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Enzymatic attributes of an L-isoaspartyl methyltransferase from *Candida utilis* and its role in cell survival

Shakri Banerjee [a], Trina Dutta [a], Sagar Lahiri [a], Shinjinee Sengupta [a], Anushila Gangopadhyay [b], Suresh Kumar Karri [b], Sandeep Chakraborty [b], Debasish Bhattacharya [c], Anil K. Ghosh [a,*]

[a] Drug Development, Diagnostics and Biotechnology Division, CSIR-Indian Institute of Chemical Biology, 4, Raja S.C. Mullick Road, Kolkata 700032, India
[b] Infectious Diseases and Immunology Division, CSIR-Indian Institute of Chemical Biology, 4, Raja S.C. Mullick Road, Kolkata 700032, India
[c] Structural Biology and Bioinformatics Division, CSIR- Indian Institute of Chemical Biology, 4, Raja S.C. Mullick Road, Kolkata 700032, India

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ABSTRACT

Backgrounds: Spontaneous deamidation and isoaspartate (IsoAsp) formation contributes to aging and reduced longevity in cells. A protein-L-isoaspartate (D-aspartate) O-methyltransferase (PCMT) is responsible for minimizing IsoAsp moieties in most organisms.
Methods: PCMT was purified in its native form from yeast *Candida utilis*. The role of the native PCMT in cell survival and protein repair was investigated by manipulating intracellular PCMT levels with Oxidized Adenosine (AdOx) and Lithium Chloride (LiCl). Proteomic Identification of possible cellular targets was carried out using 2-dimensional gel electrophoresis, followed by on-Blot methylation and mass spectrometric analysis.
Results: The 25.4 kDa native PCMT from *C. utilis* was found to have a K_m of 3.5 μM for AdoMet and 33.36 μM for IsoAsp containing Delta Sleep Inducing Peptide (DSIP) at pH 7.0. Native PCMT comprises of 232 amino acids which is coded by a 698 bp long nucleotide sequence. Phylogenetic comparison revealed the PCMT to be related more closely with the prokaryotic homologs. Increase in PCMT levels *in vivo* correlated with increased cell survival under physiological stresses. PCMT expression was seen to be linked with increased intracellular reactive oxygen species (ROS) concentration. Proteomic identification of possible cellular substrates revealed that PCMT interacts with proteins mainly involved with cellular housekeeping. PCMT effected both functional and structural repair in aged proteins *in vitro*.
General significance: Identification of PCMT in unicellular eukaryotes like *C. utilis* promises to make investigations into its control machinery easier owing to the familiarity and flexibility of the system.

1. Introduction

Cellular proteins go through spontaneous, non-enzymatic modifications like deamidation of specific asparagines or dehydration of aspartyl residues and give rise to unusual β-linked L-isoaspartyl residues (Fig. 1) [1,2]. Generation of these isoAsp residues introduces a kink in the protein backbone that may threaten to alter the functionally active structure of a protein (Fig. 1) [2]. Deamidation at asparaginyl sites and subsequent formation of isoaspartyl residues in their places poses to be potentially disruptive to protein function coupled with an increased tendency to aggregate [3]. The abnormal isoAsp residues are recognized by an enzyme, protein-L-isoaspartate (D-aspartate) O-methyltransferase or PCMT (EC 2.1.1.77). PCMT selectively methylates the α-carboxyl sidechain of L-isoaspartyl and/or naturally occurring D-aspartyl residues by transferring the methyl group from S-adenosyl L-methionine (AdoMet) and initiates the restoration of these abnormal residues back to normal amino acids (Fig. 1) [4]. The L-isoaspartyl/D-aspartyl methyl esters release one molecule of methanol to form an L-succinimide intermediate. The L-succinimide intermediate undergoes structural rearrangement and hydrolyzes into either normal L-aspartyl (30%) or abnormal L-isoaspartyl (70%), depending on the bond broken. The L-succinimide may spontaneously racemize into D-succinimide which hydrolyzes to produce a mixture of D-aspartyl and D-isoaspartyl residues. These abnormal residues then again undergo another methylation cycle by PCMT (Fig. 1) [5]. Multiple cycles of this process brings about either complete (Asp to Asp via IsoAsp) or partial (Asn to Asp via IsoAsp) repair of the age-damaged protein [6]. The success of this protein repair mechanism lies in the fact that it restores the correct conformation of the protein backbone [4]. *In vitro*, this process repairs

* Corresponding author. Fax: +91 33 2473 5197.
E-mail addresses: anilghosh51@gmail.com, aghosh@iicb.res.in (A.K. Ghosh).

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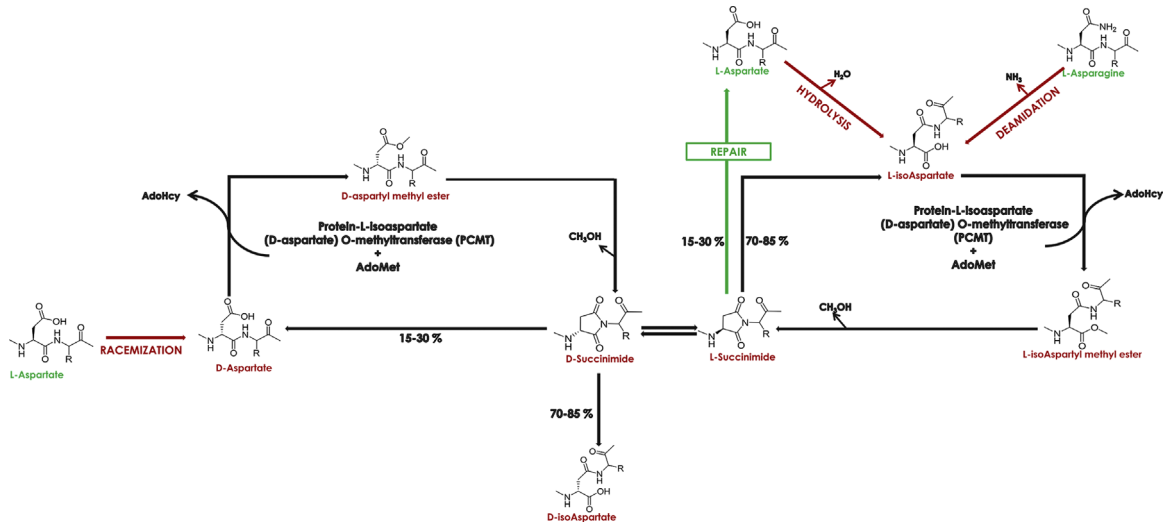

Fig. 1. Mechanism of isoAsp repair by Protein Carboxyl Methyltransferase (PCMT)**.** During prolonged storage at physiological pH, native proteins or peptides undergo either deamidation at L-asparaginyl residues or isomerisation and racemisation at L-aspartyl residues, resulting in the formation of abnormally linked L-isoaspapartates or D-aspartates. These damaged proteins or peptides exhibit reduced cellular functions. Protein L-isoaspartate (D-aspartate) O-methyltransferase (PCMT) targets these L-isoAsp or D-aspartate residues to catalyze their selective methylation with the aid of methyl group donor S-adenosyl L-methionine (AdoMet). The methylation step leads to the formation of L-isoaspartyl or D-aspartyl methyl esters along with a molecule of S-adenosyl L-homocysteine (AdoHcy). A metastable succinimide intermediate is produced when the methyl esters lose a molecule of methanol (CH_3OH) spontaneously. The L-succinimide intermediate hydrolyzes to generate a mixture of L-isoaspartyl and L-aspartyl residues, or it spontaneously gets racemized into D-succinimide which upon hydrolysis results into a mixture of D-isoaspartyl and D-aspartyl residues. Each methylation cycle reduces the total L-isoaspartate (and D-aspartate) level in a protein or peptide population by 15–30% relative to the previous cycle, resulting in a net repair of $\geq$ 85% after 10 or more cycles. The active amino acid residues and the step leading from inactive abnormally linked residue to its active form are marked in green. The inactive residues and the steps leading to formation of an abnormally linked form of amino acid are marked in red.

IsoAsp containing peptides and restores activity by 50% in structural as well as enzyme proteins [7].

Repair is the energetically efficient option for rescuing aged proteins rather than resorting to the hugely energy expensive process of protein recycling [6]. The importance of this protein repair mechanism and the enzyme PCMT is underlined by its wide distribution and its high degree of sequence conservation among varied life forms [8]. PCMT is found in most bacteria [9], plant [10], nematodes [11], flies [12], and mammals including humans [13]. Survival and longevity of certain bacteria, nematodes and mammals have been shown to be dependent on PCMT levels and was seen to be significantly reduced when deficient [14–16].

PCMT research has seen tremendous boost in the last decade. Research has focused on its physiological role in protein repair and cell survival. PCMT has been identified and isolated from higher plants, animals, but reports in fungi have not yet seen much endeavor. Owing to fast next generation sequencing techniques, a large portion of the yeast genomes have been sequenced. In most of these, the PCMT gene has been predicted but little efforts have been directed towards their isolation and characterization. Sequencing and subsequent annotation revealed that the genome of *Saccharomyces cerevisiae* lacked a PCMT gene or its homolog [17,18]. In contrast, a PCMT homolog, Pcm2, has been identified in fission yeast *Schizosaccharomyces pombe* [19]. Unicellular fungi or yeasts have been used as crucial tools in elucidating the key components of biological processes like cell cycle and apoptosis. A similar approach may work for PCMT in elucidating its regulatory mechanisms as well as downstream substrates thus underlining its role in physiological processes.

Till date, to the best of our knowledge, no published report elucidates purification of a native PCMT enzyme from any unicellular fungi or yeast. The present work is directed towards identifying and purifying a native PCMT activity in the unicellular fungi *C. utilis*. The sequence of the purified PCMT was worked out. An in depth study on the repair ability of the purified enzyme was performed with common cellular proteins, catalytic and structural, subjected to aging under physiological conditions. Role of PCMT activity was demonstrated *in vivo* in relation with cell survival under stress and cellular targets of the PCMT were identified employing 2D gel electrophoresis followed by mass spectrometric analyses. In short, our study reports the first isolation of a PCMT enzyme from yeasts in its native form along with a study of its repair activity and contributes important information to the knowledgebase of PCMT in *C. utilis*.

2. Materials and methods

2.1. Materials

Media components and agar powder were purchased from Himedia, India. Solvents of HPLC grade were procured from Merck, Germany. All chemicals were purchased from Sigma Aldrich, USA. S-Adenosyl-(methyl-^{3}H)-L-Methionine was purchased from Perkin-Elmer, USA. ISOQUANT® Isoaspartate Detection Kit and Sequencing grade Modified Trypsin was obtained from Promega, USA. All the other chemicals and media components were of analytical grade and purchased locally.

2.2. Organism and culture conditions

The wild type *C. utilis* strain was obtained from National Chemical Laboratories, Pune, India (Cat. No. NCIM Y500). Cells were grown in YPD (Yeast Extract–5%, Peptone–1% and D-glucose–2%) medium at 30 °C with mechanical shaking at 200 rpm unless otherwise mentioned [20].

A Δ*pcmt* strain of *C. utilis* was generated for the purpose of this study. The gene was disrupted using a yeast plasmid pFA6a-kanMX6 as the template for a PCR reaction [21]. Primers used for the PCR amplification reaction of the selectable marker gene (*E. coli* kanamycin resistance gene *kan*^{r}) are; forward primer-5′