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Research paper

Moonlighting protein in *Starkeyomyces koorchalomoides*: Characterization of dihydrolipoamide dehydrogenase as a protein acetyltransferase utilizing acetoxycoumarin as the acetyl group donor

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ABSTRACT

In this report we have identified for the first time a transacetylase (TAase) in a mesophilic fungi *Starkeyomyces koorchalomoides* catalyzing the transfer of acetyl group from polyphenolic acetate (PA) to a receptor protein glutathione S-transferase (GST). An elegant assay procedure was established for TAase based on its ability to mediate inhibition of GST by 7,8-diacetoxy-4-methylcoumarin (DAMC), a model PA. Utilizing this assay procedure, *S. koorchalomoides* TAase was purified to homogeneity. TAase was found to have MW of 50 kDa. The purified enzyme exhibited maximum activity at 45 °C at pH 6.8. The N-terminal sequence of purified fungal TAase (**ANDASTVED**) showed identity with corresponding N-terminal sequence of dihydrolipoamide dehydrogenase (LADH), a mitochondrial matrix enzyme and an E3 component of pyruvate dehydrogenase complex (PDHC). TAase was found to have all the properties of LADH and avidly interacted with the anti-LADH antibody. TAase catalyzed acetylation of GST by DAMC was identified by LC–MS/MS and a single lysine residue (Lys-113) was found to be acetylated. Further, recombinant LADH from *Streptococcus pneumoniae* lacking lipoyl domain was found to exhibit little TAase activity, suggesting the role of lipoyl domain in the TAase activity of LADH. These observations bear evidence for the protein acetyltransferase activity of LADH. Such an activity of LADH can be attributed as a moonlighting function of the enzyme.

1. Introduction

Acetylation like its "rival" phosphorylation has emerged as an important and versatile covalent modification of proteins [1]. Acetyl CoA dependant post-translational modification mediated by histone acetyltransferases (HATs), involves reversible transfer of acetyl group from acetyl CoA to the ε-amino group of lysine residue of histones, neutralizing its positive charge [2–4]. Lysine acetylation of histones significantly alters histone-DNA binding, interactions between nucleosomes and between histones and regulatory proteins, thus influencing cell-cycle progression, chromosome dynamics, DNA replication, recombination and repair, silencing, and apoptosis [5]. Histone acetylation is greatly influenced by acetyllysine-targeting motif in HATs (bromodomain) [6]. Another regulatory mechanism is autoacetylation observed in HATs such as PCAF, p300 and CBP,

Abbreviations: LC–MS/MS, liquid chromatography–tandem mass spectrometry; TAase, acetoxy drug: protein transacetylase; PAs, polyphenolic acetates; GST, glutathione S-transferase; DAMC, 7,8-diacetoxy-4-methylcoumarin; LADH, dihydrolipoamide dehydrogenase; PDHC, pyruvate dehydrogenase complex; NOS, nitric oxide synthase; PMSF, phenyl methyl sulphonylfluoride; DTT, 1,4-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TBS, tris-buffered saline; PVDF, polyvinylidene difluoride; rGST, recombinant glutathione S-transferase; rLADH, recombinant dihydrolipoamide dehydrogenase; HRP, horseradish peroxidase; LA, lipoic acid.

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facilitating the intramolecular rearrangements between the bromodomain and the basic loop, enabling the lysine substrate to bind to the bromodomain [7,8]. ε-lysine acetylation significantly regulates the functions of numerous and diverse proteins resulting in both "loss of function"/"gain of function". Non-histone transcription factors such as p53, E2F1, EKLF, MyoD and GATA1 have also been shown to be substrates for PCAF and p300/CBP mediated acetylation. The acetylation is also known to regulate non-nuclear proteins such as α-tubulin, Importin-α family of nuclear import factors and molecular chaperones such as Hsp90 [9].

The knowledge on protein acetylation independent of acetyl CoA was restricted to the action of aspirin that would readily acetylate Ser-530 of cyclooxygenase [10]. Snyder et al. illustrated the role of a transacetylase catalyzing the transfer of acetyl group from a small molecule to another small receptor molecule, the example being platelet activating factor (PAF)-dependent transacetylase [11] and arylamine N-acetyltransferase [12]. The persistent work carried out in our laboratory identified a microsomal TAase [13] from mammalian cells and tissues, catalyzing the transfer of acetyl group from polyphenolic acetates (PAs) to certain functional proteins such as cytosolic GST, microsomal cytochrome P-450, NADPH cytochrome *C* reductase and nitric oxide synthase (NOS), resulting in the acetylation of these proteins [14–18]. We have elaborated in detail, certain biological effects such as the antimutagenic action [19–21], enhancement of intracellular levels of nitric oxide (NO), inhibition of ADP-induced platelet aggregation and inhibition of protein kinase C in the asthmatic patients arising out of the action of TAase on PA [22,23].

We had established that isolated calreticulin, a Ca^{2+}-binding protein of endoplasmic reticulum lumen, mediated transfer of acetyl group from polyphenolic acetate to purified GST [24,25]. It was thought pertinent to look for the occurrence of such a transacetylase in organism such as *Starkeyomyces koorchalomoides* which is not known to have calreticulin, according to the available information [26].

Our laboratory is credited for the purification of mammalian TAase from the buffalo liver and human placenta for the first time and demonstrated the acetylation of GST and NOS by mass spectrometry [14–16]. The present investigation pertains to the purification to homogeneity and characterization of fungal TAase as dihydrolipoamide dehydrogenase (LADH), an E3 component of PDHC. We have unveiled the moonlighting behavior of LADH in mediating the acetylation of receptor protein GST by the application of LC–MS/MS.

2. Materials and methods

2.1. Preparation of cell free extracts

The mycelia were placed in liquid nitrogen and homogenized mechanically using a chilled mortar and pestle, suspended in lysis buffer containing 100 mM potassium phosphate buffer (pH 7.2) supplemented with 0.25 M sucrose solution having 1 mM DTT, 1 mM PMSF and 1 mM EDTA. The homogenate was centrifuged at 200 g for 20 min at 4 °C and the pellet containing the cell debris was discarded. The sample was stored at −20 °C. Protein was estimated by the methods of Lowry [27] and Bradford [28].

2.2. Assay for fungal TAase

TAase was assayed routinely using DAMC as the first substrate (unless otherwise mentioned), and cytosolic GST as the second substrate. The assay mixture in a total volume of 0.8 ml consisted of 0.25 M potassium phosphate buffer (pH 6.5), TAase (50 μg protein), cytosol (12 μg protein) and DAMC (50 μM in 50 μl DMSO) and pre-incubated for 10 min, followed by the addition of 1 mM CDNB and 1 mM GSH for the assay of GST [29]. The unit of TAase was expressed in terms of percent inhibition of cytosolic GST under the condition of the assay [18].

2.3. Purification of TAase

Fungal crude extract was subjected to ammonium sulphate fractionation. An aqueous saturated solution of $(NH_4)_2SO_4$ was added with stirring to the crude homogenate (about 120 ml) to attain 60–90% (w/v) saturation. After 30 min, the fungal extract was centrifuged and the supernatant (containing chlorophyll pigments) was discarded. The pellet was suspended in 100 mM potassium phosphate buffer (pH 7.2) and dialyzed against the same buffer containing 0.2 mM EDTA and 10 mM 2-mercaptoethanol. The dialyzed protein extract was then centrifuged at 800 g at 4 °C and the resulting supernatant was used for the next step of the purification procedure.

2.4. Solubilization of protein

The sample was then incubated in the presence of mild detergent 0.5% sodium cholate dissolved in 0.1 M potassium phosphate buffer (pH 7.2) for 30 min (2 ml/mg protein) using the method of Dey et al. [30] at 4 °C under gentle stirring. The solubilized extract was then centrifuged using ultracentrifuge (Beckman Model no L7-65 R) for 1.25 h at 100 000 g (rotor Ti-50) to remove residual non-solubilized membranes and the clear supernatant was dialyzed (12 kDa cut-off) overnight against 6 volume of 10 mM potassium phosphate buffer (pH 7.2). The dialyzed fraction was subjected to CM Sepharose, DEAE Sepharose and Q Sepharose chromatographies to purify TAase of *S. koorchalomoides* to homogeneity as described earlier [25].

2.5. SDS polyacrylamide gel electrophoresis

SDS-PAGE was performed using the discontinuous pH system of Ornstein [31] and Davis [32] as described by Laemmli [33]. The molar ratio of bisacrylamide:acrylamide was 1:29 for molecular weight determination in the presence of SDS and the continuous pH system of Weber and Osborn [34] was used. Coomassie brilliant blue G-250 was used for protein staining.

2.6. Native-PAGE

The composition of electrode buffer, stacking buffer, resolving buffer and loading dye was used as described by Ferreras [35]. The electrophoresis was carried out at 4 °C.

2.7. Zinc negative staining

In native gel electrophoresis, the gel was stained by reverse staining. The gel was incubated in 1% (w/v) sodium carbonate solution for 5 min followed by incubation of the gel for 15 min in 0.2 M imidazole and then washed with distilled water, incubated for 40 s in 0.2 M zinc sulphate and finally washed twice with distilled water. The protein was visualized as transparent band against semi-opaque background.

2.8. Elution of protein from Native gel with zinc staining method

The bands obtained from native gel were cut, crushed with glass rod and incubated in phosphate buffered saline overnight at 4 °C. After incubation for 12 h, the protein sample was centrifuged at 5000 g in sorvall centrifuge (SS-34) at 4 °C and the supernatant containing the protein was assayed for TAase activity. The sample