Biochimica et Biophysica Acta 1844 (2014) 656–662

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Characterization of inosine–uridine nucleoside hydrolase (RihC) from *Escherichia coli*

Brock Arivett [b], Mary Farone [b], Ranjith Masiragani [a], Andrew Burden [a], Shelby Judge [a], Adedoyin Osinloye [a], Claudia Minici [c], Massimo Degano [c], Matthew Robinson [a], Paul Kline [a,*]

[a] Department of Chemistry, Middle Tennessee State University, Murfreesboro, TN 37132, USA
[b] Department of Biology, Middle Tennessee State University, Murfreesboro, TN 37132, USA
[c] Division of Immunology, Transplantation and Infectious Diseases, Scientific Institute San Raffaele, Milan Italy

ARTICLE INFO

Article history:
Received 24 September 2013
Received in revised form 12 January 2014
Accepted 17 January 2014
Available online 25 January 2014

Keywords:
Nucleoside hydrolase
RihC
Escherichia coli

ABSTRACT

A non-specific nucleoside hydrolase from *Escherichia coli* (RihC) has been cloned, overexpressed, and purified to greater than 95% homogeneity. Size exclusion chromatography and sodium dodecyl sulfate polyacrylamide gel electrophoresis show that the protein exists as a homodimer. The enzyme showed significant activity against the standard ribonucleosides with uridine, xanthosine, and inosine having the greatest activity. The Michaelis constants were relatively constant for uridine, cytidine, inosine, adenosine, xanthosine, and ribothymidine at approximately 480 μM. No activity was exhibited against 2′-OH and 3′-OH deoxynucleosides. Nucleosides in which additional groups have been added to the exocyclic N6 amino group also exhibited no activity. Nucleosides lacking the 5′-OH group or with the 2′-OH group in the arabino configuration exhibited greatly reduced activity. Purine nucleosides and pyrimidine nucleosides in which the N7 or N3 nitrogens respectively were replaced with carbon also had no activity.

1. Introduction

Nucleoside hydrolases are a class of enzymes that hydrolyze the N-glycosidic bond of selected nucleosides between the base and sugar. They have been isolated from a number of sources including bacteria [1–3], parasitic protozoans [4–7], plants [8–10], marine invertebrates [11], and baker's yeast [12]. However, while nucleoside hydrolases are widely distributed, they have not been found in mammals [13].

In parasitic protozoans, the nucleoside hydrolases salvage purine ribonucleoside bases for recycling [14]. Being absent in mammals, but necessary to protozoans, they are attractive targets for drugs to treat diseases such as malaria and Chaga's disease [15]. In other organisms, such as prokaryotes and higher eukaryotes, the enzyme carries out a variety of species-specific roles [16–18]. The nucleoside hydrolases characterized to date are metalloproteins containing a Ca^{2+} ion found within a group of aspartate residues (DXDXXXDD) located at the N-terminus [13,19,20].

The most extensively studied nucleoside hydrolase is inosine–uridine nucleoside hydrolase (IU-NH) isolated from *Crithidia fasciculata*. This enzyme, part of the purine salvage pathway, has been cloned and expressed in *Escherichia coli* [21]. The transition state has been determined using kinetic isotope effects [22]. The transition state for purine nucleosides includes an oxocarbenium ion, protonation of N7, and a C3'-*exo* conformation of the sugar. A series of inhibitors based on this transition state have been synthesized [23]. An X-ray crystal structure of IU-NH complexed with *p*-aminophenyl-(1*S*)-iminoribitol has been determined containing a calcium ion bound to a group of aspartate residues at the bottom of the active site and identifying His241 as a proton donor for activation of the purine leaving group [24].

Peterson and Moller have identified three nucleoside hydrolases in *E. coli* extracts designated *rihA*, *rihB*, and *rihC* [25]. The three enzymes differ in their substrate specificity, with *rihA* and *rihB* being pyrimidine-specific and *rihC* able to hydrolyze both purine and pyrimidine ribonucleosides. Since *E. coli* recycles nucleoside bases using nucleoside phosphorylase rather than nucleoside hydrolases, the metabolic role of the nucleoside hydrolases in *E. coli* is not known. Of the three identified nucleoside hydrolases from *E. coli*, two have known crystal structures, *rihA*, also known as *ybeK*, and *rihB*, previously known as *yeiK* [26,27]. The transition state has been determined for RihC previously known as *yaaF*, the third nucleoside hydrolase [28]. The characteristics of the RihC transition state are similar to those of the transition state of IU-NH isolated from *C. fasciculata*.

Nucleoside hydrolases have traditionally been classified based on their substrate specificities into the purine-specific, the pyrimidine-

Abbreviations: BICINE, *N*,*N*-bis(2-hydroxyethyl)glycine; BSA, bovine serum albumin; CHES, 2-(cyclohexylamino)ethanesulfonic acid; CU-NH, cytidine-uridine nucleoside hydrolase; HEPES, N-(2-hydroxyethyl)piperazine-N′-4-butanesulfonic acid); IAG-NH, inosine–adenosine–guanosine nucleoside hydrolase; IG-NH, inosine–guanosine nucleoside hydrolase; IPTG, isopropyl-β-D-1-thiogalactopyranoside; IU-NH, inosine–uridine nucleoside hydrolase; LB, Luria Broth; MES, 2-(N-morpholino)ethanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid

* Corresponding author. Tel.: +1 615 898 5466; fax: +1 615 898 5182.
E-mail address: paul.kline@mtsu.edu (P. Kline).

1570-9639/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbapap.2014.01.010

specific, the 6-oxopurine-specific and the nonspecific [13]. Alternatively, the nucleoside hydrolases can be classified based on sequence similarity and active site residues [26]. In this scheme, Group I proteins contain a conserved {V,I,L,M}HD{P,A,L} tetrapeptide sequence approximately 230 amino acids from the N-terminal Ca^{2+} ion binding segment. This group contains both pyrimidine-specific and nonspecific nucleoside hydrolases. Group II nucleoside hydrolases replace the essential histidine of the Group I nucleosides hydrolases with an aromatic residue such as tyrosine or tryptophan. Group III contain an XCDX sequence in which the catalytic His239 of Group I nucleoside hydrolases is replaced with a cysteine residue. Based on its sequence, RihC from *E. coli* belongs to the group I nucleoside hydrolases along with *yeiK* and *ybeK* from *E. coli*, URH1 from *S. cerevisiae*, IU-NH from *L. major*, and IU-NH from *C. fasciculata*.

We report here the expression and purification of a full-length clone of *rihC*, along with its substrate specificity, the equilibrium constant of the inosine formation reaction, and state of oligomerization.

2. Materials and methods

2.1. Materials

Nucleosides, Amicon Ultra-15 centrifugal filter units, His-Select Ni resin, and molecular weight standards were purchased from Sigma Chemical Co. The FPLC™ Mono Q column was obtained from GE Healthcare. The pET28b vector and pUC18 positive control DNA were purchased from Novagen. BL21 (DE3) pLysS competent *E. coli* cells were purchased from Stratagene. PAGEr® precast electrophoresis gels were purchased from Fisher Scientific, while Bio-Rad protein assay dye concentrate was obtained from Bio-Rad. Phenosphere ODS reverse phase high-performance liquid chromatography (HPLC) column (150 × 4.6 mm) was purchased from Phenomenex. Erythrouridine was synthesized using the method of Kline et al. [29]. All other compounds were of reagent grade.

2.2. Expression and purification of RihC

2.2.1. Preparation of enzyme

2.2.1.1. Plasmid construct. The *rihC* gene was amplified from genomic DNA isolated from the *E. coli* K12 strain using the PCR methodology. Two gene-specific oligonucleotides were used, designed to amplify the complete gene sequence, and contain the *Nde* I and *Xho* I restriction sites at the 5′ termini. The proofreading Pfu DNA polymerase (Promega) was employed in the reaction to minimize the frequency of insertion of unwanted mutations. The blunt-ended amplicon was purified from agarose gel and ligated in the *Sma* I-digested pBluescript vector (Fermentas). The gene sequence was verified using automated dideoxy sequencing of both DNA strands.

The 912 bp *rihC* gene with its own stop codon and six histidine codons, accession number U00096, was excised from the plasmid and inserted between the *Nde* I and *Xho* I sites within the multiple cloning site of the approximately 5300 bp pET28b vector digested with the same enzymes. The insert was sequenced by GenHunter of Nashville, TN to verify the sequence.

The plasmid was then transformed into *BL21(DE3)pLysS E. coli* competent cells.

2.2.1.2. Induction of RihC in BL21(DE3)pLysS E. coli. A 25 mL overnight culture in LB broth containing kanamycin (50 μg/mL) and chloramphenicol (50 μg/mL) was used to inoculate 500 mL LB broth containing no antibiotics. The 500 mL culture was incubated at 37 °C with shaking at 220 rpm for 2–3 h until the OD_{600} reached 0.6. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 1 mM and incubation continued for 3 h leading to over-expression of RihC.

2.2.1.3. Purification of His-Tag RihC. Previously induced 500 mL cultures were centrifuged at 15,000 ×*g* at 4 °C for 15 min. The cells were washed twice with 3 mL of equilibration buffer (50 mM sodium phosphate, pH 8.0, 0.3 M sodium chloride, 10 mM imidazole) and centrifuged at 15,000 ×*g* at 4 °C for 15 min. The washed cells were then suspended in equilibration buffer and sonicated on ice with a 15 s burst at 60% amplitude followed by a 2 min incubation on ice for a total of 4 cycles. The cell debris was removed by centrifugation at 15,000 ×*g* at 4 °C for 15 min to produce a cleared lysate.

The cleared lysate was loaded onto a His-Select Ni column (10 × 100 mm) and allowed to flow through at a rate less than 1 mL/min. Unbound protein was washed from the column with equilibration buffer until the OD_{280} was 0. Bound protein was eluted by the addition of elution buffer (50 mM sodium phosphate, pH 8.0, 0.3 M sodium chloride, 250 mM imidazole) until the OD_{280} was 0. The eluate was concentrated to 2 mL using an Amicon Ultra-15 centrifugal filter unit (MWCO 10 kD). The concentrated eluate was dialyzed against 1 L 10 mM Tris pH 7.2, 0.5 mM dithiothreitol, 10 mM $CaCl_2$ at 4 °C.

Further purification was carried out on a Mono Q FPLC™ column. After loading the sample, the column was washed with 5 column volumes of 10 mM Tris pH 7.2 0.5 mM DTT. The protein was eluted with a linear gradient of 0–500 mM NaCl in 10 mM Tris pH 7.2 0.5 mM DTT. Fractions containing nucleoside hydrolase were pooled and concentrated to 2 mL, as described above. The protein was stored long-term in 10 mM Tris pH 7.2, 0.5 mM dithiothreitol, 10 mM $CaCl_2$.

2.2.2. Analysis of protein

Protein was quantitated using Bio-Rad Protein Assay Kit with BSA as the standard [30].

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the purity of the recombinant protein and its subunit molecular weight on a 10% PAGE minigel using Bio-Rad Precision Plus standards.

2.2.3. Nucleoside hydrolase activity

Nucleoside hydrolase activity was determined by HPLC. Reaction mixtures consisted of 1 mM nucleoside in 50 mM Tris pH 7.2 at 32 °C. The total volume of the reaction mixture was 1 mL. Reaction was initiated by the addition of enzyme (2.0 μg; ~60 nM). At appropriate times, 20 μL aliquots were withdrawn and the reaction quenched by addition of 20 μL of 1 M HCl. The relative amounts of the nucleoside and base were determined by HPLC. Measurements of product formation and substrate utilization were limited to 20% of possible product formation to ensure initial velocity measurements.

The relative amounts of nucleoside and base were determined on a ChromTech HPLC system, consisting of an ISO-2000 isocratic pump, Rheodyne 7725 injection valve, Model 500 UV/Vis variable wavelength detector, and PeakSimple chromatography system. Separation of the nucleosides and bases was achieved using a Phenosphere ODS reverse phase column (150 × 4.6 mm). The mobile phase was 10 mM ammonium acetate pH 5.2/methanol in either a 90/10 ratio or a 98/2 ratio with a flow rate of 1.0 mL/min. Each sample injection volume was 20 μL. Nucleosides and their corresponding bases were detected at 254 nm. The nucleoside and/or corresponding base were identified by their respective retention times. The amount of unreacted nucleoside and base produced was determined using a standard curve of concentration of nucleoside or base versus peak area. All samples were analyzed in triplicate.

For those nucleosides for which no standard base was available to determine retention time, nucleoside hydrolase activity was determined by the loss of substrate as determined by HPLC. To confirm these results the amount of ribose formed was also determined using a reducing sugar assay [4]. Reaction mixtures consisted of 1 mM nucleoside in 50 mM Tris pH 7.2, total volume 1 mL at 32 °C. The reaction was initiated by the addition of enzyme (2.0 μg: ~60 nM) and after the appropriate time terminated by the addition of 100 μL 1.0 M HCl. Copper