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

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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

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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

reagent (300 μL) consisting of 4% Na_2CO_2, 1.6% glycine, and 0.045% $CuSO_4$•$5H_2O$ and neocuproine reagent (300 μL) of 0.12% 2,9-dimethyl-1,10 phenanthroline were added and the reaction mixture was incubated in a boiling water bath for 7 min. The solution was cooled and the absorbance at 450 nm measured. The amount of sugar produced was determined using a standard ribose concentration curve.

2.2.4. Determination of equilibrium constant

Triplicate reaction mixtures consisting of 2 mM hypoxanthine and 1 M ribose in 995 μL of 10 mM Tris buffer pH 7.2, 0.5 mM dithiothreitol were prepared. The reaction was initiated by addition of 5 μL RihC nucleoside hydrolase (6.3 μg; ~188 nM) and incubated at 37 °C. The amounts of hypoxanthine and inosine were monitored by HPLC until no change in their amounts was observed over a 24 h period. The relative amounts of inosine and hypoxanthine were determined by HPLC as described earlier.

2.2.5. Methanolysis reaction

A reaction mixture consisting of [1′-^{13}C]adenosine (200 μM) in 10 mM Tris buffer pH 7.2, 0.5 mM dithiothreitol in 20% (vol %) deuterated methanol was placed in an NMR tube. The reaction was initiated by the addition of 10 μL of RihC (12 μg; ~377 nM) and the progress of the reaction monitored by ^{13}C NMR on a JEOL ECX 300 MHz NMR. The identity of the products was determined by comparison of chemical shifts to a series of standard compounds.

2.2.6. Determination of native molecular weight

The apparent molecular weight of the enzyme was determined by size exclusion HPLC on a Phenomenex TSK gel filtration column (300 × 4.6 mm) with a fractionation range of 1×10^4 to 5×10^5 daltons. The column was eluted with a mobile phase of 50 mM phosphate buffer pH 7.0 and 100 mM sodium chloride with a flow rate of 0.3 mL/min. A 20 μL sample (1 mg/mL) of RihC was injected onto the TSK gel filtration column. Proteins were detected by monitoring the absorbance at 280 nm. At the time of detection, the concentration of the enzyme was 6 μg/mL. The apparent native molecular weight of nucleoside hydrolase RihC was determined by comparing its elution position to a calibration curve constructed using the retention time of the following proteins: β-amylase sweet potato (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase bovine erythrocytes (29 kDa), and cytochrome c horse heart (12.4 kDa) (Fig. 1).

2.2.7. pH optimum

Stock buffer solutions of 300 mM of sodium acetate, MES, CHES, PIPES, HEPES, and BICINE were prepared. A combined buffer solution was prepared by adding 1 mL of each individual buffer and adjusting the pH as needed using NaOH and HCl. The pH range studied was 4–9.

Reaction mixtures (990 μL) consisting of the above combined buffer solution at the desired pH containing 1000 μM inosine were prepared. The reaction was initiated by addition of 10 μL RihC nucleoside hydrolase (12 μg; ~377 nM) and incubated for 20 min at 32 °C. The reaction was stopped by the addition of 100 μL 6 M HCl and the extent of the reaction determined by HPLC as described above.

3. Results and discussion

3.1. Protein purification

Nucleoside hydrolase from *E. coli* (RihC) was cloned into a pET28 plasmid. The insertion into the vector produced an in-frame fusion between the 5′ end of the *rihC* gene and six histidine codons located between positions 270 and 287 to create plasmid pET28-*rihC*. The enzyme was cloned with a His-Tag to aid in purification. The enzyme was overexpressed in *E. coli* BL21(DE3) cells and both the growth time and the induction time with IPTG were optimized. The fusion enzyme

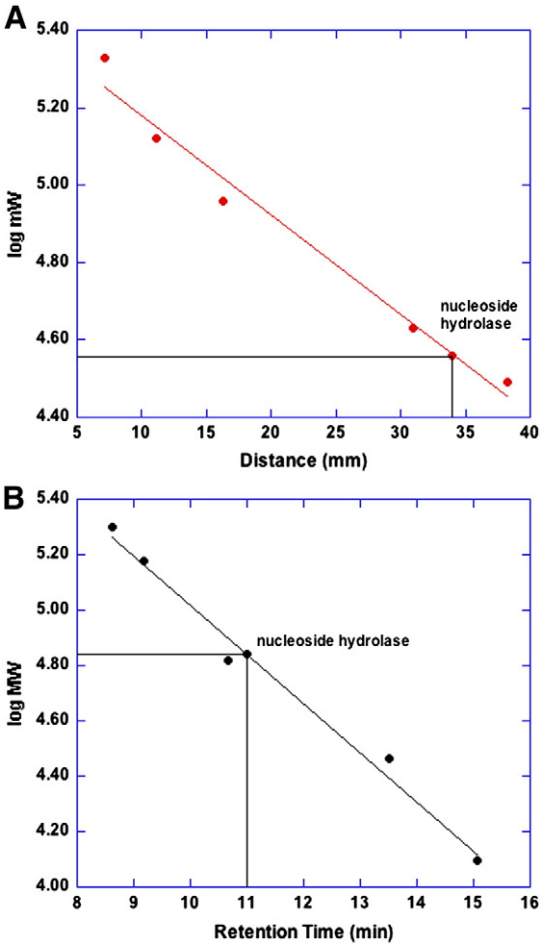

Fig. 1. A. Relative mobility of *E. coli* RihC on a 10% SDS polyacrylamide minigel to proteins of known molecular weight. The subunit molecular weight was calculated to be 36,000 Da. (B) Determination of molecular weight and subunit structure of *E. coli* RihC. Fig. 1B shows the elution position of RihC relative to proteins of known molecular weight from a Phenomenex TSK size exclusion column (retention time 11.03 min). The native molecular weight was found to be 69,000 Da. At the time of detection, the concentration of the enzyme was 6 μg/mL.

was purified using a Sigma His Ni metal affinity column followed by ion exchange chromatography using a Mono Q FPLC™ column.

The purity of the protein was assessed by SDS-PAGE. Visual inspection of the Coomassie-stained gel showed that the purity of the enzyme was approximately 95% based on the relative intensities of the bands present in the gel (data not shown).

3.2. Substrate specificity

Three nucleoside hydrolases have been identified in *E. coli*, designated *rihA*, *rihB*, and *rihC* [25]. RihA and RihB are pyrimidine-metabolizing nucleoside hydrolases exhibiting the greatest activities with cytidine and uridine. RihC is a non-specific nucleoside hydrolase hydrolyzing both purines and pyrimidines. The order of activity of RihC using *E. coli* extracts against the major nucleosides was uridine > xanthosine > inosine >adenosine > cytidine > guanosine. The same order, based on turnover number, was seen for the purified RihC from *E. coli* (Table 1). Nucleoside hydrolases can be classified according to their substrate specificity [13]. The classes include the non-specific inosine–uridine nucleoside hydrolases (IU-NH), the purine-specific inosine–adenosine–guanosine nucleoside hydrolases (IAG-NH), the pyrimidine-specific nucleoside hydrolases (CU-NH), and the 6-oxopurine specific inosine–guanosine nucleoside hydrolases. Based on its reported substrate specificity, RihC belongs to the purine non-specific inosine–uridine nucleoside hydrolase group [25].

Table 1
Kinetic analysis of *E. coli rihC*.

Nucleoside	K_m (μm)	k_{cat} per subunit (s^{-1})	k_{cat}/K_m ($M^{-1}\,s^{-1}$)
Inosine	422 ± 225	4.31 ± 0.22	1.02×10^4
Adenosine	416 ± 249	1.15 ± 0.47	2.8×10^3
Guanosine	Not determined	Not determined	Not determined
Xanthosine	454 ± 165	6.30 ± 0.05	1.3×10^4
Uridine	408 ± 184	10.85 ± 0.23	2.6×10^4
Cytidine	682 ± 298	1.12 ± 0.53	1.6×10^3
Ribothymidine	421 ± 196	1.29 ± 0.16	3.1×10^3

Other members of this group include IU-HN isolated from *C. fasciculata*, *Leishmania donovani*, and *Leishmania major* [31].

To characterize the kinetic activity of RihC from *E. coli*, the Michaelis constant, K_m, and turnover number, k_{cat}, were determined for several nucleosides (Table 1). These constants were determined by measuring the initial rates of reaction at different substrate concentrations followed by a nonlinear regression analysis based upon the Michaelis–Menten equation. All of the substrates tested followed normal hyperbolic kinetics with the exception of guanosine. Due to the low solubility of guanosine, only the linear part of the hyperbolic curve was observed and the Michaelis constant and k_{cat} values were not determined.

The Michaelis constants for the *E. coli* RihC are similar to those for the IU-NH from *L. major* compared to those of IU-NH from *C. fasciculata*. The K_m values for the pyrimidines, uridine and cytidine are comparable to those for the purines inosine, adenosine, and guanosine for both RihC from *E. coli* and IU-NH *L. major*. The Michaelis constant for ribothymidine (5-methyluridine) for RihC from *E. coli* is also comparable to the other nucleosides examined. In contrast to IU-NH *C. fasciculata,* the K_m values for the pyrimidines are significantly higher than for the purines. While the K_m values of RihC from *E. coli* are similar to those of IU-NH from *L. major*, the catalytic turnover ratio of uridine/inosine for IU-NH *E. coli* is 2.5. This is midway between the value of 5.1 for IU-NH from *C. fasciculata* and the value of 0.27 for IU-NH from *L. major* [31].

The turnover number for *E. coli* RihC is relatively constant with a variation among the common nucleosides of less than 10-fold. This is in contrast to IU-NH *L. major* and IU-NH *C. fasciculata* where the variation is 330-fold and 95-fold respectively. This may be a reflection of the relative importance of the metabolic role the enzyme plays in the organisms. In the parasitic protozoans the organism is dependent on purines derived from the host for synthesis of DNA and RNA, while in *E. coli* the functioning of the enzyme does not appear to be critical to the growth of the organism.

The activity of *E. coli* RihC was determined for a number of additional nucleosides, which tested different structural features to determine their effect on enzyme activity (Table 2). The number and position of hydroxyl groups on the ribose moiety were important in determining substrate reactivity. Nucleosides lacking the hydroxyl group in the 2′ position, including 2′-deoxyuridine, thymidine, 2′-deoxycytidine, and 2′-deoxyadenosine, exhibited no activity. This was consistent with the results of Petersen and Moller in which 2′-deoxycytidine and 2′-deoxyadenosine were reported to have no activity [25]. Arabinouridine, which retains the 2′-hydroxyl group of the sugar moiety but which has the opposite stereochemistry from that of uridine, was an extremely poor substrate with a rate of hydrolysis some four orders of magnitude lower than that of uridine. Removal of the 3′-OH group from the ribosyl moiety has the same effect as removal of the 2′-OH, a loss of activity. Cordycepin (3′-deoxyadenosine) was not a substrate for *E. coli* RihC. The effect of the 5′-hydroxyl group was studied by comparing the enzymatic rate of hydrolysis of erythrouridine versus uridine. Erythrourdine, while retaining the same configuration of the 2′ and 3′ hydroxyl groups as uridine, lacks its C5′ hydroxymethyl group. The rate of hydrolysis of erythrouridine was approximately 500 times lower than that of uridine.

Table 2
Enzymatic activity of *E. coli* nucleoside hydrolase (*rihC*).

Nucleoside	Activity × 10^2 (μmol/min mg)
2′-Deoxyuridine	No reaction
2′-Deoxycytidine	No reaction
2′-Deoxyadenosine	No reaction
Thymidine	No reaction
Arabinouridine	0.00077
Erythrouridine	0.0911
3-Deazauridine	No reaction
6-Chloropurine riboside	No reaction
Tubercidin (7-deazaadenosine)	No reaction
Cordycepin (3′-deoxyadenosine)	No reaction
Purine riboside	1.13
Allopurinol riboside (1-β-D-ribofuranosyl-1H-pyrazolo[3,4-d]pyrimidine-4-one)	No reaction
6-Benzylamino purine riboside (N^6-benzyladenosine)	No reaction
6-(γγ-Dimethylallylamino)purine riboside (N^6-(2-isopentenyl)adenosine)	No reaction

This contrasts with adenosine nucleosidase from yellow lupin in which 5′-deoxyadenosine was the best substrate tested [8].

The base specificity of RihC was low, with the common purine and pyrimidine ribonucleosides being substrates of the enzyme. However, certain structural features of the base were critical for the reaction. For the purines, tubercidin (7-deazaadenosine), in which N7 of the purine base was replaced with a carbon, had no detectable activity. The importance of the N7 position was further confirmed by the lack of activity with allopurinol riboside, another nucleoside in which N7 has been replaced with a carbon. Changing the exocyclic amino group at the C6 position also has an effect on the activity of the nucleoside with *E. coli* RihC. Addition of hydrocarbon-like substituents to the exocyclic amino group such as benzyl and isopentenyl or halogen substituents resulted in a loss of activity. 6-Benzylamino purine riboside, 6-(γ,γ-dimethylallylamino) purine riboside and 6-chloropurine riboside were not substrates (Table 2). Removal of the exocyclic amino group resulted only in a relatively small decrease in activity. Purine riboside had a specific activity of 1.13×10^{-2} μmol/min mg, while adenosine had a specific activity of 2.12×10^{-2} μmol/min mg.

3.3. Catalytic mechanism

The catalytic mechanism of IU-NH from *C. fasciculata* has been extensively studied [21,22,24]. The catalytic mechanism involves the formation of an oxocarbenium ion on the ribosyl moiety along with the formation of a negative charge on the leaving nitrogenous base. For purine bases, protonation of N7 has been proposed to activate the purine base [21]. The substrate specificity of RihC from *E. coli* supports a similar requirement. Replacement of N7 with carbon in 7-deazaadenosine and allopurinol riboside resulted in loss of activity indicating that protonation of N7 is also required for the hydrolysis of purines by RihC. A catalytic triad consisting of Tyr223, Tyr227, and His239 has been proposed to protonate N7 in the hydrolysis of purine nucleosides by Group I nucleoside hydrolases of which *E. coli* RihC is a member [20]. Sequence alignment of IU-NH from *C. fasciculata* and RihC indicated a similar catalytic triad consisting of His220, Tyr221, and His233 present in RihC [28]. The formation of an oxocarbenium ion on the ribosyl moiety during the reaction catalyzed by RihC from *E. coli* similar to the mechanism of IU-NH from *C. fasciculata* is also supported by the previously reported transition state [28].

The mechanism by which pyrimidine nucleosides undergo enzymatic hydrolysis is less clear, as the acid–base properties of the pyrimidines differ significantly from those of the purines. The same catalytic triad involving Tyr223, Tyr227, and His239 has been proposed to be involved in the activation of pyrimidine bases [20]. His239 has been proposed to directly protonate the O2 carbonyl of uracil, while Tyr227 forms a hydrogen bond to N3 in IU-NH from *C. fasciculata* [20]. In

E. coli RihC, Tyr227 has been replaced with a Ser that is also capable of forming a hydrogen bond to N3 of the leaving uracil from the hydrolyzed uridine. The importance of this interaction was supported by the lack of activity with 3-deazauridine as the substrate. Replacement of N3 with carbon results in the loss of the proposed hydrogen bond between the enzyme and substrate.

A second possibility in which uracil leaves as an anion has been proposed based on kinetic isotope effects from uracil DNA glycosylase [32]. To completely describe the substrate–enzyme interactions, additional experiments must be carried out.

3.4. Molecular weight and subunit structure

Nucleoside hydrolases come in a variety of sizes and subunits. IU-NH from *C. fasciculata* and *Leishmania* are tetramers, while IAG-NH from *C. fasciculata* and *Trypanosoma brucei brucei* are dimers. IG-NH from *C. fasciculata* is a trimer.

SDS-PAGE was carried out on the His-Tag containing RihC. Based on the calibration curve generated by the standards on the mini-gel, the major band had a molecular weight of 36 kD which is consistent with the calculated molecular weights based on the sequence of the protein (Fig. 1A). This compares to a calculated subunit molecular weight of 32,559 daltons based on the amino acid sequence of the subunit and a calculated mass of 33,642 daltons for the His-Tag protein.

Size exclusion HPLC chromatography was used to determine the native molecular weight of the enzyme. The enzyme eluted as a single peak with a retention time of 11.03 min. From the calibration curve (Fig. 1B), this yields a molecular weight of 69,000 daltons. The results are consistent with a homodimer. Based on SDS-PAGE and size exclusion chromatography, RihC from *E. coli* exists as a dimer in solution with a subunit molecular weight of approximately 36 daltons (33,642 daltons for the His-Tag protein).

Preliminary crystallographic data indicated that the protein was a partially ordered tetramer. Two of the subunits were well-defined, while two subunits were poorly defined, indicating that the state of oligomerization may be concentration dependent.

3.5. Equilibrium constant of inosine formation

Due to the high concentration of water in comparison to substrate concentrations, it can be difficult to measure the equilibrium constant in the direction of the hydrolysis reaction. Therefore, the equilibrium constant was measured in the reverse direction. The amount of inosine formed in the presence of high concentrations of hypoxanthine (2 mM) and ribose (1 M), while the temperature was maintained at 37 °C, was used to determine the equilibrium constant for RihC from *E. coli*. Under these conditions, the equilibrium constant for the inosine formation reaction was found to be 45 ± 2 M. This equilibrium constant is consistent with the results determined for other nucleoside hydrolases. The equilibrium constant for IU-NH from *C. fasciculata* was reported to be 106 ± 16 M, while that of adenosine nucleosidase from *Lupinus luteus* for adenosine was 263 ± 19 M [4,8].

3.6. Solvent reactivity

To determine the stereochemistry of the ribose initially formed during the hydrolysis of a nucleoside, i.e., α or β, the reaction was carried out in a solvent containing 20% methanol, a concentration at which *E. coli* RihC remains active. As methanol is a better nucleophile than water, methanol could compete with water as the nucleophile and trap the anomeric form of the product ribose as the methyl glycoside. The two methyl glycosides, methyl α-D-ribofuranoside and methyl β-D-ribofuranoside, are easily distinguishable by ^{13}C NMR. To increase the sensitivity of the experiment, [1-^{13}C]adenosine was used as the starting material. The ^{13}C chemical shifts of the anomeric carbons for adenosine, and the α- and β- methyl ribofuranosides were 88.6, 103.1, and 108.0 ppm respectively. After addition of RihC from *E. coli,* the progress of the reaction was monitored by ^{13}C NMR. The reaction was

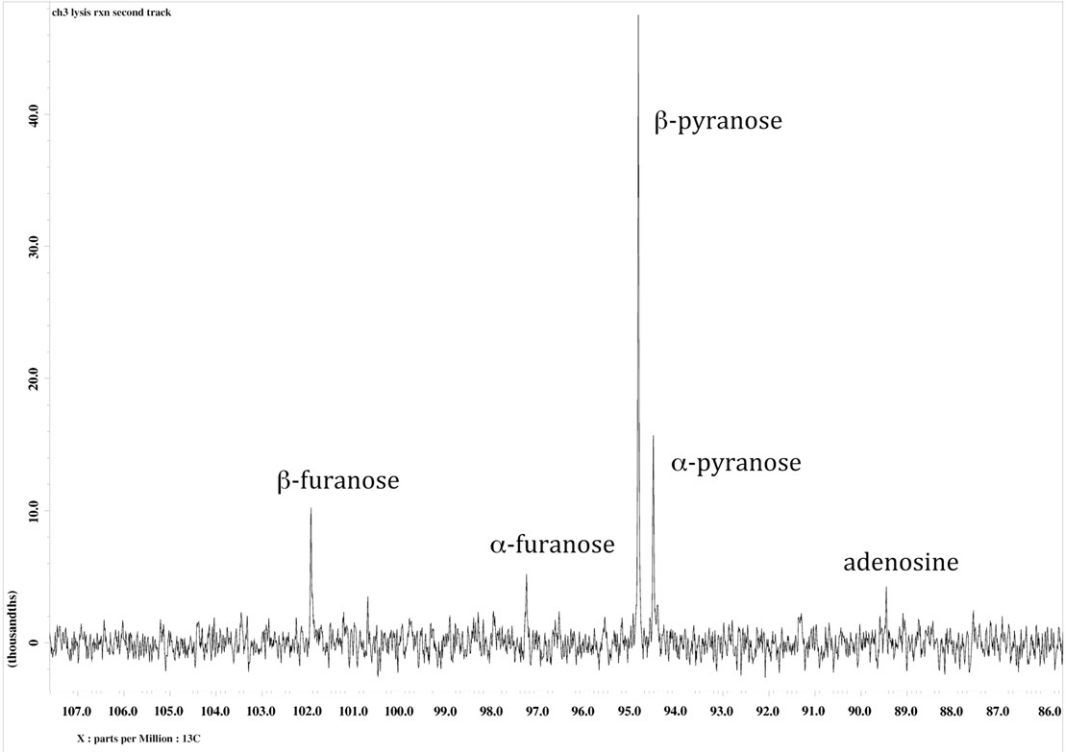

Fig. 2. ^{13}C NMR spectrum of product from reaction of [1′-^{13}C]adenosine in methanol in the presence of RihC. The peaks present are consistent with ribose and unreacted adenosine: α-furanose (97.2 ppm), β-furanose (101.8 ppm), α-pyranose (94.4 ppm), β-pyranose (94.9 ppm) and adenosine (89.5 ppm). Neither methyl α- nor β-ribofuranoside (88.6 and 103.1 ppm) was observed.

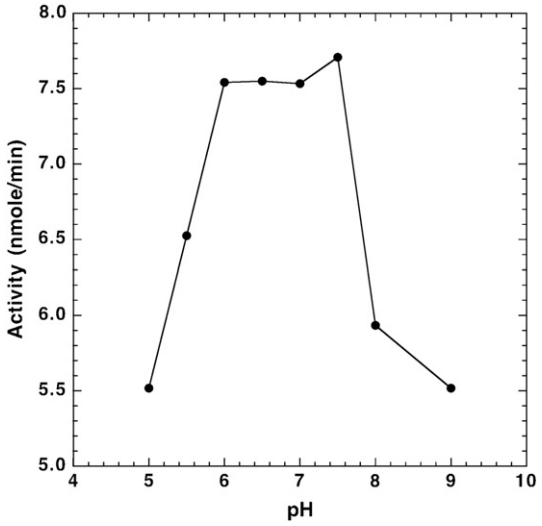

Fig. 3. Effect of pH on velocity of reaction using inosine (1000 μM) as the substrate. The pH was maintained using a mixture of sodium acetate, MES, CHES, PIPES, HEPES, and BICINE adjusted to the desired pH. The enzyme retained activity over a broad range of pH, with maximum activity observed at pH 7.5.

allowed to continue until approximately 90% of the starting adenosine had been hydrolyzed. No methyl ribofuranosides were detected. Four peaks at 97.2, 101.8, 94.4, and 94.9 ppm were observed which represent the four anomeric forms of ribose. Based on the ^{13}C NMR results, ribose rather than the methyl glycoside was formed (Fig. 2). Methanol did not act as the nucleophile. Rather, water was the nucleophile resulting in the formation of ribose. The same result was observed for nucleoside hydrolase from *C. fasciculata* and adenosine nucleosidase from *L. luteus*. This was consistent with the mechanism proposed for Group I nucleoside hydrolases in which an aspartic acid (Asp 11), acting as a base, removes a proton from an ordered water molecule. This activates the water molecule to act as a nucleophile to attack C1′ of the ribose ring, during the formation of the oxocarbenium ion as the C–N bond begins to break [20].

3.7. pH behavior

The activity of RihC from *E. coli* using inosine as the substrate as a function of pH was determined from pH 4–9. The pH profile was bell-shaped with the enzyme retaining significant activity over a wide pH range (Fig. 3). RihC retained over 50% of its specific activity at both pH 5 and 9. The enzyme exhibited maximum activity at a pH of 7.5. This is similar to the pH behavior of RihC from *Salmonella enterica serovar Typhimurium* which was also bell-shaped with an optimum at pH 7.8 [2].

4. Conclusions

RihC is one of three nucleoside hydrolases identified in *E. coli*. The enzyme has been cloned and overexpressed in *E. coli* as a fusion protein with an N-terminal His-Tag to aid in purification. The enzyme is a non-specific nucleoside hydrolase which is capable of hydrolyzing the common ribonucleosides. The substrate specificity indicates a number of important structural features that must be present for a compound to be substrate. The 2′ and 3′ hydroxyl groups must be present in the ribosyl conformation, while the 5′ hydroxyl group is important but not critical. Purines must contain N7 while pyrimidines must contain N3. The exocyclic amino group is not essential for the reaction. However, the presence of any attached substituents to the exocyclic amino group results in a loss of activity. The enzyme exists as a dimer with two identical subunits and has a pH optimum of 7.5. The reaction apparently involves an ordered water molecule, rather than an active site open to the solvent. The reaction is essentially irreversible with an equilibrium constant of 45 M for the inosine formation reaction.

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