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Biophysical and biochemical characterization of recombinant human Pop2 deadenylase

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ABSTRACT

Pop2, a component of the Ccr4–Not complex, functions as a deadenylase both *in vitro* and *in vivo*. In this research, we found that the recombinant human Pop2 (hPop2) mainly existed in a compact monomeric state with a α + β tertiary structure type. The percentages of the secondary structures evaluated from the CD spectrum were about 37% α-helix, 14% β-sheet, and 19% β-turns. The optimal condition for hPop2 catalysis was pH 7–8 at 37 °C. Mg^{2+}, Mn^{2+}, and Co^{2+} had similar effects on the deadenylation activity of hPop2, and the optimal concentration was 0.3–0.5 mM. The deadenylase activity of hPop2 was, at least partially, specific when coordinated with divalent metal ions. The enzyme was not inhibited much by the nucleotide analogs, and the product 5′-AMP was the most efficient inhibitor. The dissimilarity in the metal ion dependence and inhibitory effects of the nucleotide analogs suggested that various deadenylases might have differential regulation mechanisms.

The decay of mRNA plays a crucial role in the regulation of gene expression in all cells. In eukaryotes, the degradation of most cytoplasmic mRNAs is initiated by the shortening of the poly(A) tail at the 3′-end, followed either by the removal of the 5′ cap structure (decapping) and then 5′–3′ exonucleolytic digestion, or by an alternative 3′–5′ degradation by the cytoplasmic exosome [1–5]. In both cases, the degradation of the mRNA poly(A) tail (deadenylation) is the key and rate-limiting step during mRNA turnover and aberrant mRNA surveillance. Deadenylation is achieved by the function of deadenylases, which have the ability to specifically exo-hydrolyze the mRNA poly(A) tail from the 3′-end and release 5′-AMP as the mononucleotide product. There are several characterized eukaryotic deadenylases including poly(A)-specific ribonuclease (PARN), Pan2–Pan3, and Ccr4-Pop2 [5]. Among them, PARN has been characterized in many vertebrates and plants [6–10] but not in *Saccharomyces cerevisiae* or *Drosophila melanogaster* [11]. Pan2–Pan3 is a deadenylase depending on poly(A) binding protein (PABP) in yeast and mammals [12–14]. Ccr4 and Pop2 are the catalytic components of the large Ccr4–Not complex, which is conserved from yeast to higher eukaryotes [15,16].

Ccr4 and its associated protein Pop2 (also known as Caf1) were first characterized as transcriptional factors in *S. cerevisiae* [17]. More recently, these two proteins were found to be the major components exhibiting deadenylase activity in the Ccr4–Not complex [11,15,16,18]. It has been suggested that Ccr4 might be the primary deadenylase subunit of the Ccr4–Not complex *in vivo*. However, both Ccr4 and Pop2 contain the conserved DEDD-exoribonuclease sequence [19] and reveal catalytic activity *in vitro* [20–22]. The crystal structures of two yeast Pop2 enzymes, *Saccharomyces cerevisiae* Pop2 (scPop2)[1] and *Schizosaccharomyces pombe* Pop2 (spPop2), resolved recently [20,21] indicate that they share similar core folding patterns to the DEDDh nucleases. Furthermore, a recent study has demonstrated that Pop2 played an important role in intracellular mRNA deadenylation separate from its contact to Ccr4 [23].

The homologs of Pop2 have been characterized in many organisms [16,18,19,22,24–28]. Although the two yeast Pop2 enzymes share a similar core structure in their catalytic domains, sequence alignments indicate that these homologs have great discrepancies in their primary structures. For example, the core DDEDh consensus motif in scPop2 is replaced by an unusual SEQQt sequence, which is conserved in other Pop2 enzymes. The percentage similarity between human Pop2 (hPop2) and scPop2 is 64%, and the identity is 38% [27]. The great discrepancy in the primary structures suggested that they might have different catalytic characteristics as well as intracellular functions [19,28]. However, little is known about the *in vitro* bio-

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[1] *Abbreviations used:* CD, circular dichroism; DTT, dithiothreitol; GST, glutathione *S*-transferase; scPop2, *Saccharomyces cerevisiae* Pop2; spPop2, *Schizosaccharomyces pombe* Pop2; hPop2, human Pop2; IPTG, isopropyl-1-thio-β-D-galactopyranoside; PARN, poly(A)-specific ribonuclease; p74, the 74 kDa full-length PARN; p54, the 54 kDa N-terminal proteolytic fragment of p74 (residues 1–470); p54ΔR3H, p54 with deletion of the R3H domain; PVS, polyvinyl sulfate; SDS, sodium dodecyl sulfate; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; SEC, size exclusion chromatography.

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chemical and biophysical properties of Pop2. It is also not known why the Ccr4–Not complex requires two deadenylases, Ccr4 and Pop2. In this paper, soluble hPop2 was successfully expressed and purified in *E. coli*, which makes it possible to study the catalytic properties of hPop2 in detail. The data herein suggested that considerable difference existed in the biochemical properties, particularly in the metal ion preference and inhibitory effects by nucleotide analogs, between hPop2 and other deadenylases. This difference might be due to the differential regulation mechanisms of various deadenylases.

Materials and methods

Chemicals

Tris, methylene blue, AMP, IMP, dAMP, adenosine, isopropyl-1-thio-β-D-galactopyranoside (IPTG), and polyadenylic acid potassium salt (with an average of 200 adenosines, A_{200}) were obtained from Sigma. Dithiothreitol (DTT) and RNasin were purchased from Promega. MOPS and kanamycin were Amresco products. All other reagents were local products of analytical grade or better.

Cloning and expression

All recombinant plasmids were constructed following the standard DNA cloning procedure [29]. The full-length human hPop2 coding sequence (GenBank Accession No. AF053318) was cloned from the total cDNA of HeLa cell by RT-PCR using Pfu polymerase and the following oligonucleotide primers: Sense-primer (5′-GGGAATTCCATATGCCTGCAGCACTTGTGGA-3′), and anti-primer (5′- CGCGGATCCTCACTGCTGCATGTTGTTG-3′). After digestion of the PCR product and of plasmid pET28a with NdeI and BamHI, the *Pop2* gene was ligated into the expression vector pET28a (Novagen) and the sequence of the product pET28a-hPop2 was checked by sequencing and restriction analysis. The six-His Tag sequence of pET28a vector was fused to the N-terminus of the hPop2 open reading frame for further purification. Site-directed mutagenesis against D40 was carried out using Transformer Site-directed Mutagenesis kit (CLONTECH) with the following mutagenic primers: 5′-GTTATATTGCCATGGCCACAGAATTTCCAG-3′ and 5′-CTGGAAATTCTGTGGCCATGGCAATATAAC-3′. The recombinant plasmid pET28a-Pop2 was then transformed into *E. coli* BL21(DE3) Rossetta (Novagen). A single transformant was used for the sequent induction.

The recombinant strain was inoculated into 5 ml of Luria–Bertani medium supplemented with 50 μg/ml kanamycin and grown overnight at 200 r/min and 37 °C. The cultures were diluted (1:100) in the same medium and grown at 37 °C to reach an OD value of 0.6–0.8, and then the expression of hPop2 was induced by the addition of IPTG. The optimal condition for soluble protein expression was screened by varying the IPTG concentration and incubation temperature. It was found that the largest amounts of soluble proteins could be obtained using an IPTG concentration of 50 μM at 10 °C. At temperatures higher than 15 °C or IPTG concentrations above 0.1 mM, most proteins were found to exist as insoluble inclusion bodies. After 48 h induction, the cultures were harvested by centrifugation at 9000*g* and disrupted by sonication. The soluble and insoluble fractions were separated by centrifugation and were examined by SDS–PAGE analysis.

Protein purification

To purify the His-tagged hPop2, the cell extracts were first subjected to metal-chelated affinity chromatography using a 5 ml HisTrap HP column (GE Healthcare). The purification was performed according to the manufacturer's protocol with some modifications. To improve the purity of the His-tagged hPop2, 20 mM imidazole was added to the wash buffer to eliminate nonspecific binding of the bacterial proteins. Moreover, the column was washed again by 25 ml wash buffer containing 80 mM imidazole after being washed by 20 mM imidazole wash buffer. Most of the histidine-rich impurity proteins could be removed in this step, and the purity of products was about 90% according to the SDS–PAGE analysis. To further purify the recombinant hPop2, size exclusion chromatography (SEC) was performed using a Superdex G-200 column on an AKTA purification system. The buffer (buffer A) used for gel filtration contained 50 mM KCl, 0.5 mM DTT, 0.2 mM EDTA, 5% (v/v) glycerol, and 20 mM Tris–HCl, pH 8.0. The purified proteins were found to be homogeneous by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and the purity of the final products was about 99% as estimated by SDS–PAGE and SEC analysis. The protein concentration was determined according to the Bradford method [30] using bovine serum albumin as a standard.

The full-length PARN (p74) and the mutant with the removal of the two RNA-binding domains (p54ΔR3H) were expressed and purified as described previously [31,32]. The *Schizosaccharomyces pombe* Pop2 (spPop2) [20] was expressed and purified using the same conditions as that of hPop2.

Activity assay of hPop2

The deadenylation activity of hPop2 was measured using the methylene blue method as described before [33]. In brief, methylene blue stock buffer was prepared by dissolving 1.2 mg methylene blue into 100 ml Mops buffer (0.1 mM Mops–KOH, 2 mM EDTA, pH 7.5) and the absorbance of the Mops buffer at 688 nm was adjusted to 0.6 ± 1%. The standard reaction buffer (buffer B) contained 0.1 U of RNasin, 20 mM Tris–Cl (pH 7.0), 0.2 mM EDTA, 0.5 mM DTT, 10% (v/v) glycerol, 0.1% BSA, and 100 mM KCl. The apo-enzyme (the enzyme in the absence of divalent metal ions) was prepared by dissolving the purified hPop2 into buffer B with the addition of 5 mM EDTA. After 1 h incubation at room temperature, the sample was dialyzed against buffer B overnight. The residual EDTA concentration in the apo-enzyme solutions was 0.2 mM. Buffer B containing various concentrations of divalent metal ions was also prepared to investigate the effects of divalent metal ions on hPop2 activity. The enzyme was pre-incubated on ice, and then mixed with poly(A) substrate A_{200} dissolved in buffer B, and the reaction volume was 50 μl. The reaction was performed at 37 °C for 30 min and then terminated by 950 μl methylene blue buffer. After 15 min incubation at 30 °C in the dark, the absorbance at 662 nm or the spectrum ranging from 500 nm to 700 nm of the sample was measured on an Ultrospec 4300 pro UV/visible spectrophotometer. The final concentration of Pop2 in the activity assay was 5 μM, while that of PARN was 0.125 μM. The activity data were presented as an average of at least three repetitions. The enzymatic parameters were determined using a substrate concentration ranging from 20 μg/ml to 200 μg/ml. The data were fit to the Michaelis–Menten equation to obtain the apparent kinetic constants K_m and V_{max}, and k_{cat} was determined from V_{max} by normalizing the total enzyme concentration.

Spectroscopy

The samples used for spectroscopic experiments were prepared by dissolving the protein in buffer B with a final concentration of 0.26 mg/ml (8 μM). Far-UV circular dichroism (CD) spectra were recorded on a Jasco 725 spectrophotometer with a 1 mm pathlength cell. The resultant spectrum was baseline-subtracted *versus* the buffer spectrum. The percentages of secondary structures were determined from the CD spectrum with a wavelength range of 200–250 nm using the CONTINLL, SELCON3, and CDSSTR algo-