IDENTIFICATION OF ε-N-TRIMETHYLLYSINE IN A RAT TESTIS CALCIUM-DEPENDENT REGULATORY PROTEIN OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE

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SUMMARY: A Ca^{++}-dependent regulatory protein (CDR) of cyclic nucleotide phosphodiesterase isolated from rat testis contains 151 residues. Amino acid analysis of this protein showed one residue of an unidentified component which eluted between histidine and lysine. The elution time for this component corresponded to synthetic ε-N-trimethyllysine (TML). Natural-abundance ^{13}C Fourier transform NMR spectra of CDR showed a narrow resonance at 53.8 ppm which is characteristic of the carbon resonance of a trimethylammonium group. A single tryptic peptide of CDR was isolated which contained the residue of ε-N-TML. Digestion of the tryptic peptide with thermolysin yielded a peptide with the following sequence: Leu-Gly-Glu-TML.

A number of proteins have methylated derivatives of arginine and lysine (1). DeLange et al. (2,3) were the first to show that cytochrome C isolated from microorganisms and plants has ε-N-trimethyllysine (TML[4]); Neurospora cytochrome C and wheat germ cytochrome C contain 1 and 2 residues of ε-N-TML, respectively. Huszar (4), Huszar and Elzinga (5) and Juehl and Adelstein (6) have also identified ε-N-TML in the heavy chains of several mammalian cardiac and skeletal myosins. We recently purified a thermostable, low molecular weight protein which would regulate, in a Ca^{2+}-dependent manner, both cyclic nucleotide phosphodiesterase and skeletal muscle actomyosin ATPase (7). More detailed characterization of the rat testis Ca^{2+}-dependent regulator protein (CDR) indicated the presence of an unidentified amino acid which eluted between histidine and lysine on the amino acid analyzer. The purpose of the present report is to show that the unknown compound is ε-N-TML as identified

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4 Abbreviations used: TML, trimethyllysine; CDR, Ca^{++}-dependent regulatory protein

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by co-elution during amino acid analysis and by NMR spectral resonance. We have isolated a tryptic and thermolytic peptide of CDR and based on amino acid sequence data have aligned this peptide to various troponin C's, the calcium binding subunits of troponin (8). TLM replaces the histidine residue in skeletal muscle troponin.

MATERIALS AND METHODS

Isolation of CDR: CDR was isolated from rat testes as described by Dedman et al. (7). The purified protein yielded one major band on polyacrylamide gel electrophoresis in sodium dodecyl sulfate with a molecular weight of about 19,000.

Isolation of ε-N-TML Containing Peptide: CDR (2 μmoles) was dissolved in 10 ml of 0.1 M ammonium bicarbonate, pH 8.0, and was hydrolyzed with 1.0 mg trypsin (TPCK - Worthington) for 6 hours at 23°C. The solution was maintained at pH 8.0 by the manual addition of 1.0 M NH_4OH. The tryptic peptides were fractionated on a column (2.6 x 200 cm) of Sephadex G-50 which was equilibrated with 0.1 M NH_4HCO_3. The column was operated at flow rate of 15 ml/hr and 5 ml fractions were collected. The peptide zone which contained ε-N-TML was further fractionated by ion-exchange chromatography on a column (0.9 x 60 cm) of AA-15 (Beckman Inst., Inc.). The ion-exchange column was operated at 53°C and the effluents were monitored by the ninhydrin reaction after alkaline hydrolysis. The tryptic peptide (800 nmoles) which contained ε-N-TML was further digested with 100 μg thermolysin (EM Laboratories) and the resultant peptides fractionated by a combination of electrophoresis at pH 3.7 and chromatography on paper in pyridine:butanol:acetic acid:water. The peptides were located by spraying the paper with fluorescamine (1 mg/200 ml acetone) and were eluted from the paper with 0.1 M NH_4OH_3, 1% pyridine.

Natural Abundance ^{13}C Fourier Transform NMR Spectra: The spectra were obtained with a Varian XL-100-5 spectrometer and Nicolet TT-100 data system. A pulse sequence of 16 μsec (90°) and 1.35 sec acquisition time was repeated 56,017 times in the quadrature phase detection mode with 8196 real data points. The signal to noise ratio was enhanced digitally. The protein (40 mg) was dissolved in 2 ml of a 20% D_2O/H_2O phosphate buffer (0.01 M NaCl, 0.01 M NaH_2PO_4, 0.001 M EDTA, 0.001 M NaN_3, pH 7.4). The spectra were recorded at 25.2 MHz in a 12 mm tube at 18°C. Chemical shifts, reported in ppm are references to external Me_4Si.

Synthesis of Standard ε-N-Trimethyllysine: ε-N-Trimethyllysine was synthesized by the following procedure. t-BOC-ε-Z-lysine (2 mmole) was hydrogenated using 10% Pd/C as a catalyst under 1 atm. pressure to obtain 2-N-t-BOC-lysine. This was subjected to N-methylation conditions using $NaOH/CH_3I$ in MEOH; the solution was refluxed overnight. The solvent was evaporated under reduced pressure and the t-butoxy carbonyl group was removed with 6 N HCl. The resulting amino acid was desalted on Bio-Gel P-2 in 5% acetic acid. After lyophylization, the amino acid was crystallized from methanol-ethyl acetate. Based on the starting material, the yield was 84%. The retention time on a Beckman Model 121 amino acid analyzer using a single column methodology was 224.8 min. ^{13}C-NMR showed resonances at 67.31, 55.97, 54.44, 54.27, 54.13, 31.25, 23.34, and 22.73 ppm with respect to Me_4Si. The carbonyl resonance was not observed. The three lines at ≃ 54 ppm measured 12.0 Hz at half-height.

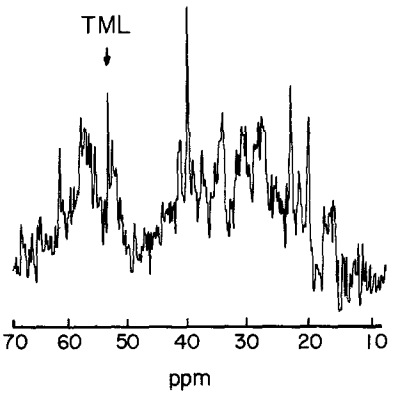

Figure 1: Region of aliphatic carbons in natural abundance ^{13}C Fourier transform NMR spectra of rat testis CDR (see Materials and Methods for details). TML designates the chemical shift of the methyl groups of trimethyllysine.

RESULTS AND DISCUSSION

The amino acid composition of CDR is given in Table I. The protein contains 151 amino acid residues with 0.7 residue of an unknown amino acid which eluted between histidine and lysine at 225.0 min; the unknown component co-eluted with synthetic ε-N-TML. The natural-abundance ^{13}C-NMR proton-decoupled spectrum of CDR (Fig. 1) showed a narrow resonance (line width at half height of 5.8 Hz) at 53.8 ppm. The chemical shift of the trimethylammonium carbons of aqueous N-hexyltrimethylammonium bromide is reported at 54.3 ppm (9) and is comparable to the resonances of the synthetic ε-N-TML described in the present study. Recently, Wilbur and Allerhand (10) reported that Candida krusei cytochrome C, a protein which contains one ε-N-TML at position 72 in the sequence, had a narrow resonance at 54.0 ppm. Eakin et al. (11) also reported values of 53.6 ppm and 54.3 ppm, respectively, for the ferro- and the ferricytochrome C. Based on the finding that a standard preparation of synthetic ε-N-TML eluted with the unknown compound, that the NMR spectra for CDR showed a resonance at ≃ 54 ppm and that the chemical shift and line width are consistent with the synthetic ε-N-TML, we have assigned the unknown amino acid residue as ε-N-TML. An unidentified compound with similar properties to that presented in the present report has been described by Watterson et al. (12) for bovine, porcine,

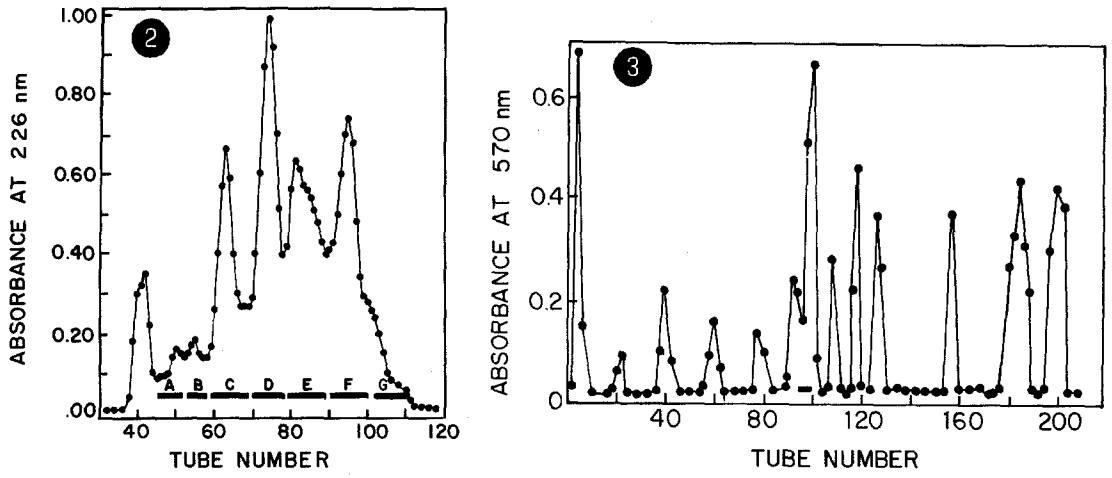

Figure 2: Separation of tryptic peptides of CDR on Sephadex G-50. The details are described in the text. By amino acid analysis, Zone D was the only fraction which contained ε-N-TML.

Figure 3: Separation of peptides in Zone D (Fig. 2) by ion-exchange chromatography. The column (0.9 X 60 cm) of AA-15 (Beckman Inst., Inc.) was equilibrated with the pH 3.1 pyridine-acetate buffer and eluted with a gradient consisting of 500 ml of the pH 3.1 buffer and 500 ml of a pH 5.1 buffer as described by Schroeder (16). The fraction indicated by the solid bar was the only one which contained ε-N-TML.

rabbit, rat and chicken brain CDR and by Stevens et al. (13) for bovine heart CDR.

To localize the single residue of ε-N-TML in CDR, the protein was treated with trypsin and the digest fractionated on Sephadex G-50 (Fig. 2). Seven zones of peptides were detected. Amino acid analysis of each zone indicated that ε-N-TML was present in Zone D. This zone of peptides was further fractionated by ion-exchange chromatography on AA-15 (Fig. 3); only one peptide was detected which contained ε-N-TML. The amino acid composition of this tryptic peptide (Table I) showed that it contained 19 amino acid residues. To isolate smaller peptides, the tryptic fragment was digested with thermolysin and the thermolytic peptides were fractionated by two-dimensional paper electrophoresis and chromatography (Fig. 4). The amino acid composition of the ε-N-TML tetrapeptide is given in Table I. Subtractive Edman degradation of

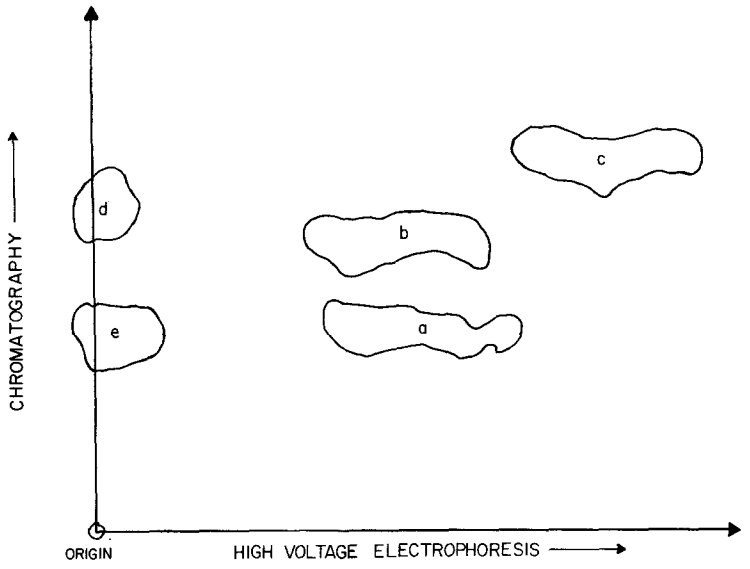

Figure 4: Separation of thermolytic peptides of the ε-N-TML containing tryptic peptide (Fig. 3). The peptides were first subjected to electrophoresis at pH 3.7 and then to chromatography in pyridine:butanol:acetic acid:water (30:45: 9:86). The peptides were located with fluorescamine.

the peptide gave the following: Step 1: Leu, 0.4; Gly, 0.9; Glu, 1.1; TML, 1.0; Step 2: Leu, 0.3; Gly, 0.4; Glu, 0.9; TML, 1.0; Step 3: Leu, 0.3; Gly, 0.4; Glu, 0.6; TML, 1.0. Aminopeptidase M digestion gave one residue of glutamic acid. Thus, the sequence is Leu-Gly-Glu-TML. Based on a detailed sequence analysis of adjacent peptides, the TML containing tetra peptide was aligned with homologous regions of skeletal muscle troponin C's of known sequence as follows:

Rat testis CDR	Leu-Gly-Glu-TML
Rabbit skeletal troponin C (8):	Ser-Gly-Glu-His 125
Chicken skeletal troponin C (14):	Thr-Gly-Glu-His 128

In CDR the biologically rare residue ε-N-TML replaces the single histidine residue found in the skeletal troponin C's.

The functional significance of ε-N-TML in CDR is not known at present. However, we have recently found that metal binding to testis CDR is markedly

TABLE I

AMINO ACID COMPOSITION OF CALCIUM-DEPENDENT REGULATORY PROTEIN OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE AND OF THE ε-N-TRIMETHYLLYSINE CONTAINING TRYPTIC AND THERMOLYTIC PEPTIDES[a]

Amino Acid	CDR	TML-Containing Tryptic Peptide	TML-Containing Thermolytic Peptide
Aspartic Acid	21.7 (22)	3.2 (3)	-
Threonine	10.2 (11)	1.1 (1)	-
Serine	4.1 (4)	-	-
Glutamic Acid	27.6 (28)	4.4 (4)	1.1 (1)
Proline	1.8 (2)	-	-
Glycine	12.2 (12)	1.2 (1)	1.0 (1)
Alanine	11.3 (11)	-	-
Cysteine	-	-	-
Valine	7.8 (8)	1.8 (2)	-
Methionine	8.9 (9)	1.8 (2)	-
Isoleucine	9.0 (9)	0.9 (1)	-
Leucine	9.8 (10)	1.9 (2)	1.0 (1)
Tyrosine	1.8 (2)	-	-
Phenylalanine	7.7 (8)	-	-
Tryptophan	-	-	-
Histidine	1.0 (1)	1.0 (1)	-
Lysine-$(CH_3)_3$ [b]	0.7 (1)	1.0 (1)	0.8 (1)
Lysine	6.9 (7)	-	-
Arginine	5.7 (6)	0.8 (1)	-
Total	151	19	4

a The values were obtained from 24 hr acid hydrolysates and are expressed as the mole amino acid per mole of protein or peptide. The hydrolysates of protein and peptides were analyzed on a Beckman Spinco amino acid analyzer, model 121, equipped with single column methodology.

b Trimethyllysine

distinct from that of skeletal muscle TnC (unpublished observation). Furthermore, TnC has a much lower affinity as compared to CDR in stimulating cyclic nucleotide phosphodiesterase (7). Tyihak et al. (1) have suggested that the methylated lysine residues of proteins may have a significant role in the regulation of cell proliferation. In this regard, Watterson et al. (15) have reported that the levels of CDR are elevated in chicken embryo fibroblast upon transformation by Rous sarcoma virus. Whether the presence of ε-N-TML in CDR is related to the increased amounts of the protein which occurs with transfor-

mation is unknown. It is also neither clear how the methylation occurs nor the effects this modification has on the active state of the protein. Studies are currently in progress to determine the regulation of the biosynthesis of ε-N-TML in CDR.

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