Identification of the Primary Structure and Post-translational Modification of Rat *S*-Adenosylmethionine Decarboxylase

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The coding region nucleotide sequences of rat, hamster, and bovine *S*-adenosylmethionine decarboxylase (AdoMetDC) cDNA exhibit over 90% homology with the human sequence. No N-terminal amino acid could be detected when either bovine or rat AdoMetDC was subjected to Edman degradation, suggesting that the β-subunit must be blocked since the pyruvate residue is located at the amino terminus of the α-subunit. In this study, we present the primary structure, including post-translational modification, of rat prostate AdoMetDC. Our strategy was to compare the molecular masses of peptides produced by five specific cleavage methods with peptides expected from the known cDNA-derived amino acid sequence of rat AdoMetDC using matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). All AdoMetDC peptide fragments produced by the five cleavage methods could be assigned to theoretical peptides based on the rat cDNA sequence except for the peptides containing the N-terminus of the β- and α-subunits. The N-terminus of the α-subunit was assigned as pyruvoyl peptide. Liberation of acetylmethionine was demonstrated when the peptide containing the β-subunit N-terminal amino acid obtained by lysylendopeptidase digestion was reacted with acylamino acid-releasing enzyme. Furthermore, N-terminal acetylation of the β-subunit was confirmed by MALDI-post source decay analysis. In conclusion, the results of the present study on amino acid full sequence of rat prostate AdoMetDC determined by the combination of five specific cleavage methods demonstrate that the N-terminus of the β-subunit is acetylated, and the expected amino acid sequence based on the rat AdoMetDC cDNA sequence is correct.

Key words *S*-adenosylmethionine decarboxylase; primary structure; N-terminal acetylation; post-translational modification; matrix assisted laser desorption ionization time-of-flight mass spectrometry

S-Adenosylmethionine decarboxylase (AdoMetDC, EC4.1.1.50) is an essential enzyme for the biosynthesis of the polyamines spermidine and spermine, which are required for normal cell proliferation and differentiation.[1—3] AdoMetDC catalyzes the removal of the carboxylate group from *S*-adenosylmethionine (AdoMet) to form decarboxylated *S*-adenosylmethionine (deAdoMet). deAdoMet is the source of aminopropyl groups for the synthesis of spermidine and spermine. AdoMetDC is one member of a small class of enzymes that contain a covalently bound pyruvate prosthetic group.[4] All known AdoMetDCs are synthesized in proenzyme forms that subsequently undergo an intramolecular cleavage at a serine residue to generate the two subunits.[2,5] In this process, the serine residue is converted to a pyruvoyl group. The cleavage site of human AdoMetDC is between Glu-67 and Ser-68 (underlined) in the sequence-Leu-Ser-Glu-Ser-Ser-Met-.[6] Proenzyme processing generates two non-identical subunits, termed β and α, which are both indispensable components of the mature enzyme.[6—8]

The coding region nucleotide sequences of rat,[7,9] hamster,[10] and bovine AdoMetDC cDNA exhibited over 90% homology with the human sequence.[7] The deduced amino acid sequences are also very similar, having only 3—7 amino acid differences. No amino acid full sequence information is available for AdoMetDC except for sequences of some peptides from lysylendopeptidase digestion of the bovine enzyme.[11]

Two apparently different forms of AdoMetDC have been purified from rat liver and rat psoas muscle, with differences observed in isoelectric point (p*I*), extent of activation by putrescine, *S*-adenosylmethionine substrate K_m, and methylglyoxal bis(guanyl)hydrazone affinity,[12] suggesting differences in post-translational modification.[5] In addition, no amino terminal residue could be detected when either the bovine[11] or rat enzyme was subjected to Edman degradation, suggesting that the β-subunit must be blocked by modification,[13] since the pyruvate residue is located at the α-subunit amino terminus.

We recently reported the primary structure of spermidine synthase purified from rat prostate using matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) coupled with three specific cleavage methods combined with Edman degradation for sequence analysis.[14] This is a useful technique for ascertaining the known cDNA-derived amino acid sequence of certain proteins using a very small amount of the homogeneous form. Therefore, we applied this method to examine the primary structure of rat prostate AdoMetDC.

Here, we present the primary structure, including post-translational modification, of purified rat prostate AdoMetDC. We also report results of experiments to determine whether the seven cysteine residues of rat AdoMetDC exist in the sulfhydryl or disulfide form.

MATERIALS AND METHODS

Chemicals Dithiothreitol (DTT), 2-nitro-5-thiocyanobenzoic acid (NTCB), tris(2-carboxyethyl) phosphine hydrochloride (TCEP), 2,5-dihydroxybenzoic acid (DHB), and α-cyano-4-hydroxycinnamic acid (CHCA) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). *S*-Adenosyl [carboxy-^{14}C] methionine was obtained from GE Healthcare (Little Chalfont, U.K.). Lysylendopeptidase (*Achromobactor* protease I) was obtained from Wako Pure Chemical Indus-

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tries (Osaka, Japan). V8 protease (*Staphylococcus aureus* protease) was purchased from ICN Pharmaceuticals Inc. (Aurora, OH, U.S.A.), and modified trypsin (sequencing grade) from Promega (Madison, WI, U.S.A.). Arginylendopeptidase (mouse submandibular protease) and acylamino acid-releasing enzyme (AARE)[15,16] were obtained from Takara Bio Inc. (Shiga, Japan). Double-distilled deionized water (Milli-Q, Millipore, Bedford, MA, U.S.A.) was used for HPLC and MALDI-TOF MS. All other reagents and organic solvents were of commercial analytical grade. 5′-{[(*Z*)-4-Amino-2-butenyl]amino}-5′-deoxyadenosine (MDL74038) was synthesized according to the method.[17]

Purification of AdoMetDC from Rat Prostate Glands AdoMetDC was purified from rat prostate glands as described previously.[12] Enzyme activity was measured using *S*-adenosyl [carboxyl-^{14}C] methionine as a substrate,[18] and protein concentration was measured using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.) using bovine serum albumin (Sigma-aldrich, St. Louis, MO, U.S.A.) as a standard protein. Purified AdoMetDC (about 1.9 μmol/mg/min) was stocked in 10 mM Tris–HCl buffer (pH 7.5) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA), 2.5 mM DTT, and 2.5 mM putrescine at 0 °C.

Protease Digestion Purified AdoMetDC (0.28 nmol) was denatured and reduced in a solution containing 6 M guanidine hydrochloride, 21 mM DTT, 10 mM EDTA, and 500 mM Tris–HCl buffer (pH 8.5), under N_2 for 2 h. A monoiodoacetic acid solution was added to the reaction mixture at a final concentration of 45 mM. The carboxymethylation reaction proceeded for 30 min at room temperature, and then 2-mercaptoethanol was added to halt the reaction. The reaction mixture was desalted by extensive dialysis against a buffer needed for protease digestion described below. Carboxymethylated AdoMetDC was digested for 18 h at 37 °C by four proteases, lysylendopeptidase, arginylendopeptidase, trypsin, and V8 protease. The buffer for lysylendopeptidase digestion contained 10 mM Tris–HCl buffer (pH 8.5), 1 mM EDTA, and 5% (v/v) acetonitrile. The molar ratio of AdoMetDC to protease was 100 : 1. Arginylendopeptidase buffer contained 50 mM Tris–HCl buffer (pH 8.0). The molar ratio of AdoMetDC to arginylendopeptidase was 10 : 1. For trypsin digestion, the buffer contained 50 mM ammonium carbonate, and had a weight ratio of AdoMetDC to trypsin of 40 : 1. Finally, for V8 protease, the digestion buffer contained 100 mM ammonium carbonate, and had a molar ratio of AdoMetDC to V8 protease of 30 : 1.

Chemical Cleavage at Cysteine Residues by NTCB Cleavage at cysteine residues by NTCB was carried out basically according to the literature.[14,19]

Transamination of Pyruvate Prosthetic Group To analyze the N-terminus of the AdoMetDC α-subunit using a gas phase sequencer, AdoMetDC was transaminated according to the literature.[8] We used an irreversible inhibitor, MDL74038, by which the α-subunit is believed to be transaminated to an alanine residue.[8,20]

Reversed-Phase HPLC and AARE Treatment Peptides of purified AdoMetDC obtained from digestion with lysylendopeptidase were separated on a reversed-phase HPLC system using a TSK gel ODS 120T column (4.6 mm i.d. ×250 mm) (Tosoh Co., Ltd., Tokyo, Japan) and a linear gradient between 5% acetonitrile containing 0.1% trifluoroacetic acid (TFA) (solvent A) and 80% acetonitrile containing 0.1% TFA (solvent B), from 0% B to 75% B over 90 min (flow rate: 1 ml/min; detection: 220 nm). Then, 50 μl of 10 mM Tris–HCl buffer (pH 7.5) containing 0.1 mM EDTA, 2.5 mM DTT, 2.5 mM putrescine, and AARE (*ca.* 7.5 munits) was added to the peptide fraction obtained by reversed-phase HPLC. The mixture was incubated for 16 h at 37 °C.

Carboxymethylation of Cysteine Residues to Identify the Existence of Disulfides Purified rat prostate AdoMetDC (0.58 nmol) was treated with or without 21 mM DTT at room temperature for 2 h under N_2 in a 400 μl solution containing 6 M guanidine hydrochloride, 10 mM EDTA, and 500 mM Tris–HCl buffer (pH 8.5). Monoiodoacetic acid was added to a concentration of 45 mM, and the solution was incubated in the dark for 1 h at room temperature. The reaction mixture was divided in two equal parts, each of which was digested by lysylendopeptidase and trypsin as described above.

Sample Preparation by ZipTip$_{C4}$ and ZipTip$_{C18}$ Proteins and peptides were extracted with ZipTip$_{C4}$ and ZipTip$_{C18}$ (Millipore, Bedford, MA, U.S.A.), respectively, desalted by washing with 0.1% TFA, and eluted with 80% acetonitrile containing 0.1% TFA.

Mass Spectrometry MALDI mass spectra were obtained in reflector mode on a Finnigan MAT Vision 2000 TOF mass spectrometer (ThermoQuest, San Jose, CA, U.S.A.) using DHB as the matrix. The peaks with the mass error between the observed and theoretical mass lower than 1 Da were selected from the detected peaks with signal to noise (S/N) higher than 5. The theoretical mass was calculated using EXPASy-PeptideMass. MALDI-post source decay (PSD) spectra were obtained in reflector mode on an AXIMA-CFR (Shimadzu/Kratos, Manchester, U.K.) using CHCA as the matrix. Ionization was achieved by irradiation with a nitrogen laser (337 nm). The mass spectra were calibrated with angiotensin I (MW 1296.5), substance P (MW 1347.6), insulin B (MW 5733.5), and cytochrome *c* (MW 12360.1).

Protein Sequencing Excess inhibitor was removed from the reaction mixture and sample protein was concentrated by ultrafiltration using a Microcon YM-10 column (Millipore, Bedford, MA, U.S.A.). The concentrated sample was sequenced using a Shimadzu PPSQ-21A gas-phase sequencer (Shimadzu, Kyoto, Japan).

RESULTS AND DISCUSSION

The MALDI-TOF MS spectrum of AdoMetDC purified from rat prostate is shown in Fig. 1. Two main peaks were observed. The peak at *m*/*z* 30498.8 was close to the calculated value of 30503.9, corresponding to the molecular weight of the α-subunit of rat AdoMetDC in which Ser-68 has been converted into a pyruvate residue. However, the major observed peak at *m*/*z* 7678.9 was 42.4 daltons greater than the calculated value of 7636.5 for the β-subunit of rat AdoMetDC. This result suggests the possibility of an error in the expected amino acid sequence or a post-translational modification in the β-subunit.

In order to identify the primary structure of rat prostate AdoMetDC, including any post-translational modification, we performed micro analyses using MALDI-TOF MS for the

peptide fragments liberated by five specific cleavage methods, namely, lysylendopeptidase digestion, arginylendopeptidase digestion, trypsin digestion, V8 protease digestion, and a chemical method using NTCB for cleavage at the cysteine residues. Peptides obtained by these cleavage reactions were compared with the calculated masses of expected peptides (Fig. 2). Most of the fragments could be assigned to theoretical peptides based on the rat cDNA sequence, except for the peptides containing the N-terminus of the α- and β-subunits, although the N-terminus of the α-subunit was assigned as pyruvoyl peptide. In addition, a peak at an *m*/*z* value 42 daltons greater than the calculated value of the β-subunit N-terminal peptide was observed in all cleavage reactions. These results suggest that the N-terminus of the β-subunit is acetylated.

We also examined the N-terminus of the α-subunit. Because the pyruvate residue is located at the α-subunit amino terminus, the amino acid sequence of the α-subunit cannot be determined by Edman degradation. It has been reported that the pyruvoyl group of recombinant human AdoMetDC is transaminated through inactivation by the substrate *S*-adenosylmethionine itself or one type of irreversible inhibitor having a *cis*-amino-2-butenyl moiety.[8,20] Therefore, we analyzed the enzyme with a gas phase sequencer after inactivation by treatment with a similar irreversible inhibitor, MDL74038. The detected amino acid sequence corresponded to the expected amino acid sequence of rat AdoMetDC cDNA with Ser-68 converted into alanine (Fig. 2).

We then confirmed the sequence of the N-terminus of the β-subunit. One of the peptides obtained by lysylendopeptidase digestion, peptide K1, with an observed *m*/*z* of 1438.8, corresponded to M1-K12 (containing acetylated M1, calculated *m*/*z* 1438.6). Peptide K1 was separated by reversed-phase HPLC and subjected to reaction with AARE. AARE specifically cleaves the *X*–*Y* bond of *R*CO-*X*-*Y*-type peptides shorter than about 40 residues (*R*: alkylgroup, *X*/*Y*: L-amino acid).[16] Liberation of acetylmethionine from peptide K1 was clearly demonstrated (Fig. 3). In addition, the N-terminal acetylation of the β-subunit was confirmed by MALDI-PSD analysis. As shown in Fig. 4, the fragment ions (y-series) at *m*/*z* 1396.30 (y12) and 1266.08 (y11) provided sufficient confirmation of the formation of acetylated Met.

Rat AdoMetDC has six cysteine residues (C82, C148, C156, C226, C292, C310) in the α-subunit, and one cysteine residue (C49) in the β-subunit. To examine whether these seven cysteine residues exist in the sulfhydryl or disulfide state, rat AdoMetDC was carboxymethylated with monoiodoacetic acid in the presence or absence of DTT, and then subjected to lysylendopeptidase and trypsin digestion. Among the peptides obtained by lysylendopeptidase digestion, five peptides corresponding to D46-K56 (containing carboxymethylated C49, calculated *m*/*z* 1250.6), T81-K89 (containing carboxymethylated C82, calculated *m*/*z* 1007.5), D202-K234 (containing carboxymethylated C226, calculated *m*/*z* 3617.1), C292-K301 (containing carboxymethylated C292, calculated *m*/*z* 1176.6) and R307-K327 (containing carboxymethylated C310, calculated *m*/*z* 2563.9) were detected in both the presence and absence of DTT. In addition, among the peptides obtained by trypsin digestion, five pep-

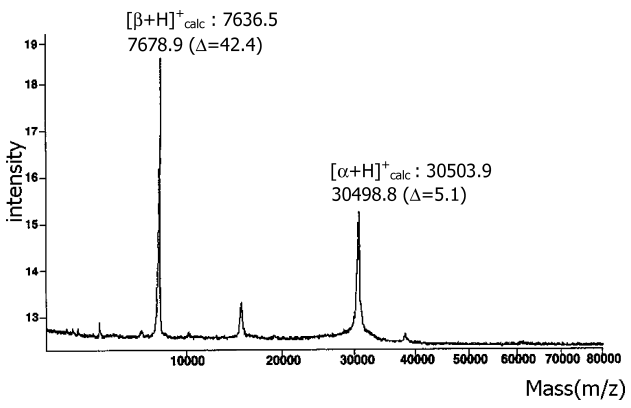

Fig. 1. MALDI-TOF MS Spectrum of Purified AdoMetDC from Rat Prostate

$[\alpha+H]^+_{calc}$ indicates the calculated mass of the α-subunit based on rat AdoMetDC with Ser-68 converted into a pyruvate residue. $[\beta+H]^+_{calc}$ indicates the calculated mass of the β-subunit based on the rat AdoMetDC sequence.

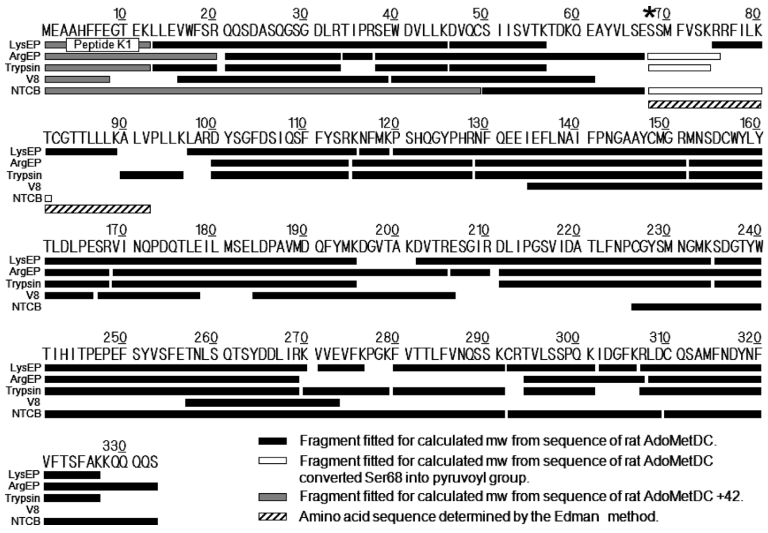

Fig. 2. Amino Acid Sequence of AdoMetDC Based on the Rat cDNA Sequence and MALDI-TOF MS Data from Purified Rat Prostate AdoMetDC

Asterisk indicates the site of pyruvate residue formation in the self-cleavage reaction. LysEP, lysylendopeptidase digestion; ArgEP, argynylendopeptidase digestion; V8, V8 protease digestion; NTCB, chemical method using NTCB.

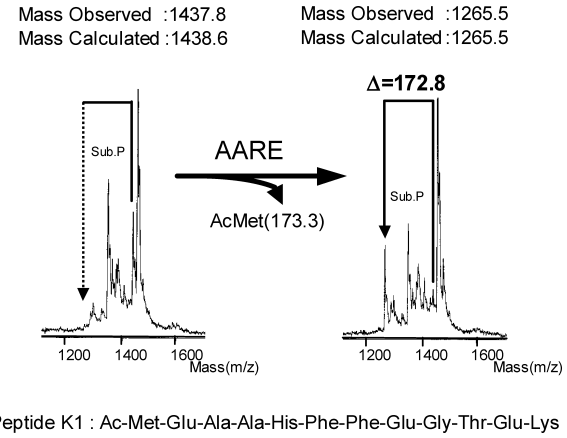

Fig. 3. Liberation of Acetylmethionine from the Peptide K1 by Reaction with AARE

The lysylendopeptidase digestion product of purified rat prostate AdoMetDC, peptide K1, was incubated with AARE as described in Materials and Methods. The incubation mixture was analyzed by MALDI-TOF MS.

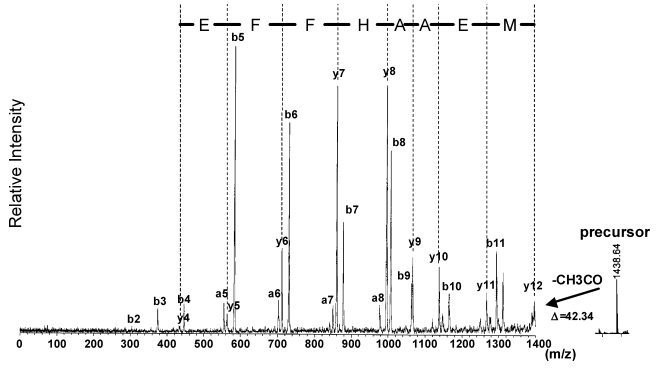

Fig. 4. MALDI-PSD Spectrum of the Peptide Containing the β-Subunit N-Terminus of AdoMetDC, Derived from Tryptic Digestion

tides corresponding to D46-K56 (containing carboxymethylated C49, calculated *m*/*z* 1250.6), N129-R151 (containing carboxymethylated C148, calculated *m*/*z* 2694.0), M152-R168 (containing carboxymethylated C156, calculated *m*/*z* 2163.9), D211-K234 (containing carboxymethylated C226, calculated *m*/*z* 2603.0), and L308-K327 (containing carboxymethylated C310, calculated *m*/*z* 2406.0) were detected in both the presence and absence of DTT. These results indicate that all cysteine residues of rat prostate AdoMetDC exist in the sulfhydryl form.

We observed that purified rat prostate AdoMetDC stored for more than four months on ice formed a disulfide bound between C148 and C156. The three-dimensional structure of human AdoMetDC indicates that C148 is situated close to C156.[21,22] Our result suggests that C148 is also situated close to C156 in solution. This phenomenon occurred in a test tube, but the disulfide bond between C148 and C156 could also be formed within the cell depending on the redox condition.

In conclusion, the results of the present study on amino acid full sequence of rat prostate AdoMetDC determined by the combination of five specific cleavage methods demonstrate that the N-terminus of the β-subunit is acetylated, and the expected amino acid sequence based on the rat AdoMetDC cDNA sequence is correct, indicating the existence of AdoMetDC protein having no post-translational modifications other than N-terminal acetylation of the β-subunit in rat prostate.

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