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Structural and biochemical characterization of the yeast HD domain containing protein YGK1 reveals a metal-dependent nucleoside 5′-monophosphatase

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ABSTRACT

HD-domain is a conserved domain, with the signature of histidine and aspartic (HD) residues doublets. HD-domain proteins may possess nucleotidase and phosphodiesterase activities, and they play important roles in signaling and nucleotide metabolism. In yeast, HD-domain proteins with nucleotidase activity remained unexplored. Here, we biochemically and structurally characterized two HD domain proteins YGK1 (YGL101W) and YB92 (YBR242W) from *Saccharomyces cerevisiae* as nucleoside 5′-monophosphatases, with substrate preference for deoxyribonucleoside 5′-monophosphatase over ribonucleoside 5′-monophosphatase. By determining the crystal structure of YGK1, we unveiled that YGK1 structure resembled as the crystal structure of YfbR from *E. coli*. Size-exclusion chromatography and crosslinking studies suggested that YGK1 and YB92 existed in the form of a dimer, respectively, which were consistent with structural observation of YGK1. Site-directed mutagenesis demonstrated that more extensive conserved residues near the divalent metal coordinating active site were essential for YGK1 activity than previous suggested. The metal coordinating His89 and Asp90, and the neighboring conserved Glu93, Glu114 and Glu145 were individually critical for catalysis. In addition, alignments suggested that three flexible loops with hydrophobic residues might be implicated in substrate selectivity to nucleoside moiety. Together, our comparative structural and mutational studies suggested that YGK1 and YB92 functioned as 5′-nucleotidases in *S. cerevisiae*.

1. Introduction

In bacteria and eukaryotes, the pool sizes of nucleotides and nucleosides are maintained at constant levels to favor the efficient synthesis of DNA, RNA, and biosynthesis of cell wall [1] [2,3]. In cooperating with the enzymes ribonucleotide reductase and nucleoside kinases, various nucleotidases balance the intracellular pool of nucleotides and nucleosides during nucleotide metabolism [4,5]. In particular, intracellular 5′-nucleotidases regulate the cellular levels of nucleoside monophosphates by conversion of 5′-nucleotides to nucleosides and a phosphate, and thereby controlling all ribo- and deoxyribonucleotide pools [6,7].

Organisms ubiquitously encode multiple intracellular 5′-nucleotidases different in substrate specificities to deal with nucleotide homeostasis. In *Escherichia coli* for instance, at least six proteins (NagD, YrfG, YjjG, SurE, YfbR, and YfdR) were reported to possess cytosolic 5′-nucleotidases that hydrolyze the phosphate group of 5′-nucleotides, to eliminate nucleotide accumulation and nucleotide analog toxic potential [8,9]. Generally, 5′-nucleotidases reported so far were enzymes with broad specificity on NMP and dNMP. NagD and YrfG preferentially hydrolyzed purine nucleotides (GMP and IMP), and YjjG preferred pyrimidines (UMP, dUMP, and dTMP) [8,10]. In contrast, YfbR and YfdR preferred 5′-deoxyribonucleotides to 5′-ribonucleotides [11]. In human, seven major cytosolic 5′-nucleotidases with different substrate

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preferences and subcellular localization were found. cN-Is and cN-II member of 5′-nucleotidases hydrolyzed AMP and IMP/GMP respectively, while cN-IIIs were pyrimidine nucleotidases. mdN and cdN were the mitochondrial and cytosolic 5' (3′)-deoxyribonucleotidases, respectively. All these eukaryotic soluble 5′-nucleotidases studied so for were members of the haloacid dehalogenase superfamily (HADSF) of enzymes, representing the major nucleotidase activity in cultured human cells involved in purine and pyrimidine salvage pathways, nucleic acid repair, cell-to-cell communication, and signal transduction, among others [12,13].

Till now, only a few 5′-nucleotidases from the model organism *Saccharomyces cerevisiae* were characterized as the HADSF enzymes, including ISN1, PHM8, and SDT1 [14–17]. Notably, they all favored 5′-ribonucleotides over 5′-deoxyribonucleotides. To identify additional 5′-nucleotidases in yeast, we carried out biochemical enzymatic assays and structural studies on two stand-alone HD-domain proteins YGK1 (YGL101W) and YB92 (YBR242W) from *S. cerevisiae*. HD domain was found widely conserved catalytic domain in almost 5000 proteins. HD domain contains a pair of histidines and aspartates (HD) of conserved residues that bind active site metal ions, mostly executing phosphohydrolase (nucleotidase and phosphodiesterase) activities [11,18–20]. We showed here that recombinant HD domain proteins YGK1 and YB92 possessed phosphohydrolase activities against general phosphatase substrate *p*-nitrophenyl phosphate (*p*NPP) and selected canonical nucleoside 5′-monophosphate substrates. By structural and mutational studies, we suggested that YGK1 and YB92 functioned as 5′-nucleotidases in *S. cerevisiae*.

2. Materials and methods

2.1. Plasmids and constructs

The open reading frames of *ygk1* and *yb92* were PCR-amplified with specific primers and *S. cerevisiae* genomic DNA template. Restriction-digested PCR products were conjugated into a modified pET28a vector (Novagen) for generating the construct pET28a-YGK1 and pET28a-YB92 for over-expression of protein YGK1 and YB92 in fusion with an N-terminal hexa-histidine and SUMO tag. All constructs were confirmed by DNA sequencing.

2.2. Protein expression and purification

To produce the YGK1 and YB92 proteins, Rosetta2 (Novagen) strains harboring the respective pET28a-YGK1 and pET28a-YB92 constructs were cultured. The expression strains was grown in shaker flasks to approximately OD_{600} 0.8 at 37 °C and induced at 30 °C for 4 h with 0.5 m*M* isopropyl *β*-D-thiogalactopyranoside. After induction, cells were harvested, and cell pellet was flash-frozen. For protein purification, cell pellet was lysed in 50 m*M* Tris-Cl (pH 8.0), 20% (*w/v*) sucrose, 500 m*M* NaCl, 10 m*M* imidazole, 0.2% Tween 20, 1 m*M* PMSF, 1 m*M* DTT, and 20 μg ml^{-1} DNase, by a high-pressure homogenizer. Cleared supernatant was subjected to nickel-nitrilotriacetic acid bead column for tagged protein purification. To rid of the tagged His-SUMO fusion, the eluate protein was digested by Ulp1 enzyme over night at 4 °C, followed by a Superdex 200 column (GE Healthcare) and a HiTrap Q column (GE Healthcare) purifications. Protein fractions were detected by SDS-PAGE.

2.3. Site-directed mutagenesis

Six conserved residues of YGK1 in or near the catalytic site (His89, Asp90, Glu93, His111, Glu114 and Glu145) were selected and mutated to alanine. For constructing all these mutant plasmids, the site-directed mutagenesis method was followed according to the manufacturer's protocol, with a pair of specific primers and the template pET28a-YGK1. All mutations were verified by DNA sequencing.

2.4. Phosphatase activity assays

The general phosphatase activity assays of YGK1 and YB92 were spectrophotometrically performed against *p*-nitrophenyl phosphate (*p*NPP) in 1-mL reaction mixture, containing 50 mM HEPES-Na buffer pH8.0, 5 mM $MgCl_2$ or 0.1 mM other metal ions, 5 mM *p*NPP and 1 μM protein. After 20 min of incubation at 30 °C, the reaction was stopped by the addition of 0.1 ml of 0.5 M EDTA. The concentration of the *p*-nitrophenol product was determined by the absorbance at 410 nm.

Phosphatase activity toward canonical nucleotide was assayed in an 80-μL reaction mixture containing 50 mM HEPES pH 8.0, 1 mM substrate, 0.1 mM $CoCl_2$ and 0.1 μM protein. Metal-dependence activity was analyzed in the presence of various divalent cations (5 mM Mg^{2+} or 0.1 mM of Mn^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, Ca^{2+}, or Cu^{2+}). Reactions were carried out at 30 °C for 20 min of incubation. By the addition of the Malachite Green reagent, the level of Pi released was measured by absorbance at 630 nm.

For determination of the K_m and V_{max}, the phosphatase assays contained substrates at concentrations of 0.01–5 mM. Kinetic parameters were determined by non-linear curve fitting from the Oringin software hyperbole model.

2.5. Crystallization and structure determination

The crystallization screening was done in 96-well crystallization plates at 20 °C, with 1 μL of protein solution mixed with 1 μL of reservoir solution. Crystals of selenomethionine-substituted (Se-Met) YGK1 were grown in 0.1 M Sodium acetate trihydrate pH 4.5, 22% w/v PEG 3350, 0.01 M $CdCl_2$ at 20 °C with a concentration of 20 mg/ml. Before flash-cooling in liquid nitrogen, crystals were harvested into crystallization solution with 25% (v/v) glycerol as cryoprotectant.

Diffraction data were collected at 100 K on the beamline BL17U1 of Shanghai Synchrotron Radiation Facility (SSRF). Data were processed with the HKL2000 program package. Phenix. AutoSol was used to locate the Se site and generate the initial phase, and interpretable electron density was produced from these phases. After several cycles of manually correction using Coot, phases were transferred into Buccaneer for performing further model building. All structures above were refined with the program Phenix. refine and manually corrected in Coot. The qualities of the final models were checked with the program MolProbity. The program PyMOL was used to prepare all structural figures. The coordinate of the YGK1 has been deposited under accession code 5YOX in the Protein Data Bank.

2.6. Chemical cross-linking

Chemical cross-linking was carried out in a 200-μl reaction, containing 50 mM HEPES pH 8.0, 50 mM KCl, 5 mM $MgCl_2$, 8 μg YGK1 (or YB92) and 10 mM dimethyl pimelimidate (DMP). Reactions were incubated at 22 °C, 20-μl aliquots were taken out at indicated times (1, 5, 10, 30 and 60 min), immediately stopped by the addition of 1 μL of 0.5 M Tris-HCl (pH 6.8). The cross-linking samples were analyzed by SDS-PAGE.