Amino Acids (2008) 35: 355–358
DOI 10.1007/s00726-007-0613-1
Printed in The Netherlands

Amino Acids

Identification and characterisation of arsenite (+3 Oxidation State) methyltransferase (AS3MT) in mouse neuroblastoma cell line N1E-115

J. P. P. John, J.-E. Oh, A. Pollak, and **G. Lubec**

Department of Pediatrics, Medical University of Vienna, Vienna, Austria

Received August 23, 2007
Accepted October 1, 2007
Published online December 21, 2007;

Summary. Handling and detoxification of metals by enzymes is a major issue that is not in the focus of current biomedical research concepts. The finding of the presence of arsenic (+3 Oxidation State) methyltransferase (AS3MT) in neuroblastoma cells NE-115 as a high abundance protein made us investigate primary structure of AS3MT reflecting an example of metal-handling in eucaryotes. Proteins extracted from NE-115 cells were run on 2-DE followed by two different mass spectrometrical methods. High sequence coverage was obtained by multiple protease digestion and a sequence conflict was solved at arginine 335.

These findings are important when future studies on this enzyme are designed at the protein level and in particular, when antibodies against this protein will be generated.

Keywords: Arsenite methyltransferase – MALDI-TOF-TOF – Nano-LC-ESI-MS/MS – Neuroblastoma cells

Introduction

Methylation of inorganic arsenic (iAs) to methylarsonic acid (MMA) and dimethylarsinic acid (DMA) has been considered to be the major pathway of inorganic arsenic biotransformation and detoxification (Crecelius, 1977). Methylated products of inorganic arsenite biotransformation were initially described by Challenger while characterizing arsenic metabolism in fungi (Challenger, 1951). Among several arsenite methyltransferases that have been identified, arsenic (+3 oxidation state) methyltransferase (AS3MT) is the best characterized at the genetic and functional level. Recent evidence shows that a protein AS3MT catalyzes the multi-step process that converts iAs to its methylated metabolites (Lin et al., 2002; Waters et al., 2004); i.e. this enzyme catalyses S-adenosylmethionine (AdoMet)-dependent methylation and the reduction of As^{V} to As^{III}. Like other arsenate reductases (Mukhopadhyay and Rosen, 2002), it uses thioredoxin or glutaredoxin to reduce AS^{V} to As^{III}. The gene for mouse arsenite methyltransferase is localised on mouse chromosome19D1. In the mouse AS3MT is expressed in different tissues, such as kidney, liver, lung and testis (Healy et al., 1998). It consist of 376 amino acids and predicted molecular weight is 41792.66 Da. Nucleotide sequences were reported by Phillips et al. (1999) and Gerhard et al. (2004) and there are sequence differences found in the standard databases (http://www.expasy.org/uniprot/Q91WU5; release 39; July 2007). Healy et al. (1998) reported that specific activity of this enzyme in the testis was 3.6 times greater than that of the liver and the specific activity of the kidney was 1.8 times greater than that of the liver in the mouse. Based on reverse transcription-polymerase chain reaction, Lin et al. (2002) detected this enzyme as mRNA of rat tissues and in HepG2 cells. AS3MT was initially purified from the cytosolic fraction of rat liver, using pH-dependent fractionation, chromatofocusing and S-adenosylhomocysteine-affinity chromatography (Lin et al., 2002).

The fact that AS3MT was not fully characterized in protein chemical terms along with nucleotide sequence conflicts, the detection of this protein in N1E-115 (Mouse neuroblastoma cells) cells, and the possibility of an important biological role for arsenic biotransformation and detoxification made us investigate the primary protein sequence. And indeed, tandem mass spectrometry with liquid chromatography coupled to mass spectrometry and the use of two different proteolytic enzymes enabled us to solve conflicts and characterize about 77% of the protein sequence.

Materials and methods

Cell culture

N1E-115 (mouse neuroblastoma cells) cells were obtained from ATCC (CRL 2263) and maintained in DMEM (Gibco) containing 4500 mg/l glucose, L-glutamine, without pyruvate and with 10% fetal bovine serum (FBS), antibiotics penicillin, streptomycin at concentrations of 60 mg/ml and 100 mg/ml, respectively and incubated in a humidified incubator with 5% CO_2 at 37 °C (Oh et al., 2006).

Sample preparation and 2-DE

N1E-115 cells were washed three times in 10 ml of phosphate-buffered saline (Gibco BRL), centrifuged for 10 min at 800 × *g* at room temperature samples were collected using standard techniques as described (Oh et al., 2006). Samples were centrifuged for 150,000 × *g* for 60 min and the supernatant transferred into Ultrafree-4 centrifugal filter units (Millipore, Bedford, MA), for desalting and concentrating proteins. The protein concentration was determined using Bradford assay (Bradford, 1976). 2-DE was performed essentially as reported (Chen et al., 2006). Samples of 800 µg protein were applied on immobilized pH 3–10 nonlinear gradient strips: The second-dimensional separation was performed on 10–16% gradient SDS-PAGE. The gels were stained with colloidal Coomassie blue (Novex, San Diego, CA, U.S.A.) (Oh et al., 2005).

In-gel digestion and extraction

Gel spots were excised and digested with trypsin and Asp-N with concentration of 40 ng/µl and 25 ng/µl, respectively. For MALDI-TOF-TOF analysis, peptides were extracted with 10 µl of 1% TFA in 5 Mm OGP and nano-LC-ESI-MS/MS analysis, peptides were extracted with 15 µl 0.1% formic acid, 2% acetonitrile (Oh et al., 2004).

MALDI analysis

MS and MS/MS experiments were performed on an Ultraflex™ TOF/TOF (Bruker Daltonics) mass spectrometer in reflectron mode. Peptide standard was used as external calibration. Mass spectra were analyzed using the FlexAnalysis 2.4 software (Suckau et al., 2003). PMF and MS/MS datasets were interpreted with MASCOT (Matrix Science Ltd., London, U.K.) software searched against the MSDB 20051115 database (ftp://ftp.ncbi.nih.gov/repository/MSDB/) via BioTools 2.2 software (Bruker Daltonics). A mass tolerance of 25 ppm and one missing cleavage site for PMF and MS/MS tolerance of 0.5 Da and one missing cleavage site were allowed and oxidation of methionine was considered.

nano-ESI-LC-MS/MS analysis

The Peptide mixture obtained from trypsin and Asp-N digests were separated by an UltiMate 3000 dual nano-LC system (Dionex) and analyzed using QSTAR XL (Applied Biosystems, Foster City, CA) equipped with a nano electrospray ionization source. For nano-LC-ESI-MS/MS, the digest was loaded onto a PepMap 100 C18 precolumn (300 µm i.d., 5 mm long cartridge, from Dionex, Amsterdam, The Netherlands) from 0 to 30 min and then separated by a PepMap 100 C18 analytical column (75 µm i.d., 150 mm long cartridge, from Dionex) using a linear gradient of 4% B (solvent A, 0.1% FA; solvent B, 80% ACN/0.08% FA) to 60% B from 0 to 30 min, 90% B constant from 30 to 35 min, and 4% B from 35 to 60 min using the Ultimate micropump at a flow rate of 300 nl/min. As the peptides eluted from the LC system, they were electrosprayed into QSTAR XL. Each cycle consisted of one full scan mass spectrum ($m/z = 350$–1600) followed by MS/MS spectra on the three most abundant peptide ions in the full MS scan. The derived MS datasets were converted to MASCOT generic format flat files by a script (mascot.dll 1.6b21; Matrix Science, Boston, MA) supplied with the AnalystQS 1.1 software (Applied Biosystems) and searched against in-house-licensed MSDB 20051115 and Expasy/TrEMBL databases (Chen et al., 2006).

Results

For determination of the identity of AS3MT, the spot was excised from two-dimensional electrophoresis gels and digested with trypsin and Asp-N. The masses of the tryptic peptide fragments were measured using MALDI-TOF-TOF and nano-ESI-LC-MS/MS analysis. Observed ions were searched with MASCOT against Expasy/TrEMBL and NCBI Protein Database. Peptide mapping and MS/MS measurements on tryptic and Asp-N digested fragments led to confirmation of mouse arsenic (+3 oxidation state) methyltransferase (AS3MT) (NCBI protein database accession no: NP_065602). Thirty-seven percent of sequence was verified with MS/MS analysis and combined with peptide mapping sequence coverage was increased to 77%. Table 1 summarize the peptide sequence determined by MS, MS/MS analysis of the AS3MT digests. Based on hematopoietic stem cell regulatory genes from liver tissue, Phillips et al. (1999) published a sequence

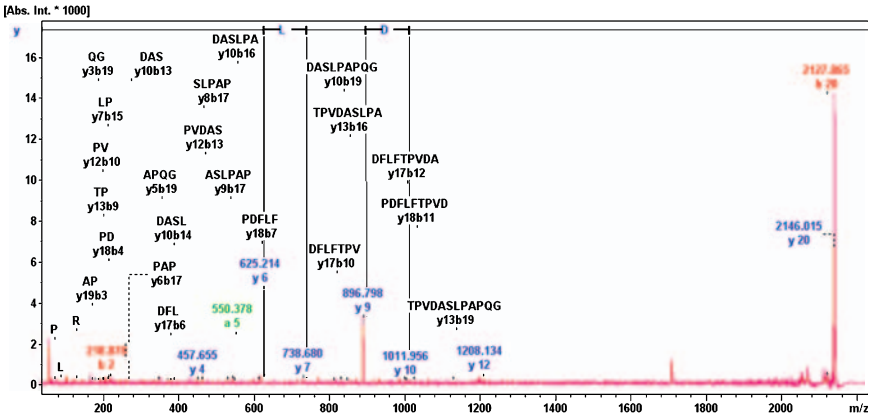

Fig. 1. LID-LIFT-TOF/TOF spectrum of m/z 2146.06 obtained from tryptic peptides and subsequent mascot search confirmed the presence of Arginine at position 335 (for an interpretation of the reference to colour in this figure, the reader is referred to the online version of this paper under www.springerlink.com)

Table 1. Identification of AS3MT peptides by a combination of MALDI-TOF-TOF and nano-ESI-LC-MS/MS mass spectrometry analysis

Start–end	Observed	Mr (expt)	Mr (cal)	Delta	Miss	Sequence	Enzyme[c]
6–23	2121.03	2120.02	2120.02	0.01	1	DADEIHKDVQNYYGNVLK	T
24–36	719.346	1436.67	1436.65	0.02	0	TSADLQTNACVTR Deamidated (NQ)[a,b]	T
27–45	2189.08	2188.07	2188.14	0.07	0	DLQTNACVTRAKPVPSYIR	A
54–70	657.94	1970.80	1970.95	0.15	1	DVSSRYYGCGLTVPERL[a,b]	A
59–69	657.81	1313.61	1313.60	0.01	0	YYGCGLTVPER[a,b]	T
75–84	487.77	973.53	973.51	0.01	0	ILDLGSGSGR[a,b]	T
85–96	705.86	1409.70	1409.68	0.02	0	DCYVLSQLVGEK[a,b]	T
85–102	1974.96	1973.95	1973.99	0.04	1	DCYVLSQLVGEKGHVTGI	A
107–122	1940.95	1939.94	1939.98	0.04	1	VQVEVAKTYLEHHMEK	T
121–137	1960.99	1959.98	1960.03	0.05	0	EKFGFQAPNVTFLHGRI	A
121–141	2402.20	2401.20	2401.29	0.10	1	EKFGFQAPNVTFLHGRIEKLA	A
123–136	1590.82	1589.81	1589.81	0.00	0	FGFQAPNVTFLHGR[a]	T
166–174	581.824	1161.63	1161.61	0.01	0	QQVLQEVYR[a,b]	T
171–185	1796.88	1795.87	1795.93	0.05	1	EVYRVLKHGGELYFS	A
178–198	2368.14	2367.13	2367.13	0.00	0	HGGELYFSDVYASLEVPEDIK	T
178–201	2720.25	2719.25	2719.31	0.07	1	HGGELYFSDVYASLEVPEDIKK	T
196–215	2360.13	2359.13	2359.21	0.09	1	DIKSHKVLWGECLGGALYWK	T
202–215	1651.84	1650.83	1650.82	0.01	0	VLWGECLGGALYWK	T
216–234	2083.10	2082.10	2082.17	0.07	0	DLAIIAQKIGFCPPRLVTA	A
224–230	423.72	845.42	845.42	0.00	0	IGFCPPR[a,b]	T
243–252	574.28	1146.54	1146.53	0.01	0	ELEGVLGDCR[a,b]	T
250–266	2086.05	2085.04	2085.12	0.08	0	DCRFVSATFRLFKLPKT	A
250–269	2383.18	2382.18	2382.25	0.08	1	DCRFVSATFRLFKLPKTEPA	A
270–285	1901.93	1900.92	1900.97	0.05	1	ERCRVVYNGGIKGHEK	A
286–296	672.85	1343.69	1343.67	0.01	0	ELIFDANFTFK	T
297–312	815.92	1629.82	1629.80	0.01	0	EGEAVAVDEETAAVLK[a,b]	T
286–296	1344.70	1343.69	1343.68	0.02	0	ELIFDANFTFK[a,b]	T
304–318	549.90	1646.69	1646.82	0.13	2	DEETAAVLKNSRFAP[a]	A
316–335	2146.07	2145.06	2145.09	0.03	0	FAPDFLFTPVDASLPAPQGR[a]	T
342–349	987.57	986.57	986.59	0.02	1	VLIRDPFK	T

[a] Denotes sequence identified with MS/MS analyis
[b] Denotes sequence identified with nano-ESI-LC-MS/MS
[c] *T* trypsin; *A* Asp-N digests

which contains the presence of Glycine instead of Aspartic acid at amino acid number 151 and also the sequence start from 335 to 376 was missing. A close examination of PMF data of tryptic peptides determined ion at $m/z = 986.57$ (observed $m/z = 987.57$), indicating that the protein sequence of AS3MT contains the sequence (342–349) VLIRDPFK. Tryptic peptides from AS3MT protein spot were analyzed by MALDI-TOF-TOF, and the presence of Arginine at amino acid 335 was confirmed. The MS/MS analysis of a tryptic peptide at $m/z = 2146.06$ and subsequent database search with MASCOT search against nucleic acid database perfectly matched to AS3MT 316–335 (FAPDFLFTPV DASLPAPQGR) with high score and confirmed the presence of Arginine at amino acid number 335 (Fig. 1).

Discussion

Understanding the pathway for conversion of iAs to methylated products, depends on development strategies to purify and characterize the enzymes that are involved in each step of the pathway. Walton et al. (2003) reported that sodium selenite was the most potent inhibitor of the methylation of arsenite by recombinant rat AS3MT and showing direct interactions between selenite and AS3MT. Two conceptual models are currently available to describe the action of AS3MT. The first model states that alternative oxidative methylation and reduction of As^{V} to As^{III}, yield a series of intermediates and products (Cullen et al., 1984). The second conceptual model takes a different approach to the reactions involved in the methylation of arsenicals (Hayakawa et al., 2005). Here, thiol-containing complexes of arsenicals containing As^{III} are substrates for sequential reactions that transfer methyl groups to the arsenical. In this model, methylation occurs by a nonoxidative mechanism. Therefore identification and characterisation of AS3MT protein is of importance to better understand reaction mechanisms. The major outcome of the study is to demonstrate the use of proteolytic digestion

using trypsin and Asp N for the determination of primary structure of AS3MT and solving an existing sequence conflict in standard databases. Detection of AS3MT in 2D-Gels from mouse N1E-115 cells along with a possible important biological role for arsenic biotransformation and detoxification made us investigate the primary sequence. Combination of multi-enzyme digestion and mass spectrometry allowed for high sequence coverage of AS3MT (Table 1). Based on this result we confirmed the sequence AS3MT (NCBI protein database accession no: NP_065602) protein sequence. Moreover the published AS3MT protein sequence shown by Phillips et al. (1999) (NCBI protein database accession no: AF166383) differs from the NP_065602 protein sequence that may represent isoforms/splice variants. We were not able to identify the presence of Glycine or Aspartic acid at amino acid number 151, this is due to proteolytic enzymes were not covered in this region or technical limitation of mass spectrometry and also we could not able to identify any post translation modification. Taken together a potentially important protein involved in arsenic metabolisms has been analysed at the chemical level by two different mass spectrometric approaches and the corrected sequence information is relevant for further studies at the protein level. Moreover, it is intriguing to show that in the neuroblastoma cell line used arsenic (+3 oxidation state) methyltransferase is a high abundance protein although its role remains elusive.

Acknowledgment

We are highly indebted to the Verein zur Durchführung der wissenschaftlichen Forschung auf dem Gebiet der Neonatologie und Kinderintensivmedizin "Unser Kind".

References

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254

Challenger F (1951) Biological methylation. Adv Enzymol 12: 429–491

Chen WQ, Kang SU, Lubec G (2006) Protein profiling by the combination of two independent mass spectrometry techniques. Nat Protoc 1: 1446–1452

Crecelius EA (1977) Changes in the chemical speciation of arsenic following ingestion by man. Environ Health Perspect 19: 147–150

Cullen WR, McBride BC, Reglinski J (1984) The reduction of trimethylarsineoxide to trimethylarsine by thiols: a mechanistic model for the biological reduction of arsenicals. J Inorg Biochem 21: 45–60

Gerhard DS, Wagner L, Feingold EA, Shenmen CM, Grouse LH, Schuler G, Klein SL, Old S, Rasooly R, Good P, Guyer M, Peck AM, Derge JG, Lipman D, Collins FS, Jang W, Sherry S, Feolo M, Misquitta L, Lee E, Rotmistrovsky K, Greenhut SF, Schaefer CF, Buetow K, Bonner TI, Haussler D, Kent J, Kiekhaus M, Furey T, Brent M, Prange C, Schreiber K, Shapiro N, Bhat NK, Hopkins RF, Hsie F, Driscoll T, Soares MB, Casavant TL, Scheetz TE, Brown-stein MJ, Usdin TB, Toshiyuki S, Carninci P, Piao Y, Dudekula DB, Ko MS, Kawakami K, Suzuki Y, Sugano S, Gruber CE, Smith MR, Simmons B, Moore T, Waterman R, Johnson SL, Ruan Y, Wei CL, Mathavan S, Gunaratne PH, Wu J, Garcia AM, Hulyk SW, Fuh E, Yuan Y, Sneed A, Kowis C, Hodgson A, Muzny DM, McPherson J, Gibbs RA, Fahey J, Helton E, Ketteman M, Madan A, Rodrigues S, Sanchez A, Whiting M, Madari A, Young AC, Wetherby KD, Granite SJ, Kwong PN, Brinkley CP, Pearson RL, Bouffard GG, Blakesly RW, Green ED, Dickson MC, Rodriguez AC, Grimwood J, Schmutz J, Myers RM, Butterfield YS, Griffith M, Griffith OL, Krzywinski MI, Liao N, Morin R, Palmquist D, Petrescu AS, Skalska U, Smailus DE, Stott JM, Schnerch A, Schein JE, Jones SJ, Holt RA, Baross A, Marra MA, Clifton S, Makowski KA, Bosak S, Malek J, MGC Project Team (2004) The status, quality, and expansion of the NIH full-length cDNA project: the mammalian gene collection (MGC). Genome Res 14: 2121–2127

Hayakawa T, Kobayashi Y, Cui X, Hirano S (2005) A new metabolic pathway of arsenite: arsenite-glutathione complexes are substrates for human arsenic methyltransferase Cyt19. Arch Toxicol 79: 183–191

Healy SM, Casarez EA, Ayala-Fierro F, Aposhian H (1998) Enzymatic methylation of arsenic compounds. V. Arsenite methyltransferase activity in tissues of mice. Toxicol Appl Pharmacol 148: 65–70

Lin S, Shi Q, Nix FB, Styblo M, Beck MA, Herbin-Davis KM, Hall LL, Simeonsson JB, Thomas DJ (2002) A novel S-adenosyl-L-methionine: arsenic(III) methyltransferase from rat liver cytosol. J Biol Chem 29: 10795–10803

Mukhopadhyay R, Rosen BP (2002) Arsenate reductases in prokaryotes and eukaryotes. Environ Health Perspect 110: 745–748

Oh JE, Krapfenbauer K, Fountoulakis M, Frischer T, Lubec G (2004) Evidence for the existence of hypothetical proteins in human bronchial epithelial, fibroblast, amnion, lymphocyte, mesothelial and kidney cell lines. Amino Acids 26: 9–18

Oh JE, Karlmark Raja K, Shin JH, Hengstschläger M, Pollak A, Lubec G (2005) The neuronal differentiation process involves a series of antioxidant proteins. Amino Acids 29: 273–282

Oh JE, Karlmark Raja K, Shin JH, Pollak A, Hengstschlager M, Lubec G (2006) Cytoskeleton changes following differentiation of N1E-115 neuroblastoma cell line. Amino Acids 31: 289–298

Phillips et al. (1999) Genebank submission, available under (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nuccore&val=6006812)

Suckau D, Resemann A, Schuerenberg M, Hufnagel P (2003) A novel MALDI LIFT-TOF/TOF mass spectrometer for proteomics. Anal Bioanal Chem 376: 952–965

Walton FS, Waters SB, Jolley SL, LeCluyse EL, Thomas DJ, Styblo M (2003) Selenium compounds modulate the activity of recombinant rat AsIII-methyltransferase and the methylation of arsenite by rat and human hepatocytes. Chem Res Toxicol 16: 261–265

Waters SB, Styblo M, Thomas DJ (2004) Endogenous reductants support the catalytic function of recombinant rat cyt19, an arsenic methyltransferase. Chem Res Toxicol 17: 404–409

Authors' address: Prof. Gert Lubec, Department of Pediatrics, Medical University of Vienna, Währinger Gürtel 18, A-1090 Vienna, Austria, Fax: 143-1-404003200, E-mail: gert.lubec@meduniwien.ac.at