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Yihx-encoded haloacid dehalogenase-like phosphatase HAD4 from *Escherichia coli* is a specific α-D-glucose 1-phosphate hydrolase useful for substrate-selective sugar phosphate transformations

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ABSTRACT

Phosphomonoester hydrolases (phosphatases; EC 3.1.3.) often exhibit extremely relaxed substrate specificity which limits their application to substrate-selective biotransformations. In search of a phosphatase catalyst specific for hydrolyzing α-D-glucose 1-phosphate (αGlc 1-*P*), we selected haloacid dehalogenase-like phosphatase 4 (HAD4) from *Escherichia coli* and obtained highly active recombinant enzyme through a fusion protein (Z_{basic2}-HAD4) that contained Z_{basic2}, a strongly positively charged three α-helical bundle module, at its N-terminus. Highly pure Z_{basic2}-HAD4 was prepared directly from *E. coli* cell extract using capture and polishing combined in a single step of cation exchange chromatography. Kinetic studies showed Z_{basic2}-HAD4 to exhibit 565-fold preference for hydrolyzing αGlc 1-*P* ($k_{cat}/K_M = 1.87 \pm 0.03\ mM^{-1}\ s^{-1}$; 37 °C, pH 7.0) as compared to D-glucose 6-phosphate (Glc 6-*P*). Also among other sugar phosphates, αGlc 1-*P* was clearly preferred. Using different mixtures of αGlc 1-*P* and Glc 6-*P* (e.g. 180 mM each) as the substrate, Z_{basic2}-HAD4 could be used to selectively convert the αGlc 1-*P* present, leaving back all of the Glc 6-*P* for recovery. Z_{basic2}-HAD4 was immobilized conveniently using direct loading of *E. coli* cell extract on sulfonic acid group-containing porous carriers, yielding a recyclable heterogeneous biocatalyst that was nearly as effective as the soluble enzyme, probably because protein attachment to the anionic surface occurred in a preferred orientation via the cationic Z_{basic2} module. Selective removal of αGlc 1-*P* from sugar phosphate preparations could be an interesting application of Z_{basic2}-HAD4 for which readily available broad-spectrum phosphatases are unsuitable.

1. Introduction

Approximately half of the intracellular carbohydrates in the *Escherichia coli* metabolome contain a phosphate moiety [1]. A plethora of enzymes is involved in the dynamic regulation and homeostasis of sugar phosphate metabolite levels. One key enzyme subset is the phosphatases [2]. They catalyze phosphomonoester group hydrolysis to release inorganic phosphate from phosphorylated substrate.

The known sugar phosphate phosphatases represent a large diversity in protein structures, catalytic mechanisms, and substrate specificity. However, many of them are evolutionary related to haloacid dehalogenase (HAD)-like proteins [3–5]. Originally named after the activity of its first structurally characterized member (L-2-HAD from *Pseudomonas* sp. YL), the HAD-like protein superfamily currently comprises mainly phosphatases, with about 79% of the classified and biochemically studied proteins exhibiting this activity [6].

Common structural element of HAD-like phosphatases is a Rossmann fold-like α/β core domain that contains the active site. According to the presence and structure of an additional domain, the so-called CAP, HAD-like phosphatases are subdivided into subfamilies CI (α-helical CAP), CII (mixed α/β CAP), and CIII (no CAP) [7]. The catalytic centre is highly conserved. It features a key Asp that is proposed to function as catalytic nucleophile in a double displacement-like enzyme reaction mechanism. Phospho-monoester hydrolysis would accordingly proceed in two catalytic steps via a covalent aspartyl-phosphate enzyme intermediate [6–8]. HAD-like phosphatases require divalent metal ion (typically Mg^{2+}) bound in the active site for their full activity [9,10]. Phosphatase substrate specificity is dictated by flexible

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loop structures on the α/β core domain and on the CAP domain [11]. The spectrum of phosphoryl substrates hydrolyzed is usually very broad, and it includes both sugar and non-sugar phosphates [12]. Relationships between structure and specificity are only weakly defined for HAD-like phosphatases. Substrate binding and product release involve domain-closing and opening movements of the CAP [9,13–15]. Protein conformational flexibility further complicates efforts to infer substrate specificity from sequence and three-dimensional structure information alone. Purposeful selection or molecular design of a HAD-like phosphatase for biocatalytic conversion of specific target substrates is therefore difficult. Evidence from biochemical characterization is vital for efficient development.

Selectivity is a prime feature of many enzymatic transformations [16]. A growing number of examples show that in a comparison of conventional-chemical and biocatalytic process options in organic synthesis [17,18], it is usually the bio-based selectivity that provides a decisive advantage for process development. In this paper, we address the problem of substrate-selective hydrolysis of α-D-glucose 1-phosphate (αGlc 1-*P*) by phosphatases and report identification of HAD4 (*yihx* gene product) from *E. coli* for that purpose. Major demand for the reaction is in reactive processing of mixtures of sugar phosphates to eliminate all of the αGlc 1-*P* present in the starting material. Sample work-up for analytics, in the field of sugar phosphate metabolomics for example, and facilitated sugar phosphate product recovery constitute interesting applications of selective αGlc 1-*P* converting phosphatases. The readily available broad-spectrum phosphatases are however not suitable for these applications, and discovery of new phosphatase catalysts is therefore required. HAD4 was obtained as highly active recombinant enzyme through a specially designed fusion protein (Z_{basic2}-HAD4) that contained Z_{basic2}, a strongly positively charged three α-helical bundle module, at its N-terminus [19]. Z_{basic2} facilitated functional expression of soluble protein in *E. coli* and enabled protein capture and polishing efficiently combined in a single step of cation exchange chromatography. Furthermore, the Z_{basic2} module had an instrumental role in the development of an immobilized HAD4 biocatalyst where Z_{basic2}-HAD4 was bound directly from crude bacterial cell extract on sulfonic acid group-containing porous carriers. Attachment of fusion protein to the negatively charged carrier surface was not only highly selective in that accompanying *E. coli* protein only showed marginal binding under the conditions used, but it also appeared to have occurred in a strongly preferred orientation via the cationic binding module: immobilized Z_{basic2}-HAD4 was nearly as effective as the free enzyme. Kinetic characterization of Z_{basic2}-HAD4 with αGlc 1-*P* and the competing sugar phosphate substrate D-glucose 6-phosphate (Glc 6-*P*) is reported. Utilization of different sugar phosphates as substrates for hydrolysis by Z_{basic2}-HAD4 is described, and some basic properties of the enzyme fused to Z_{basic2} are reported. Clear-cut separation of Glc 6-*P* from αGlc 1-*P* through selective hydrolysis of the sugar 1-phosphate at high concentration (up to 180 mM) and in the presence of up to 9-fold excess of competing substrate is shown.

2. Materials and methods

2.1. Chemicals and reagents

Unless stated otherwise, all chemicals were of highest purity available from Sigma–Aldrich (Vienna, Austria) or Roth (Karlsruhe, Germany). Sugar phosphates were obtained as sodium or ammonium salts. Oligonucleotide primers were from Sigma–Aldrich (Vienna, Austria). DNA sequencing was done at LGC Genomics (Berlin, Germany).

2.2. Expression and purification of His_6-HAD4

Expression vector pCA24N-yihx encoding HAD4 equipped with N-terminal hexahistidine tag was kindly provided by Dr. Alexander Yakunin. The vector was transformed into electro-competent cells of *E. coli* BL21 (DE3), and single-colony transformants were selected on agar plates containing 0.025 mg/mL chloramphenicol. Recombinant protein was produced in 1-L baffled shaking flasks at 37 °C using an agitation rate of 110 rpm (Certomat BS-1 shaking incubator, Sartorius, Göttingen, Germany). Flasks contained 250 mL Lennox-medium supplemented with 0.025 mg/mL chloramphenicol. At OD_{600} of 0.8, temperature was decreased to 18 °C, 0.01 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added, and cultivation was continued for 20 h. Centrifuged (4 °C, 30 min; 4,420 g) and washed cells were suspended in 10 mL of 50 mM Mes pH 7.0 buffer and disrupted by triple passage through French pressure cell at 150 bar (American Instruments, Silver Springs, USA). After removal of cell debris (4 °C, 30 min; 20,000 g) and filtration through a 1.2 μm cellulose-acetate syringe filter (Sartorius, Göttingen, Germany). The pre-treated cell lysate (350 mg; 8 mL) was loaded on a 14 mL copper-loaded affinity column (Chelating Sepharose Fast Flow; XK 16/20 column, GE Healthcare, Little Chalfont, U.K.), beforehand equilibrated with buffer A (50 mM Tris HCl, pH 7.0 containing 300 mM NaCl) and mounted on an Bio-Logic DuoFlow™ system (Biorad, Hercules, CA, USA). Differential elution was performed with buffer B (50 mM Tris HCl, pH 7.0 containing 300 mM NaCl and 400 mM imidazole) at 10 °C and a flow rate of 3 mL/min. The elution protocol comprised 5 steps, whereas concentration of buffer B was stepwise increased to 0, 10, 30, 60 and 100% in a volume of 100, 90, 60, 30 and 75 mL, respectively. All buffers were degassed and filtered using 0.45 μm cellulose-acetate or 0.2 μm polyamide filters. Protein elution was monitored spectrophotometrically at 280 nm, and collected fractions were assayed for protein concentration and phosphatase activity against *p*-nitrophenyl phosphate (pNPP; see Assays). Active fractions were pooled and buffer was exchanged to buffer A_2 (50 mM KH_2PO_4-buffer, pH 7.0) with Amicon Ultra-15 Centrifugal Filter Units (Millipore, Billerica, USA). Pooled fractions were further purified using a 50 mL Fractogel EMD-DEAE column (XK 26/20, Merck, Darmstadt, Germany) mounted on a BioLogic DuoFlow™ system. Protein elution was performed with buffer B_2 (50 mM KH_2PO_4, 1 M NaCl, pH 7.0) applying a five-step purification protocol at a constant flow rate of 5 mL/min: step 1, isocratic flow of 100% buffer A_2 for 50 mL; step 2, linear increase to 15% buffer B_2 in 40 mL; step 3, isocratic flow of 85% buffer A_2 and 15% buffer B_2 for 80 mL; step 4, linear increase to 100% buffer B_2 in 180 mL; step 5, isocratic flow of 100% buffer B_2 for 70 mL. Active fractions were pooled and buffer was exchanged to 50 mM Hepes pH 7.0 with Amicon Ultra-15 Centrifugal Filter Units. Protein purification was monitored by SDS PAGE and phosphatase activity measurements.

Alternatively, the pre-treated cell lysate (280 mg; 8 mL) was loaded on 1.6 cm × 2.5 cm; 5 mL HisTrap FF column (GE Healthcare), equilibrated with buffer C (50 mM MES, pH 7.4 containing 125 mM NaCl, 20 mM imidazole) and mounted on an ÄKTA prime plus (GE Healthcare) system. Protein was eluted using an imidazole gradient from 0 to 80% buffer D (50 mM MES, pH 7.4 containing 125 mM NaCl and 500 mM imidazole) at 10 °C and at a flow rate of 4 mL/min. At 20% buffer D, the linear gradient was halted for 2 column volumes to facilitate differential elution of bound protein. Active fractions were pooled and buffer was exchanged to 50 mM MES, pH 7.0. Protein purification was monitored by SDS PAGE and phosphatase activity measurements. Densitometry evaluation of the SDS PAGE was performed with the GelAnalyzer software (http://www.gelanalyzer.com/index.html).