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Carnitine and Calmodulin N-Methylation in Rat Testis: Calmodulin May be Involved in Carnitine Biosynthesis

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Abstract

Rat testis known to contain all of the enzymes required for carnitine biosynthesis also contains high concentrations of calmodulin, a protein which may or may not contain trimethyllysine, the major substrate in carnitine biosynthesis. The purpose of this study was to investigate the levels of carnitine and the state of calmodulin N-methylation in rat testes, and to discuss the possibility of the involvement of calmodulin in carnitine biosynthesis. Nonesterified carnitine, acid soluble acyl carnitine, and acid insoluble acyl carnitine of rat testes were 273 nmole, 62 nmole, and 4 nmole/g tissue, respectively. Total carnitine level was 339 nmole/g testes tissue. Calmodulin purified from rat testes was assayed for methylation potential using N-methyltransferase from the rat testes. Rat testes calmodulin showed no ^{3}H-methyl incorporation indicating that the calmodulin was trimethylated already by endogenous calmodulin N-methyltransferase. Amino acid composition analysis revealed that the rat testes calmodulin contained one mole of trimethyllysine per mole of calmodulin. These data suggest that testes calmodulin could provide the trimethyllysine needed for the synthesis of carnitine in the rat testes.

Key words: carnitine, calmodulin, trimetyllysine, testis

INTRODUCTION

Carnitine (β-hydroxy-γ-trimethyl-amino butyric acid) is ubiquitous non-protein amino acid-like trimethylamine which is synthesized in mammals. It is an essential cofactor for facilitating fatty acid transport into the mitochondrial matrix where β-oxidation takes place (1). Carnitine is synthesized in a sequencial reaction as follows: lysine → protein-bound lysine → protein-bound trimethyllysine → free trimethyllysine → γ-butyrobetaine → carnitine (2,3). LaBadie et al. (4) found that only ε-N-trimethyllysine residues in protein are of value for carnitine biosynthesis, whereas ε-N-mono- and ε-N-dimethyllysine residues once freed by proteolysis do not appear to be further methylated to the trimethyl level and thus can not be utilized for carnitine biosynthesis (5). Since the involvement of protein-bound N-trimethyllysine as a metabolic precursor for carnitine biosynthesis was reported (4), the biological significance of protein methylation has been continuously explored (6).

Calmodulin is a highly conserved, multifunctional calcium-modulated protein that interacts with a variety of enzymes and proteins (7,8). N-methylated lysine in calmodulin was first identified from the amino acid sequence and composition analysis of bovine brain calmodulin (9). This analysis showed that bovine brain calmodulin contains ε-N-trimethyllysine position 115. Since then, many attempts have been made understand the nature of the enzyme responsible for the ecific methylation (10-13) and to gain insight into the biogical relevance of the modification (14-18). The biological role of calmodulin methylation in mammals is, however, unclear. With respect to the regulatory activities of calmodulin, unmethylated calmodulins are not significantly different from methylated calmodulins in their ability to bind calcium and activate enzymes (10,19). However, one calmodulin-dependent enzyme, plant NAD kinase, is sensitive to calmodulin methylation (14).

Because rat testes contain all of the enzymes of carnitine biosynthesis (20,21) it was postulated that, if calmodulin in rat testes contain trimethylated lysine residues, the degradation of testes calmodulin might provide a local labile source of trimethyllysine for local carnitine biosynthesis. Carnitine fractions in rat testes and liver and the state of calmodulin methylation in rat testis were determined and the possible involvement of calmodulin in carnitine biosynthesis in rat testes was discussed.

MATERIALS AND METHODS

Animals

Five, normal adult (300 g body weight) male Sprague-Dawley rats (Institute of Bioscience and Biothecnology, Taechon, Korea) were used in this study. There were no special treatments or diets provided the animals. Aniaml care and use in this study complied with the guidelines as provided by the Guide for the Care and Use of Laboratory Animals (22).

Chemicals

[1-^{14}C]acetyl CoA and [^{3}H-methyl]S-adenosylmethionine

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were purchased from Amersham (Buckinghamshire, England). Carnitine acethyltransferase, Dowex 1×8, DEAE-cellulose, CM Sephadex, calmodulin agarose, and phenyl Sepharose were purchased from Sigma (St. Louis, USA). All chemicals and reagents used were of the highest grade commercially available.

Carnitine analysis

Rat testis or liver (100 mg each) was homogenized with glass homogenizer in 1 ml of 0.3 mol/L perchloric acid, and was centrifuged in a microfuge for 10 min at 4000 rpm and supernatant collected. The fractionation and quantitation of carnitines in the supernatants were performed by a modified method of Cederblad and Lindstedt (23). Briefly, 100 µl of sample was mixed with 100 µl of 8% bovine serum albumin and 200 µl of 0.6 mol/L perchloric acid and the mixture vortexed. The samples were centrifuged in a microfuge for 10 min at 1500 rpm. The supernatants were used for the determination of nonesterified carnitine (NEC) and acid soluble acylcarnitine (ASAC), and the pellets were used for the determination of acid insoluble acylcarnitine (AIAC). For ASAC, acylcarnitine in the supernatants was hydrolyzed with 125 µl of 0.5 N KOH and then carnitine was assayed. ASAC was calculated by substracting NEC from the KOH hydrolyzed sample carnitine. For AIAC determination, the pellets were washed twice with 0.6 mol/L perchloric acid and the pellets were resuspended by 200 µl of 0.5 N KOH to hydrolyzed acylcarnitine, and then carnitine was assayed. Radiometric carnitine assay procedures were done as previously described (24). Reaction mixtures (total volume of 500 µl) contained carnitine fraction (100 µl), 40 µmol/L [1-^{14}C] acetyl CoA (0.025 µCi), 4 µmol/L ethylene glycol bis-(β-aminoethyl ether) N, N, N', N'-tetraacetic acid (EGTA), 4 µmol/L sodium tetrathionate and 25 mmol/L 3-(N-morpholino)prophanesulfonic acid (MOPS) (pH7.4). The reaction was initiated by the addition of carnitine acetyltransferase and the mixture was incubated for 30 min at 37°C. ^{14}C acetyl carnitine was separated from the other reaction components by ion-exchange column with Dowex 1×8 resin and counted in a Beckman model LS 3801 liquid scintillation counter. L-carnitine for the determination of NEC and ASAC and L-palmityl carnitine for the determination of AIAC were used as standard for the carnitine determination.

Protein purification and analysis

Calmodulins used in this study were purified from rat testes, tobacco plants, and *E. coli* carrying calmodulin expression vectors (14) by hydrophobic phenyl-Sepharose column chromatography of Gopalakrishna and Anderson (25). SDS-PAGE of the fractions of the phenyl-Sepharose columns was done by using the method of Laemmli (26). EGTA (1 mmol/L) was added to the gel for the analysis to prevent the anomalous migration of calmodulin (27). The fractions containing calmodulin were pooled and dialyzed against 20 mmol/L NH_4HCO_3 and then extensively against deionized water. The dialized sample was frozen at -80°C and then lyophilized in an acid washed container. The lyophilized calmodulin was resuspended in of 10 mmol/L NH_4HCO_3 and was stored frozen at -80°C. Calmodulin methyltransferase was purified from rat testes by a combined protocol of the procedures of Rowe et al. (10) and Han et al. (12). The enzyme was initially purified by homogenization using Biohomogenizer (Biospec Products Inc., USA) and differential ammonium sulfate precipitation (35-70%). The sample was then chromatographed on DEAE-cellulose, CM-Sephadex, and calmodulin-agarose. Active fractions were pooled and concentrated by pressure ultrafiltration (Amicon PM-10 membrane), and quickly frozen to -80°C. For methylation analyses, a radiometric assay (28) based on purified calmodulin methyltransferase was used. The reaction mixtures for the assay contained, in 100 µl final volume, calmodulin (0.8 µg), 12 µmol/L [^{3}H-methyl]S-adenosylmethionine (0.5 µCi), 0.1 mol/L NaCl, 2 mmol/L $MgCl_2$, 5 mmol/L dithiothreitol (DTT), 0.01% (w/v) Triton X-100, 0.1 mmol/L $CaCl_2$, 0.1 mol/L glycylglycine-NaOH, pH 8.0. Reactions were initiated by the addition of enzyme. After 20 min reaction at 37°C, the reaction was terminated by heating at 90°C for 3 min. Then 200 µl of phenyl-Sepharose slurry in 50 mmol/L Tris-HCl buffer (pH 8.0) containing 0.1 mmol/L $CaCl_2$, 0.3 mol/L NaCl were added. After vortexing at 5 min intervals, the samples were centrifuged and the resin was collected. Then the resin was washed with 4 ml of 50 mmol/L Tris-HCl buffer (pH8.0), 0.1 mmol/L $CaCl_2$, 0.3 M NaCl. [^{3}H]calmodulin was eluted with 0.1 mol/L NH_4HCO_3 (pH8.0), 2 mmol/L ethylenediaminetetraacetic acid (EDTA) and radioactivity was measured by liquid scintillation counter (Beckman LS 3801).

Statistical analysis

The data are expressed as the mean with ± standard deviation. Statistical significance between rat testis and liver were analyzed by Student's t-test ($p<0.05$ considered significant) (29).

RESULTS

To compare the levels of carnitine in testes with those in liver, the best known as a carnitine synthesizing organ the carnitine contents of rat testes and liver were determined. The levels of testes and liver total carnitine were 339 nmole and 444 nmole/g tissue, respectively (Table 1). Previously, Rowe et al. (10) showed that rat testis have the highest calmodulin and calmodulin N-methyltransferase among seven rat tissues. The purifications of calmodulin and calmodulin N-methyltransferase from rat testes were performed to test the state of calmodulin N-methylation. Calmodulins from rat testes and from other systems including tobacco plants and *E. coli* carrying calmodulin expression plasmid were purified by Ca^{2+}-dependent hydrophobic chromatography on phenyl-Sepharose column. SDS-PAGE analysis of the fractions of the phenyl-Sepharose column

Table 1. Carnitine concentrations in rat testis and liver[1]

Carnitine	nmole/g testis	nmole/g liver
Nonesterified carnitine	273.4±15.8[a]	322.0±21.2[b]
Acid soluble acylcarnitine	61.6±13.6[a]	115.4±11.2[b]
Acid insoluble acylcarnitine	3.7± 0.4[a]	7.0± 2.0[b]
Total carnitine	338.7±14.4[a]	444.4±13.8[b]

[1]All values are means±SD of five rats.
Values with the different superscripts within a row are significantly different ($p<0.05$).

show that the only detectable protein was calmodulin (Fig. 1). Calmodulin N-methyltransferase was purified 957-fold with 22% yield from rat testes through ammonium sulfate precipitation and chromatography on DEAE-cellulose, CM-Sephadex and calmodulin-agarose (Table 2).

The ability of rat testes calmodulin, tobacco calmodulin, recombinant DNA-derived calmodulin (lys 115), and calmodulin mutants (arg 115, lys to arg 115; ile 115, lys to ile 115) to serve as substrates for the methyltransferase was tested by using the radiometric assay (28). The recombinant calmodulin that contains an unmethylated lysine at position 115 serves as a good substrate for the methyltransferase (Table 3). However, mutant calmodulins and rat testes calmodulin are no longer substrates for the calmodulin methyltransferase (Table 3). These data suggest that the calmodulin methyltransferase from rat testes is highly specific for the position of 115 of calmodulin and that the rat testes calmodulin contains trimethyllysine at position 115. The analysis of amino acid composition confirmed that the rat testes calmodulin contains one trimethyllysine (data not shown).

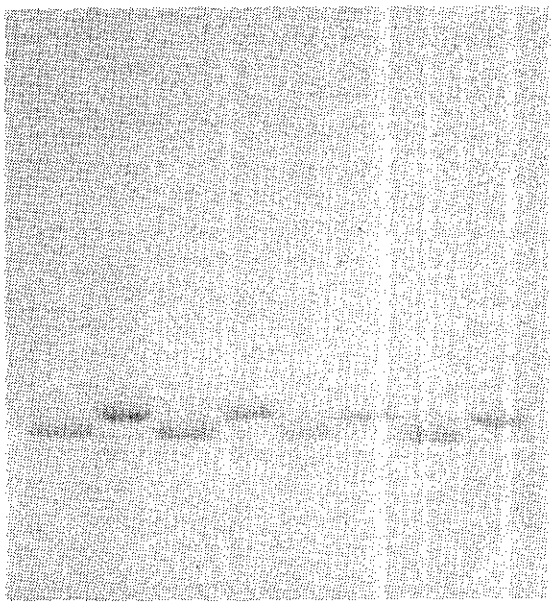

Fig. 1. SDS-PAGE analysis of the purified calmodulin by phenyl-Sepharose column chromatography. 10 μl of sample mixtures were loaded to the wells of 15% SDS-polyacrylamide gel containing 1 mmol/L EGTA. After electrophoresis, the proteins were stained with Coomassie Brilliant Blue. Lane 1, 3, 5 and 7, tobacco calmodulins; lane 2, rat testes calmodulin; lane 4, recombinant DNA-derived calmodulin (lys 115); lane 6 and 8, recombinant DNA-derived calmodulin mutants (arg 115 and ile 115, respectively). Testes calmodulin, recombinant DNA-derived calmodulin and calmodulin mutants show slower mobility than tobacco calmodulins. Previously, it was shown that animal calmodulins, recombinant DNA-derived calmodulin and the calmodulin mutants show slower mobility than plant calmodulins (27,28).

Table 2. Purification of calmodulin methyltransferse from rat testes with recombinant DNA-derived calmodulin as a substrate

Step	Total protein (mg)	Total activity[1] (pmole/min)	Specific activity[2]	Purification fold	Recovery (%)
Crude extract	559	14,532	26	1	100
$(NH_4)_2SO_4$ 35~75%	247	13,944	56	2.2	95
DEAE-cellulose	31.8	10,062	316	12.2	69
CM-Sephadex	8.8	6,663	757	29	45
Calmodulin-agarose	0.13	3,234	24,876	957	22

[1]Expressed as pmole of [^{3}H]-methyl groups incorporated into recombinant calmodulin (lys 115) per min.
[2]Standardized to mg of protein in each sample.

DISCUSSION

N-methylation is one of the major posttranslational modifications. It has been demonstrated that only protein-bound lysine is valuable for trimethyllysine and carnitine biosynthesis in mammalian systems (4). Protein N-methyltransferase catalyzes the methylation of specific residues within specific recognition sites (6). ε-N-trimethyllysine is a posttranslational modification that is found at position 115 of many calmodulins (9,10- 13). The lysine 115 is located in between the third and the fourth calcium binding domain, is the most solvent exposed lysine residue, and has no interaction with other residues in the structure (30). Methylated amino acid residues such as ε-N-trimethyllysine are released as free amino acids

Table 3. Substrate specificity of various proteins for the calmodulin methyltransferase measured by a radiometric assay[1]

Substrate	^{3}H-methyl incorporated (dpm/30 min)[2]
Tobacco calmodulin	3,200± 65
Recombinant calmodulin (lys 115)	43,000±980
Rat testes calmodulin	N.D.
Recombinant calmodulin (arg 115)	N.D.
Recombinant calmodulin (ile 115)	N.D.

[1]0.8 μg of each calmodulin was incubated with 12 μmol/L [^{3}H-methyl] S-AdoMet (0.5 μCi per reaction) and 1.0 μg of purified calmodulin N-methyltransferase from five rat testes.
[2]N. D., not detectable; plus calmodulin reaction was indistingushable from blank. Values are the means of four independent determinations with the standard deviation of the mean.

when methylated proteins are subjected to metabolic breakdown. Calmodulin can be coupled by coupling to ubiquitin *in vitro* in the presence of micromolar calcium concentrations (17,18). However, calmodulin with trimethyllysine was not coupled to ubiquitin in the absence of calcium (15,16). These observations suggest that : i) the Ca^{2+}-bound form of calmodulin is preferred by calmodulin N-methyltransferase; ii) the methylated calmodulin can be degraded by the cytoplasmic ubiquitin-dependent proteolytic pathway.

The data showing that a substantial amounts of carnitine is found in rat testes (similar, though less, to the concentrations in liver, the best known carnitine synthesizing organ) raises the following interesting question: what are the roles of carnitines in testis? Testis is one of the most important organs characterizing male. The sex hormone testosterone is synthesized in testes. Therefore, testis should have all the materials for the sex hormone production. It is known that testosterone can be produced from acetyl CoA via cholesterol (31). Since carnitine provides a means by which long chain fatty acids enter mitochondria, where they are oxidized to provide a metabolite, acetyl CoA, and carnitine also buffers the acylation state of the mitochondrial CoA by providing a means of removing the excess acetyl and long chain acyl groups from the mitochondrial matrix (32), it can be expected that carnitine in testes may have a role for testosterone production. Trimethyllysine released from protein is the precursor of carnitine (2-4). Although calmodulin is one of major proteins found in rat testes (7,33) and rat testes calmodulin has trimethyllysine (this study), the question that remains to be answered in future studies is what other methylated proteins can provide trimethyllysine to meet the needs of carnitine biosynthesis. Other naturally occuring proteins with ε-N-trimethyllysine are histone, cytochrome C, myosin, and ribosomal proteins (5,6). However, the methylation state of these proteins in rat testes is not determined yet. Interestingly, it has been shown that calmodulin also stimulates lysine methylation in other proteins (34,35). For example, Siegel and Wright (34) found that calmodulin stimulates the *in vitro* N-methylation of several proteins in cytosolic extracts of testis, liver and kidney. In rat testes, calmodulin stimulated the methylation of M_r 24,000, 47,000 and 48,000 proteins. Calmodulin, also, stimulated the methylation of M_r 29,000, 32,000 and 45,000 proteins in rat liver and kideny. Thus, it can be suggested that calmodulin may have a role in providing trimethyllysine for carnitine synthesis in rat testes and other tissues by both providing trimethyllysine from its own degredation as well as by stimulating lysine methylation in other proteins.

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