthetic cAPK C α N-terminal peptides: myrGD_{iso}AAAAK (a), myrGDAAAAK (b), and mixture of myrGD_{iso}AAAAK and myrGDAAAAK (c).

		-----10-----20-----30-----40-----50
C α	1	GNAAAAK GS EQESVKEFLA KAKEDFLKKW ENPAQNTAHL DQFERIKTLG
C β		GNAAATAK -GS EVESVKEFLA KAKEDFLKKW ENPAFPNAGL EDFERIKTLG
		T1 T3-4 T7-8, T8, T8-9
	51	TGSFGRVMLV KHKETGNHFA MKILDQKQV KLKQIEHTLN EKRILOAVNF
		TGSFGRVMLV KHKATEQYVA MKILDQKQV KLKQIEHTLN EKRILOAVNF
		T20-21, T21
	101	PFLVKLEYSF KDNSNLYMVM EYVPGGEMFS HLR-----
		PFLVRLFEFSF KDNSNLYMVM EYVPGGEMFS HLRRIGRFSE PHAREYAAQI
		T22
	151	-----DLKPE NLLIDQQGYI QVTDGFGFAK VKGRWTWLCG
		VLTPFYLHSL DLIIYRDLKPE NLLIDHOGYI QVTDGFGFAK VKGRWTWLCG
		T27-28, T28, T28-29
	201	TPEYLAPEII LSKGYNKA VD WVALGVLIYE MAAGYPPFFA DQPIQIYEKI
		TPEYLAPEII LSKGYNKA VD WVALGVLIYE MAAGYPPFFA DQPIQIYEKI
	251	VSGKVRFP SH FSSDLKDLLR NLLQVLDLTKR FGNLKNQVND IKNHKWFATT
		VSGKVRFP SH FSSDLKDLLR NLLQVLDLTKR FGNLKNQVND IKTHKWFATT
	301	DWIAIYQRKV EAPFIPKFKG PGDTSNFDYD EEEIIRVSIN EKCQKEFSEF
		DWIAIYQRKV EAPFIPKFRG SGDTSNFDYD EEDIRVSIT EKCQKEFCEP
		T49

Fig. 8. Sequence of porcine C α and C β . Sequences in bold type represent tryptic peptides sequenced by tandem mass spectrometry. Underlined and italicized sequences represent peptides located by mass as reported in Table 4. Sequences of porcine C α and C β are from published partial protein and nucleic acid sequencing (Adavani et al., 1987; Buechler et al., 1989; Swiss-PROT data bank accession nos. P05383, P36887).

Table 4. Identified tryptic peptides (T) from porcine heart cAPK C β ^a

Tryptic peptide	Calculated peptide mass (Da)	Observed peptide mass in fraction A (Da) ^b	Observed peptide mass in fraction B (Da) ^b
T1	843.5, 842.5	843.6	842.7
T3-4	1,422.8	1,422.4	1,422.8
T8	1,858.8	1,859.2	1,859.1
T7-8, T8-9	1,986.9	1,986.7	1,987.3
T20-21	1,572.9	1,571.8	nd ^c
T21	1,416.8	1,417.0	1,417.2
T22	770.4	770.3	770.3
T27-28	5,321.1	5,320.9	5,321.4
T28	2,763.1	2,762.8	2,762.6
T28-29	2,919.3	2,918.4	2,918.8
T49	676.4	676.9	676.8

^aBold indicates tryptic peptides sequenced by tandem mass spectrometry.

^bAll peptide masses reported are singly protonated with error in mass of 0.005% total peptides identified in fraction B, 10; in fraction A, 11 of 27 unique C β tryptic peptides.

^cnd, not detected.

and 2*) and no radiolabeled rC α could be detected in fraction A (lanes 1 and 1*). Together, these results clearly indicate that purification procedures are not responsible for Asn 2 deamidation of native C-subunit. Thus, the observed Asn 2 deamidation of C-subunits depends on processes occurring in the muscle tissue.

In vitro deamidation of synthetic model peptides

The detection of peptides characteristic of iso-aspartate-containing isomers in the A fractions suggests deamidation of the catalytic subunits via the β -aspartyl shift mechanism. According to reports on deamidation via this mechanism in the literature (e.g., Aswad & Johnson, 1987; Geiger & Clarke, 1987; Lura & Schirch, 1988), however, a much higher isoAsp to Asp ratio would be expected than was actually detected. Therefore, we analyzed the degree of nonenzymatic deamidation and isoAsp relative to Asp formation in synthetic myristoylated model peptides. Incubation of the myrGNAAAK heptapeptide under conditions (50 mM NH₄HCO₃, pH 8 at 60 °C) that are known to aid deamidation (Lura & Schirch, 1988) indicated 16% deamidation after 18 days (data not shown). Analysis of the peak areas of selected mass chromatograms corresponds to 11% of the peptide population containing isoaspartate, and only 5% containing aspartate. A 2:1 ratio of isoaspartate to aspartate corresponds to a ratio expected for a β -aspartyl shift mechanism of deamidation. Possible reasons for the observed lower isoAsp to Asp ratios in the mammalian C-subunit will be discussed.

Expression of an rC α (Asn 2 Asp) mutant in E. coli

It could not be excluded completely that Asp 2-containing C α and C β enzymes may be distinct gene products. A negatively charged residue following the N-terminal glycine, however, is known to be inhibitory for NMT (for review Towler et al., 1988). To test in C α

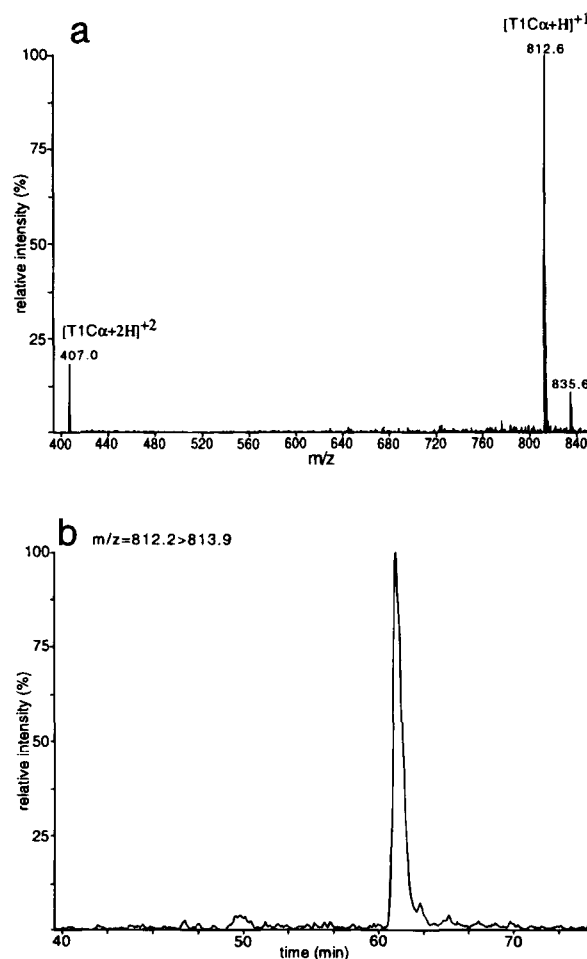


Fig. 9. Electrospray mass spectrum of the protonated N-terminal tryptic peptides from μ LC-ESI-MS analysis (a) and selected mass chromatogram (b) of recombinant myristoylated rC α , indicating the absence of Asn 2 deamidation.

whether or not substantial myristoylation of a genetically encoded Gly-Asp sequence can be achieved, a bovine rC α (Asn 2Asp) mutant was coexpressed with yeast NMT in *E. coli*. The purified enzyme was indistinguishable in its chromatographic behavior on an FPLC MonoS column from mutant protein expressed in the absence of NMT (data not shown). In contrast, myristoylated and nonmyristoylated wild-type proteins can be clearly distinguished by their elution profile. Myristoylated protein elutes at higher salt concentrations mainly in one major peak, whereas nonmyristoylated mutant and wild-type enzymes elute in three peak fractions, indicating two-, three- and fourfold phosphorylation (not shown, but see Herberg et al., 1993). This confirms that, at least in the case of bovine rC α and yeast NMT, the presence of an aspartate residue following the initial glycine inhibits myristoylation. Hence, distinct gene products appear unlikely to be responsible for the myristoylated Asp 2-containing subunits found in the mammalian samples.

Discussion

Analysis of the catalytic subunit of cAMP-dependent protein kinase by electrospray mass spectrometry in combination with cap-

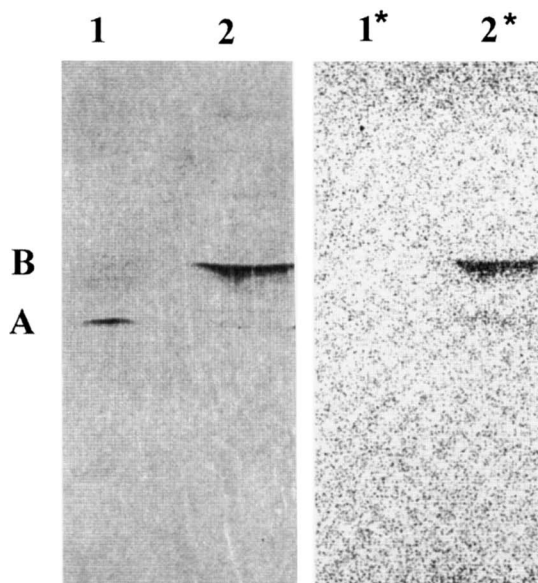


Fig. 10. Copurification of ^{35}S -methionine-labeled rC α and fractions A and B from bovine heart. Lanes 1 and 2, Coomassie blue-stained IEF gel of fractions A, and B, respectively. Lanes 1* and 2*, corresponding autoradiography exposed for 10 days on a Fuji Phosphorimager.

illary liquid chromatography revealed that approximately one third of the enzyme isolated from muscle tissue has a site-specific modification that is conserved among the two major C-isozymes in four mammalian species. This modification, the replacement of an asparagine residue by aspartate, appears to account for the long-known isoelectric variants in C-subunit preparations, formerly named C_A and C_B (Van Patten et al., 1986, 1988; Kinzel et al., 1987). Because of the demonstrated complex composition of the C-subunit fractions A and B, which do not represent defined single C-isoforms, we suggest avoiding the use of the over-simplified designations, C_A and C_B.

Without exception, the amino-terminal tryptic peptides derived from the enzyme that eluted in fraction A contained an aspartate instead of the conserved Asn 2 residue, whereas isozymes with unmodified N-termini coeluted in fraction B. This was true for both major isozymes C α and C β in preparations of heart and/or skeletal muscle from pig, cattle, rabbit, and rat. C β in certain mammalian species contains three cysteine residues instead of the two known to exist in C α . Thus, the relative abundance of C β may explain previous results from SH-group titration experiments that indicated the existence of more than two cysteines (Bechtel et al., 1977; Peters et al., 1977; Kupfer et al., 1979; Jiménez et al., 1982).

Chromatographic separation of the cAPK subunits into fractions A and B seems to depend critically on the nature of the second residue and not on other modifications. This is indicated by the highly similar total molecular masses obtained for the enzyme in fractions A or B for each species, and by data from IEF of fractions A and B at pH 7.1 and 7.5, respectively (data not shown, and Kinzel et al., 1987), corresponding to a theoretical shift of the isoelectric point of the bovine C α subunit by -0.4 pH units after replacement of an asparagine residue by aspartate.

The aspartate residue found in position two in C α and C β from fraction A is unlikely to originate from distinct genes because negatively charged residues in this position are known to inhibit

myristoylation by N-myristoyl transferase (for review see Towler et al., 1988), a cotranslational event (Wilcox et al., 1987). This was confirmed by the myristoylation negative phenotype of an rC α (Asn 2 Asp) mutant in the *E. coli* coexpression system, and is further indicated by the lack of N-terminal myr-Gly-Asp sequences in sequence databases. A mechanism known to replace an asparagine residue by aspartate to create the sequence myr-Gly-Asp is deamidation. The occurrence of deamidation is supported here by the detection of trace amounts of N-terminal peptides characteristic of isoaspartate-containing isomers. Isoaspartate is one of the products of the so-called isoaspartyl shift mechanism of deamidation (for review see Wright, 1991). Deamidation via this mechanism involves the formation of cyclic imide intermediates that open at either one of two bonds forming either isoAsp or Asp (Bornstein & Balian, 1977; Aswad & Johnson, 1987; Lura & Schirch, 1988; Artigues et al., 1990).

A key question is whether the C-subunits are deamidated *in vivo* or *in vitro*, as an artifact of the preparation procedures. Deamidation during the course of the purification could be excluded by two experiments. First, affinity-purified myristoylated recombinant bovine C α , otherwise indistinguishable from native C-subunit, did not contain Asn 2 deamidation products, as controlled by mass spectrometry of myr-rC α tryptic digests. Second, an affinity purification of free C-subunit from fresh bovine heart in the presence of ^{35}S -labeled myr-rC α showed that the radiolabel comigrated with the C-subunit in fraction B only, but not with the C-subunit in fraction A.

Deamidation *in vivo* may be further supported by the observed ratios of β -aspartyl-containing isomers to those containing aspartate. Deamidation via a β -aspartyl shift mechanism usually produces isoAsp in excess of Asp, often in a 3:1 ratio (Bornstein & Balian, 1977; Aswad & Johnson, 1987; Geiger & Clarke, 1987; Lura & Schirch, 1988; Artigues et al., 1990). Indeed, *in vitro* deamidation of the synthetic N-terminal myristoylated C α heptapeptide resulted in a 2:1 isoAsp:Asp ratio. A 1:10 and 1:5 isoAsp:Asp ratio as observed for amino-terminal peptides from heart tissues and skeletal muscle tissues, respectively, could indicate either (1) an additional mechanism of deamidation that does not result in isoaspartate formation, (2) a selective degradation of isoaspartate-containing isoforms, or (3) the action of PCMT. PCMT, ubiquitous in all mammalian tissues (Boivin et al., 1995), is specific for L-isoAsp- and D-Asp-containing substrates (Aswad, 1984; Murray & Clarke, 1984), and catalyzes their partial conversion to aspartate *in vivo* (for review of the mechanism, see Clarke 1985, 1988; Aswad & Johnson, 1987; Wright, 1991). It is interesting to note the reciprocal correlation between the abundance of this "repair" enzyme and the observed ratios of isoAsp:Asp in enzyme from the corresponding tissues: the reported cellular levels of PCMT are much higher in heart muscle than in skeletal muscle, at both the level of mRNA and protein (Boivin et al., 1995). The action of PCMT thus may provide an explanation for the observed isoAsp:Asp isomer ratios in the selected tissues, although this possibility requires further investigation. The proposed cyclic succinimide intermediate, for example, is prone to racemization (Geiger & Clarke, 1987). A resulting D-isoaspartate, which, in contrast to D-aspartate, is not a substrate for PCMT, would be expected to accumulate as a consequence of PCMT action (Brennan et al., 1994) in fraction A. Analyzing the ratios of D- to L-forms in fraction A thus will be helpful to evaluate a participation of this enzyme.

Deamidation of proteins may arise from the spontaneous degradation of labile Asn and Gln residues. This process usually is a

matter of days or weeks in vitro (Robinson & Rudd, 1974; Wright, 1991). Spontaneous, nonenzymatic deamidation is most often, but not exclusively, observed on Asn residues followed by a glycine residue (Wright, 1991). The absence of a side chain facilitates nucleophilic attack of the Asn side-chain carbonyl by the backbone nitrogen of the ensuing residue, to form a cyclic imide intermediate by releasing NH_3 . Although deamidation on Asn-Ala sequences is observed, deamidation seems to occur at a much slower rate. Tyler-Cross and Schirch (1991) report a 10-fold longer half life ($t_{1/2}$ 66 h at 60°C, pH 7.3) of the Asn residue of a pentapeptide VSNAV compared to VSNGV ($t_{1/2}$ 5.8 h). This is supported by the relative resistance to deamidation of the myr-GNAAAAK peptides (16% iso-Asp and Asp isomers after 18 days at 60°C). Temperature has a main effect on the rate of deamidation: lowering the temperature from 60 to 37°C slows the reaction by about a factor of 10 (Geiger & Clarke, 1987; Lura & Schirch, 1988). These conditions are in contrast to the reported half life of the catalytic subunit in cell lines of between 4.5 and 9 h (Lee & Steinberg, 1996). To explain the observed high level of Asn 2 deamidation in the native C-subunit, one has to assume either a much longer half life of the C-subunit in striated muscle tissue compared to culture cells, or deamidation of the C-subunit at an unusually high rate. Location of Asn residues in flexible regions of a protein may facilitate deamidation (Ota & Clarke, 1989). This may be true for Asn 2 of the C-subunit. In the high-resolution crystal structure of myristoylated porcine C-subunit (Bossemeyer et al., 1993), the first seven N-terminal residues have little electron density, presumably because they are rather mobile. In view of the stability of the B fraction during storage (Kinzel et al., 1987), and the failure to detect significant Asn 2 deamidation in the enzyme expressed in bacteria, we cannot exclude at present that other factors could contribute to the observed high degree of C-subunit deamidation at Asn 2 in mammalian tissue. The conditions for the bacterial expression of soluble protein at 24°C, however, may have slowed spontaneous Asn 2 deamidation. Currently, little is known about enzymatic protein deamidation. Only recently, enzymes that deamidate N-terminal asparagine residues have been isolated from porcine liver (Stewart et al., 1994) and yeast (Baker & Varshavsky, 1995). Also, induction of deamidation by cytotoxic necrotizing factors, leading to the constitutive activation of a signaling protein, p21Rho, has been reported (Flatau et al., 1997; Schmidt et al., 1997).

The high level and the conservation of C-subunit deamidation at Asn 2 suggests a biological purpose for this modification. So far, two relevant observations have been reported. The first is that enzyme in fraction A appears to be more resistant to a kinase-splitting membrane protease from brush border than enzyme from fraction B (Kinzel et al., 1987). The other finding is that enzyme from fraction A can autophosphorylate at Ser 10 in vitro, whereas fraction B enzyme cannot (Toner-Webb et al., 1992). The latter is supported by our own observations: phosphate-modification specific mass spectrometric analysis of native porcine enzyme indicated phosphorylation at Ser 10 exclusively in enzyme from fraction A (P.T. Jedrzejewski, V. Kinzel, & D. Bossemeyer, unpubl.). Both findings suggest structural differences between deamidated and unmodified enzyme. The crystal structure of porcine native, myristoylated fraction A enzyme has been solved at high resolution (Bossemeyer et al., 1993; Protein Data Bank entry 1CDK). The distal portion of the myristate occupies a hydrophobic pocket formed by residues from the N-terminal helix and the large C-terminal lobe. The overall structure is highly similar to the structures of bovine (Engh et al., 1996; Protein Data Bank entries 1YDR, 1YDS,

1YDT) or mouse (Knighton et al., 1991; 2CPK) nonmyristoylated recombinant enzyme. If structural differences exist due to deamidation of Asn 2, they are likely to be limited to those N-terminal residues not visible in the crystal structures because of their high mobility.

A functional role of the observed Asn 2 to Asp 2 conversion in a cellular context has not been elucidated yet; however, the primary effect of this deamidation clearly is an increase in negative charge close to the myristoyl moiety. In vitro autophosphorylation studies, deamidation of Asn 2 coincided with a further negative charge in the form of a phosphorylated Ser 10 (Toner-Webb et al., 1992), which may be added subsequently. For several myristoylated proteins, changes in electric charge, usually due to phosphorylation of serine residues or changes in the pH, are part of a myristoyl electrostatic switch mechanism for the modulation of enzyme translocation and membrane interaction (McLaughlin & Aderem, 1995; Hanakam et al., 1996). Deamidation of the myristoylated amino terminus of the cAPK catalytic subunit, which shares several structural features with known myristoyl switch proteins, may serve a regulatory purpose as well.

The results in this study may have more general significance, because not only the $\text{C}\alpha$, $\text{C}\beta$, and $\text{C}\gamma$ isozymes of cAPK, but several other myristoylated enzymes and signaling proteins, such as ARF, calcineurin, recoverin, or NO-synthase (Table 5), share this N-terminal myr-Gly-Asn sequence and thus may also be subject to partial deamidation. Our present and future work thus fo-

Table 5. Examples of N-terminal sequences of N-myristoylated proteins with asparagine as second residue from the Swiss Protein Data Bank, as potential candidates for site-specific deamidation^a

N-terminal sequence	Protein	Origin	Access no.
myrGNAAAAK	cAPK $\text{C}\alpha$	H	P17612
myrGNAATAK	cAPK $\text{C}\beta$	H	P22694
myrGNAPAKK	cAPK $\text{C}\gamma$	H	P22612
myrGNNATTS	cAPK C	FF	P12370
myrGNGESQL	OA-synthetase	H	P29728
myrGNRHAKA	Annexin XIII	H	P27216
myrGNIFANL	ARF 1	H	P32889
myrGNIFGNL	ARF 3	H	P16587
myrGNEASYP	Calcineurin B	H	P06705
myrGNRAFKS	Hisactophilin 1	SM	P13231
myrGNRAFKA	Hisactophilin 2	SM	P42526
myrGNLKSVA	NO-synthase	H	P29474
myrGNSTSSD	G polyprotein	MEV	P12296
myrGNSSSKS	PP-Z1	Y	P26570
myrGNSGSKQ	PP-Z2	Y	P33329
myrGNSKSGA	Recoverin	H	P35243
myrGNTKSGA	S-modulin	BF	P31227
myrGNSRSSA	Visinin	CH	P22728
myrGNSPSYN	GAG	BLV	P25058
myrGNKWSKG	Negative factor	HIV	P05859

^aARF, ADP-ribosylation factor; OA-synthetase, (2'-5')oligoadenylate synthetase; PP-Z, serine/threonine phosphatase Z; GAG, genome polyprotein. Origin: H, human; FF, *Drosophila melanogaster*; SM, *Dictyostelium discoideum*; MEV, Meningo encephalomyocarditis virus; Y, *Saccharomyces cerevisiae*; BF, *Rana catesbeiana*; CH, *Gallus gallus*; BLV, bovine leukemia virus; HIV, human immunodeficiency virus 1.

cuses on the search for protein components responsible for C-subunit deamidation and on the structural and biological aspects of this conserved deamidation.

Materials and methods

Materials

TFA and acetic acid were purchased from Sigma-Aldrich (Deisenhofen, Germany). HPLC-grade solvents were obtained from E. Merck, Darmstadt, Germany.

Protein purification and isolation of the isoenzymes

The catalytic subunit from porcine heart (2 kg), bovine heart (2 kg), bovine skeletal muscle (2 kg), rabbit skeletal muscle (1.6 kg), and rat skeletal muscle (0.8 kg) was isolated by modification of methods described earlier (Kinzel & Kübler, 1976; Kübler et al., 1979; Nelson & Taylor, 1981). All steps were performed at 4 °C. For 1 kg of tissue, 1.5 L of 50 mM potassium phosphate, 2 mM DTT, 1 mM EDTA, pH 6.5, was used for homogenization. Soluble proteins from 1 kg were bound to 1.25 L DE cellulose (Whatman). After washing with 80 mM potassium phosphate, pH 6.5, protein was eluted in 300 mM potassium phosphate, diluted to 50 mM with H₂O, and bound to 0.5 L DE cellulose. The cellulose was washed with 15 L of 50 mM potassium phosphate, 2 mM DTT, 1 mM EDTA, pH 6.5, poured into a column and washed with an additional 10 L of 15 mM potassium phosphate, pH 6.1. The catalytic subunit was eluted with 35 mg of cAMP and 35 mg 8-bromo-cAMP in the same buffer and bound directly to CM-Cellulose (Whatman). The buffers contained 1 mM EDTA and 0.1 mM DTT if not stated differently. After elution with a gradient from 15 mM potassium phosphate, pH 6.1, to 100 mM MOPS-NaOH, 150 mM KCl, pH 7.0, peak fractions were diluted twofold with H₂O for loading onto a MonoS HR 5/5 (Pharmacia) column for separation of the catalytic subunit isoforms. Two peaks containing the A or B fractions eluted with a gradient from 50 to 300 mM LiCl, in 20 mM BisTrisPropane-HCl, pH 8.5.

Phosphate determination

The phosphate content of fractions A and B was determined according to Itaya and Ui (1966).

Enzymatic digest

All cAPK C-subunit samples were chromatographed using a Smart HPLC system (Pharmacia LKB Biotechnology AB, Freiburg, Germany) equipped with a Vydac C₄ column (2.1 × 150 mm) from MZ Analysentechnik (Mainz, Germany). Chromatographic solvents were 0.065% TFA in water (solvent A) and 0.05% TFA in 80% MeCN in water (solvent B). Protein samples were eluted with a gradient from 10 to 100% solvent B over 30 min at a flow rate of 0.1 mL/min. Fractions were concentrated using a Speedvac vacuum concentrator (Savant Instruments, Farmingdale, NY). Digestion of catalytic subunit was performed with modified trypsin (Promega, Madison, Wisconsin) by incubating with protein samples (enzyme:substrate ratio of 1:50) in 10% MeCN and 50 mM NH₄HCO₃, pH 8.5, overnight at 37 °C. For infusion, analyses samples were diluted (1:1) with 50% MeCN, 1% acetic acid.

Mass spectrometric analysis

Electrospray mass spectra were acquired on a Finnigan MAT TSQ7000 triple quadrupole mass spectrometer (San Jose, California) equipped with an ESI ion source. The mass spectrometer was operated at unit mass resolution with acquisition scan range set to 350–2,200 at a rate of 4 s/scan with a step of 0.1 Da. For all μ LC-ESI-MS studies, the ESI needle potential was set at +1.9 kV. Chromatography was performed with an Applied Biosystems (San Jose, California) model 140A dual syringe pump system.

For the molecular mass analysis of the C-subunit, aliquots of Mono-S fractions A and B were individually analyzed by μ LC-ESI-MS. The chromatography was performed using a fused silica capillary column (0.2 × 250 mm) packed with 10- μ m, 300-Å pore, C₄ packing material from Vydac (The Separations Group, Hesperia, California) prepared in house (Davis & Lee, 1992; Moritz et al., 1994). A flow rate of 1 μ L/min through the column was established by a precolumn split from a delivery flow rate of 50 μ L/min. Protein samples (20–50 pmol) were eluted with a linear gradient from 10 to 100% solvent B over 60 min at a flow rate of 1.0 μ L/min.

Analyses of tryptic digests was performed using μ LC-ESI-MS. Digest (approximately 50 pmol per analysis) was separated on a fused silica capillary column (0.2 × 250 mm) packed in house with Vydac 5- μ m, 300-Å pore, C₁₈ packing material. A flow rate of 1 μ L/min through the column was established by a precolumn split from a delivery flow rate of 50 μ L/min. Tryptic peptides were eluted with a linear gradient program from 10 to 60% solvent B in 90 min followed by a gradient from 60 to 100% in 15 min. Analysis of synthetic peptides was performed accordingly.

For tandem mass spectrometric analysis, total digest samples (approximately 2 pmol per analysis) were delivered at a flow rate of 150 nL/min with a syringe pump (model 22, Harvard Apparatus, South Natick, Massachusetts). Solutions were sprayed from a fused silica needle (25- μ m ID × 220- μ m OD, SGE, Weiterstadt, Germany) with a needle potential of +1.5 kV. Tandem mass spectrometry analyses were performed with 2.0 m Torr argon pressure in the collision cell. Acquisition of daughter ion spectra was accomplished using a custom acquisition algorithm that collected 150–250 spectra (10 s/scan, 0.1 Da step) while varying the collision energy from 18 to 38 eV. Because of the low amounts of these samples, rabbit- and rat-derived C-subunits were analyzed using nanoelectrospray ion source (Wilm & Mann, 1996) with typical sample flow rate of 25 to 50 nL/min. Solutions were sprayed from in house-prepared needles according to the protocol described in Wilm and Mann (1996). Spray conditions were achieved by application of +0.7 kV to the needle.

Solid-phase synthesis of N-terminal myristoylated peptides

The peptides GNAAAAK, GDAAAAK, and GD_{iso}AAAAK were synthesized on an ABI 433 peptide synthesizer (Applied Biosystems, Weiterstadt, Germany) employing HBTU (2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) activation and standard Fmoc (9-fluorenylmethoxycarbonyl) methodology. The first amino acid was attached to HMP-resin (*p*-alkoxybenzylalcohol) (0.45 mmol/g). Side-chain protecting groups were O^tBu (*tert*-Butyl) for Asp, Boc (*tert*-butoxycarbonyl) for Lys, and Trityl (triphenylmethane) for Asn. Myristic acid (0.16 mmol) was pre-activated by treatment with 0.16 mmol HOBt (1-Hydroxybenzotriazole) and 0.15 mmol DIC in 2 mL DCM/DMF for 3 h at 4 °C.

After activation, 20 mg of peptide resin with unprotected N terminus was added to this solution and stirred for 4 h at room temperature. This procedure was repeated once again with stirring overnight. After washing the resin with DMF, DCM, and diethylether, the myristoylated peptide was cleaved from the solid support by treatment with 90% TFA/8% triisopropylsilane/2% water for 2 h at room temperature. The peptide was precipitated with cold diethylether, purified by RP-HPLC, and the masses were confirmed by mass spectrometry. They were chromatographed under the same condition as the tryptic digests (see above).

In vitro deamidation of N-terminal peptides

Myristoylated peptides corresponding to the N-terminal tryptic fragment of C α were synthesized and incubated in 50 mM NH₄HCO₃, pH 8, for 18 days at 60°C (Lura & Schirch, 1988). Samples were withdrawn and subjected to μ LC-ESI-MS as before.

Expression and purification of recombinant myr-rC α

Recombinant bovine catalytic C α (Wiemann et al., 1992) was expressed at 24°C for 18 h by using vector pT7-7 in *E. coli* strain BL21(DE3) and purified by affinity chromatography on immobilized PKI(5-24) as described (Olsen & Uhler, 1989; Girod et al., 1996), except that the vector pBB131 for the expression of yeast NMT was coexpressed together with C α (Duronio et al., 1990), leading to virtually complete myristoylation of the catalytic subunit. Purified enzyme was digested with trypsin and subjected to μ LC-ESI-MS as before.

Preparation of ³⁵S-methionine-labeled myr-rC α

For radioactive labeling of myr-rC α , 100 mL LB-medium were inoculated with *E. coli* strain BL21(DE3), which was cotransformed as before with plasmids for the expression of bovine rC α and yeast NMT. Cells were grown until the OD₆₅₀ reached 0.7. After 5 min on ice, the cells were washed four times in M9 medium, resuspended in 100 mL methionine assay medium (DIFCO, Detroit, Michigan) with 0.2% glucose, 50 μ g/mL carbenicillin, 50 μ g/mL kanamycin, and further incubated under aerobic shaking at 24°C. After 1 h, the cells were induced with 0.5 mM IPTG for 1.5 h before 2.5 mCi ³⁵S-methionine were added. After 2.5 h, 1 mM cold L-methionine was added and the incubation proceeded overnight. Cells were harvested and C-subunit prepared as before. Labeled myr-rC α was concentrated to a final volume of 35 μ L in a centricon 30 concentrator (Amicon).

Copurification of ³⁵S-methionine-labeled myr-rC α and native bovine C-subunit

A 400-g sample of fresh bovine heart from a local slaughterhouse, transported on ice and used within 45 min (all steps at 4°C), was first finely chopped using a blender (3 min at low speed) with 600 mL 50 mM potassium phosphate, 0.1% Chaps, 0.5 mM PMSF, 1 mM EDTA, and 100 μ M cAMP, pH 6.5. Two-hundred fifty milliliters of this slurry were substituted with 25 μ L ³⁵S-labeled myr-rC α , and homogenized using an ultra turrax. After a 30-min low-speed centrifugation step, the supernatant was ultracentrifuged at 42,000 rpm using a Ti45 Beckmann rotor for 45 min. One millimolar ATP, 3 mM MgCl₂, another 100 μ M cAMP, and 1 mL PKI-affinity matrix [protein kinase inhibitor peptide PKI(5-24)

coupled agarose (Olsen & Uhler, 1989)] were added to 45 mL of the supernatant and incubated under shaking for 2 h at 4°C. The matrix was washed two times with TMN 50 (Olsen & Uhler, 1989), 250 μ M ATP, and stored overnight at 4°C. The matrix was then transferred to a column and washed again with TMN 200, 250 μ M ATP, 3 mM DTT. C-subunit was eluted with TMN 50 plus 200 mM arginine, 3 mM DTT. In TMN buffers containing DTT, PMSF was omitted. The eluate was diluted fourfold with H₂O and chromatographed into fractions A and B on a Mono S HR5/5 column as before. The A and B peak fractions were concentrated in centricon 30 concentrators and subjected to flat bed IEF using ampholine PAGplate 3.5–9.5 (Pharmacia).

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