**TITLE**

**ABSTRACT**

**INTRODUCTION**

**MATERIALS AND METHODS**

*Protein Expression and Purification.* Salmonella enterica PncA was amplified using primers to add 5’-EcoR1 and 3’-Not1 restriction sites and cloned into plasmid pAB-6xHis-MBP to yield plasmid pPNC1([*13*](file:///C%3A%5CUsers%5Cxguan%5CDocuments%5Cgxy%5CData%5CPublication%20to%20be%5COutline%20of%20Biochemistry_091012.doc#_ENREF_13)), which encodes PncA protein with an N-terminal maltose-binding protein-hexahistidine (6xHis-MBP) tag. Plasmid pPNC1 was moved into Escherichia. Coli strain BL21 (DE3) by chemical transformation. The resulting strain was grown overnight and subcultured 1:100 (v/v) into 2 liters of lysogenic broth containing ampicillin (100 g/ml). The culture was grown shaking at 37 °C to A600 ~0.7, and MBP-H6-PncA synthesis was induced with isopropyl-1-thio--D-galactopyranoside (1mM). The culture was grown overnight at 25 °C. Cells were harvested and MBP-H6-PncA purified as following: Cells expressing MBP-H6-PncA were harvested by centrifugation at 10,500 xg at 4oC for 12 min in a SORVALL Legend x 1R centrifuge with a FiberLite F15-6x100y rotor (Thermo Scientific). Cell pellets were resuspended in buffer A (sodium phosphate buffer (20 mM, pH 7.5, at 24 °C), NaCl (0.5 M), imidazole (20 mM)) and broken by sonication using a CL-18 Sonic Dismembrator (Fisher Scientific) for 3 min (50% duty). Cell debris was removed by centrifugation at 35,000 xg for 30 minutes. MBP-H6-PncA was purified by affinity chromatography using a 5-ml HisTrap HP column. After equilibration with buffer A and loading of cell-free extract, the column was washed with 50 ml of buffer A, followed by 40 ml of 8% buffer B (sodium phosphate buffer (20 mM, pH 7.5, at 24 °C), NaCl (0.5M), imidazole (0.5 M)). A 50-ml linear gradient increased buffer B to 100%. PncA eluted at 30% buffer B and was xxx pure and was stored in tris(hydroxymethyl) amino methane buffer (Tris-HCl, 50mM, pH 7.5, at 25 °C) containing KCl (100 mM) and 50% glycerol (v/v) at -80 °C. MBP-h6-PncA-containing fractions were pooled and H6-rTEV protease added to reach a 1:50 H6-rTEV protease:MBP-H6-PncA ratio. The cleavage reaction mixture was incubated at room temperature for 3 h and dialyzed overnight against two liters of buffer A at 4oC. Tagless PncA protein was resolved from the raction mixture using the 5-ml His Trap HP column, which did not bind tagless PncA. Protein was stored in Tris-HCL (50 mM, pH 7.5 at 25oC) containing KCl (100 mM) and 20% (v/v) glycerol at –80oC

*Measurement of Deacetylation activity using an enzyme coupled continuous assay.* SIRT3 activity was measured continuously using a Multiskan Ascent microplate reader (LabSystems; Franklin, MA, USA). Typical assay mixtures contained 20 to 800 mM AcH3, 20 to 1000 mM NAD+, 0.2 mM NAD(P)H, 1 mM dithiothreitol (DTT), 3.3 mM a-ketoglutarate, 1 to 2 mM MBP–PncA (nicotinamidase), 2 units of glutamate dehydrogenase from proteus (with 1 unit defined by the manufacturer as reducing 1.0 mmol of a-ketoglutarate to glutamate per minute), and 0.2 to 1 mM Sirt3 in 20 mM potassium phosphate at pH 7.5. Nicotinamide inhibition reactions contained 80 mM NAD+ and 100 mM AcH3, with inhibitor concentrations varying from 12.5 to 300 mM with 0.5 mM SIRT3 for the positive control and no SIRT3 for the negative control. SIRT3 reactions were carried out in a final volume of 300 ml per well in a clear, flat-bottomed, 96-well plate. All assay components except SIRT3 or NAD+ were pre-incubated at 25 oC for 5 min or until absorbance at 340 nm stabilized, and the reaction was initiated by the addition of SIRT3 or NAD+. The rates were analyzed continuously for 10, 20, 30, or 60 min by measuring NAD(P)H consumption at 340 nm. Alternatively, NAD(P)H was quantified by its intrinsic fluorescence with excitation at approximately 340 nm and emission at 460 nm in a solid black, flat-bottomed, 96-well plate. Rates were determined from the slopes of the initial linear portion of each curve using an extinction coefficient for NAD(P)H of 6.22 mM-1cm-1 and a pathlength of 0.9 cm for 300 ml reactions. The background rates of reactions lacking either SIRT3 or NAD+ resulting from the spontaneous formation of nicotinamide or ammonia were subtracted from the initial velocities of the SIRT3-catalyzed reactions.

**RESULTS**

**DISCUSSIONS**

**CONCLUSIONs**