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Bacterial expression, folding, purification and characterization of soluble NTPDase5 (CD39L4) ecto-nucleotidase☆

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Abstract

The ecto-nucleoside triphosphate diphosphohydrolases (eNTPDases) are a family of enzymes that control the levels of extracellular nucleotides, thereby modulating purinergically controlled physiological processes. Six of the eight known NTPDases are membrane-bound enzymes; only NTPDase 5 and 6 can be released as soluble enzymes. Here we report the first bacterial expression and refolding of soluble human NTPDase5 from inclusion bodies. The results show that NTPDase5 requires the presence of divalent cations (Mg^{2+} or Ca^{2+}) for activity. Positive cooperativity with respect to hydrolysis of its preferred substrates (GDP, IDP and UDP) is observed, and this positive cooperativity is attenuated in the presence of nucleoside monophosphate products (e.g., GMP and AMP). In addition, comparing the biochemical properties of wild-type NTPDase5 and those of a mutant NTPDase5 (C15S, which lacks the single, non-conserved cysteine residue), also expressed in bacteria, suggests that Cys^{15} is not essential for either proper refolding or enzymatic activity (indicating this residue is not involved in a disulfide bond). Moreover, the substrate profile of bacterially expressed NTPDase5, as well as the C15S mutant, was determined to be similar to that of full-length, membrane-bound and soluble NTPDase5 expressed in mammalian COS cells.

Keywords: Nucleoside triphosphate diphosphohydrolase; NTPDase5; CD39L4; Bacterial expression; Protein refolding; Enzymatic characterization; Positive cooperativity

1. Introduction

The ectonucleoside triphosphate diphosphohydrolases (eNTPDases) are a family of enzymes that hydrolyze nucleotides and require divalent cations (e.g. Ca^{2+} or Mg^{2+}) for activity. These enzymes share seven highly conserved regions of homology named apyrase conserved regions (ACRs) [1–4], which are essential for catalytic activity. The six well-established enzymes, NTPDase1–6, consist of four membrane-bound enzymes and two soluble, secreted enzymes [5]. NTPDase5 and NTPDase6 can be extracellularly released as soluble enzymes following cleavage of their respective N-terminal signal sequences [6–11]. Additionally, two other NTPDases have recently been described, NTPDase7 (also known as LALP1 [12]) and NTPDase8 [13]. NTPDase8 is a novel plasma membrane enzyme cloned and characterized from mouse, which is highly homologous to the previously characterized [14,15] and cloned [16] chicken ecto-ATPDase.

NTPDase5 (CD39L4) was first identified as a member of the CD39/NTPDase family in 1998 [17]. Later, NTPDase5 was shown to be identical to PCPH [18], a human proto-

Abbreviations: NTPDases, *n*ucleoside *trip*hosphate *d*iphosphohydrolases; NTPDase5, nucleoside triphosphate diphosphohydrolase type 5; ACRs, apyrase conserved regions; B-PER, bacterial protein extraction reagent; MWCO, molecular weight cut off; P_i, inorganic phosphate; MOPS, 3-[*N*-morpholino]propanesulfonic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; DTT, dithiothreitol; DMEM, Dulbecco's modified Eagle medium; NTA, nitrilotriacetic acid; KLH, keyhole limpet hemocyanin; TBS, Tris-buffered saline; SEC, size exclusion chromatography

☆ The sequence of the cDNA encoding the NTPDase5 expressed in this study has been submitted to GenBank and assigned accession no. AY430094.

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oncogene product that was discovered after its activation upon treatment with a chemical carcinogen [19].

To date, two studies on the characterization of "NTPDase5" have been published [9,10]. Additionally, a study on an enzyme termed the "ER-UDPase", a soluble, mammalian, endoplasmic reticulum (ER) localized nucleoside diphosphatase, has been published [11]. This mouse liver ER-UDPase (GenBank Accession No. AJ238636 [11]) is approximately 88% identical to human NTPDase5 at the amino acid level. Interestingly, these previous studies have produced some seemingly contradictory information about NTPDase5, especially in relation to its hydrolytic activity towards ADP.

The primary sequence of the soluble portion of NTPDase5 encodes five cysteine residues, four of which are conserved amongst the NTPDases. In NTPDase6, the other soluble NTPDase, it was shown that these four conserved cysteine residues form two disulfide linkages [7], which are very likely to be conserved in other NTPDases, including NTPDase5. This suggests that the single, non-conserved cysteine residue in the soluble NTPDase5 (C15) is not involved in a disulfide bond.

Physiologically, the NTPDases are involved in many important functions including smooth muscle contraction, pain perception, and the modulation of platelet aggregation (for a review, see [20]). The NTPDases function in these processes by modulating the concentrations of nucleotide agonists in the extracellular space, thus regulating the purinergic receptors in control of each respective process.

Recent studies have focused on the therapeutic potential of these enzymes. Hydrolysis of ADP (that regulates platelet aggregation and blood clotting) by the soluble portion of NTPDase1 has been shown to decrease damage caused by ischemic stroke [21]. Thus, naturally occurring, soluble nucleotidases such as the NTPDase5 enzyme studied in this work may be useful clinically, and serve as a starting point for the optimization of enzymatic properties via mutagenesis, in order to generate enzymes with modified nucleotide specificities that may be more useful as therapeutic proteins. To this end, Dai et al. [22] have recently reported that site-directed mutagenesis of an unrelated nucleotidase, the human soluble calcium activated nucleotidase (SCAN), can be used to engineer a protein much better suited as an anticoagulant protein than the naturally occurring protein. To accomplish this goal for the NTPDases, a better biochemical understanding of this class of soluble nucleotidases is necessary, and thus, this study strives to address that goal for the soluble NTPDase5/CD39L4 protein.

2. Materials and methods

2.1. Materials

The QuikChange site-directed mutagenesis kit and *Escherichia coli* XL-1 Blue competent cells were purchased from Stratagene. The DNA Core Facility at the University of Cincinnati produced the synthetic oligonucleotides and performed DNA sequencing. Quality Control Biochemicals performed the peptide synthesis and conjugation to keyhole limpet hemocyanin (KLH). Rabbit antisera to the NTPDase5 C-terminal synthetic peptide ((C)ALGATFHLLQSLGISH) conjugated to KLH (via its N-terminal cysteine) were generated by Lampire Biologicals. Plasmid purification kits and Ni-NTA agarose were purchased from Qiagen. *Nhe*I, *Xho*I, and *Hin*dIII restriction endonucleases, T4 DNA ligase, and the mammalian expression vector pcDNA3 were obtained from Invitrogen. The bacterial expression vector pET28a and the expression host *E. coli* BL21 (DE3) and BL21 (DE3) Star were purchased from Novagen. Glycerol and dialysis tubing were from Fisher. Bacterial protein extraction reagent (BPER), Enhanced Chemiluminescent Reagents, and bovine serum albumin (BSA) standard were purchased from Pierce. 4–15% acrylamide Tris–Glycine gels, the S-200 size exclusion chromatography (SEC) matrix, SDS, SEC molecular weight standards, protein assay reagent, and the anion-exchange QMA cartridge were from Bio-Rad. DMEM was a generous gift from Deb Marsh at Hyclone and subsequently has been purchased from Gibco BRL/Invitrogen. Lipofectamine, Plus reagents and antibiotics/antimycotics were from Gibco BRL/Invitrogen. Kanamycin, nucleotides, IPTG, glucose, DTT, oxidized glutathione, reduced glutathione, the protease inhibitor cocktail and other reagents were from Sigma.

2.2. NTPDase5 cloning and sequencing

NCBI BLAST searches of human EST databases identified an IMAGE clone (ID# 4817229) that was highly homologous to CD39L4 (NTPDase5). The clone was purchased from ATCC and completely sequenced (with T7, T3, and three custom-synthesized internal primers). This 1997 base pair IMAGE clone contained the full-length open reading frame encoding CD39L4 (NTPDase5), and was submitted to GenBank under accession number AY430094.

Computer analysis of the NTPDase5 insert predicted that cleavage of the signal peptide would occur after Arg in the sequence SAVSHRNQQTWF. In order to express only the soluble portion of NTPDase5 in the bacterial expression vector, pET28a two endonuclease sites flanking the coding region of the soluble insert (which begins with amino acid residues NQQTWF…) were introduced with site-directed mutagenesis.

To allow cloning of NTPDase5, a 5′ *Nhe*I restriction site (indicated by boldface type) was introduced before the start of the soluble sequence with the following primer: 5′ GTCTCCCACAGG**GCTAGC**AACCAGCAGACTTG3′. A 3′ *Xho*I site (indicated in boldface) was introduced after the stop codon using the following primer: 5′ GGCCACGTACTTCCTTG**CTC**GAGACCTGCATTTGCC3′. The mutated NTPDase5 insert was digested with *Nhe*I and *Xho*I (1 h at 37 °C) and ligated into the pET28a bacterial expression