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Research paper

Enzymatic characterization of a monomeric isocitrate dehydrogenase from *Streptomyces lividans* TK54

Beibei Zhang [a], Baojuan Wang [a], Peng Wang [a], Zhengyu Cao [a], Enqi Huang [a], Jiasheng Hao [a,b], Antony M. Dean [c], Guoping Zhu [a,b,*]

[a] Key Laboratory of Molecular Evolution and Biodiversity and Institute of Molecular Biology and Biotechnology, College of Life Sciences, Anhui Normal University, Wuhu 241000, China
[b] Key Laboratory of Biotic Environment and Ecological safety in Anhui Province, Anhui Normal University, Wuhu 241000, China
[c] BioTechnology Institute and Department of Ecology, Evolution, and Behavior, University of Minnesota, St. Paul, MN 55108, USA

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ABSTRACT

Isocitrate dehydrogenase (IDH) is one of the key enzymes in the citric acid cycle, which involves in providing energy and biosynthetic precursors for metabolism. Here, we report for the first time the enzymatic characterization of a monomeric $NADP^+$-dependent IDH from *Streptomyces lividans* TK54 (*Sl*IDH). The *icd* gene (GenBank database accession number EU661252) encoding IDH was cloned and overexpressed in *Escherichia coli*. The molecular mass of *Sl*IDH was about 80 kDa, typical of a monomeric NADP-IDH, and showed high amino acid sequence identity with known monomeric IDHs. The optimal activity of the 6His-tagged *Sl*IDH was found at pH values 8.5 (Mn^{2+}) and 9.0 (Mg^{2+}), and the optimal temperature was around 46 °C. Heat-inactivation studies showed that about 50% *Sl*IDH activity was preserved at 38 °C after 20 min of incubation. The recombinant *Sl*IDH displayed a 62,000-fold (k_{cat}/K_m) preference for $NADP^+$ over NAD^+ with Mn^{2+}, and a 85,000-fold greater specificity for $NADP^+$ than NAD^+ with Mg^{2+}. Therefore, *Sl*IDH is a divalent cation-dependent monomeric IDH with remarkably high coenzyme preference for $NADP^+$.

1. Introduction

Both monomeric and dimeric $NADP^+$-dependent isocitrate dehydrogenases (IDHs) belong to the metal-dependent β-decarboxylating dehydrogenase family and catalyze the oxidative decarboxylation from 2R,3S-isocitrate to yield α-ketoglutarate, CO_2, and NADPH. However, the biochemical analyses, crystal structures and catalytic mechanism of monomeric IDH are not yet well known as dimeric IDH [1–3].

IDHs belong to a large ancient family of enzymes that play central roles in energy metabolism, amino acid biosynthesis and vitamin production [1,2]. Based on coenzyme specificity, the IDH family can be divided into the NAD^+-dependent IDH (EC 1.1.1.41, NAD-IDH) and the $NADP^+$-dependent IDH (EC 1.1.1.42, NADP-IDH). Both enzymes play important roles in central metabolism. NAD-IDH participates in respiratory ATP production, while NADP-IDH is involved in generating NADPH and α-ketoglutarate for biosynthesis [4,5]. NADP-IDH is further classified into homodimeric and monomeric forms. All eukaryotes and most prokaryotes have a homodimeric NADP-IDH with two subunits of 40–50 kDa each, whereas monomeric NADP-IDH, with a molecular mass of approximately 80 kDa, has been found only in bacterial species [3–5]. Dimeric and monomeric IDHs are only 7–8% identical in amino acid sequences, values so low that they may have evolved convergently [6].

The homodimeric NADP-IDHs from prokaryotes and eukaryotes have been extensively studied with respect to structural, kinetic, catalytic and regulatory characteristics: from *Escherichia coli* (*Ec*IDH) [7–10], porcine heart mitochondria (*Pm*IDH) [11] and human cytosol (*Hc*IDH) [12], as well as IDHs from hyperthermophilic *Aeropyrum pernix* [13]. By contrast, the monomeric IDHs in only a few species of bacteria have been purified and characterized: *Azotobacter vinelandii* [14], *Colwellia maris* [6], *Corynebacterium glutamicum* [3,4,15], *Desulfobacter vibrioformis* [16], *Rhodomicrobium vannielii* [17] and *Vibrio parahaemolyticus* Y-4 [18]. Recent genome projects have identified possible open reading frames encoding monomeric IDHs in more than 50 additional bacterial species. However, structures are available for only two monomeric IDHs: *A. vinelandii* IDH (*Av*IDH, PDB 1ITW and 1J1W) and *C. glutamicum* IDH (*Cg*IDH, PDB 2B0T) [1,5]. Structural studies of *Av*IDH suggested that monomeric NADP-IDH evolved from the

* Corresponding author at: College of Life Sciences, Anhui Normal University, No.1 Beijing East Road, Wuhu 241000, Anhui, China. Tel./fax: +86 553 3883592.
E-mail address: gpz1996@yahoo.com (G. Zhu).

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dimeric IDHs through a partial gene duplication and subsequent accumulation of substitutions, insertions and deletions [1,19]. In addition, several features revealed that the monomeric IDHs are structurally more related to the eukaryotic dimeric IDHs than to the bacterial dimeric IDHs [1]. Further characterizations of monomeric IDHs are needed to confirm this hypothesis.

Streptomycetes are high *G* + *C* Gram-positive, antibiotic-producing, mycelial soil bacteria. Unlike unicellular bacteria, streptomycetes exhibit complex multicellular development, forming dense interconnected networks of mycelia that share many characteristics in common with fungi – an example of convergent evolution caused by adaptation to similar ecological niches. Streptomycetes are also responsible for producing most natural antibiotics used in human and veterinary medicine, including anticancer agents and immunosuppressants [20]. Because of their phylogenetic importance and commercial value, they have become a model prokaryote for the study of evolution and secondary metabolism. In this study, a new gene encoding monomeric NADP-IDH from *Streptomyces lividans* TK54 was successfully cloned and expressed in *E. coli* as a fusion protein. Furthermore, the protein was purified and its enzymatic properties were characterized.

2. Materials and methods

2.1. Strains and reagents

S. lividans TK54 is the focus of our studies. *E. coli* Rosetta (DE3) and plasmid pET-28*b*(+) (a generous gift from Professor Congzhao Zhou, School of Life Sciences, University of Science and Technology of China, CAS, Hefei 230027, China) were used for protein expression. PrimeStar™ HS DNA polymerase was obtained from TaKaRa (Dalian, China). Restriction enzymes and protein molecular weight standards were purchased from Fermentas (Shanghai, China).

2.2. Plasmid construction

Genomic DNA was extracted from *S. lividans* TK54 cultured in YEME liquid medium [21]. Based on the genomic sequence from *Streptomyces coelicolor* A3(2) (GenBank accession No. NC_003888), several pairs of homologous primers were designed [22]. Of these, sense primer 5′-GACCCCAATCTAGAATGTTG CAGACGTGGACGCG AG-3′ (*Xba*I site underlined) and antisense primer 5′-AAAAT ACTCGAGGGACGGCAGACAGACGGACACC-3′ (*Xho*I site underlined) were used to obtain the complete *icd* gene by PCR. The PCR product was digested and ligated into pBluescript II SK(+) to generate the recombinant plasmid pBZ54. The presence of a gene encoding a monomeric IDH was confirmed by DNA sequencing. Sense primer 5′-GACGTGCGCATATGACTGACTCGACCATCATCTATACAC-3′ (*Nde*I site underlined) and antisense primer 5′-AAAAATCTCGAGGACGGC AGACAGAC GGACAC-3′ (*Xho*I site underlined) were used to amplify the *icd* gene from pBZ54. The PCR product was digested and inserted into pET-28*b*(+) to generate the recombinant expression vector pEZ54.

2.3. Overexpression and purification of 6His-tagged *Sl*IDH

E. coli Rosetta (DE3) cells harboring pEZ54 were used to express 6His-tagged *Sl*IDH. Cells cultured overnight in LB medium with 30 μg/ml kanamycin and 30 μg/ml chloramphenicol were inoculated (1:100) into 50 ml of fresh LB medium with the same antibiotics and grown in 250 ml flask at 225 rpm, 37 °C, until the culture density reached an OD_{600} of 0.5–0.6. IPTG was added to the culture at a final concentration of 0.1, 0.3, 0.5, 0.7 and 0.9 mM with subsequent incubation for 4 h. Cells were harvested by centrifugation and re-suspended in sonication buffer (10 mM KH_2PO_4 (pH 7.7), 500 mM NaCl, 2 mM $MgCl_2$ and 2 mM β-mercaptoethanol). After sonication, cell debris was removed by centrifugation at 11,000 rpm for 10 min. IDH activities of unfractionated extracts indicated the optimal concentration of IPTG for expression was 0.5 mM. A 1.5 L cell culture grown under optimal conditions for expression was prepared and lysed in BD TALON Tractor buffer (Clontech, LaJolla, CA, USA) with 0.75 mg/ml of lysozyme and 2 U/ml of DNase. Cell debris was removed by centrifugation at 11,000 rpm for 20 min and the 6His-tagged IDH was purified using BD TALON Metal Affinity Resin according to the manufacturer's instructions.

2.4. Purity and western blot analysis

Enzyme purity was determined by SDS-PAGE. For western blot analyses, samples (25 μg each) were separated by 12% SDS-PAGE and transferred onto nitrocellulose membranes (Amersham Biosciences, Germany) by electroblotting. The membrane was blocked for 1 h at room temperature in TBS–T buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl and 0.2% Tween-20) and 5% nonfat milk. His-tagged polyclonal antibody (Cell Signaling Technology Inc., Beverly, MA, USA) and alkaline phosphatase conjugated anti-rabbit IgG (Promega, Madison, WI, USA) were then applied to the blots, followed by incubation with the Lumi-Phos™ WB Chemiluminescent Substrate (Pierce, Rockford, IL, USA). The chemiluminescence signal, corresponding to specific antibody/antigen reaction on the blots, was visualized using X-ray film.

2.5. Gel filtration chromatography

The molecular mass of *Sl*IDH was estimated by gel filtration chromatography on a HiLoad™ 16/60 Superdex 200 column (Amersham Biosciences), equilibrated with 0.05 M potassium phosphate buffer (pH 7.0) containing 0.15 M NaCl and 0.01% sodium azide. Protein standards for calibrating gels were 1.5 mg/ml Myoglobin (17 kDa), 4 mg/ml Ovalbumin (45 kDa), 5 mg/ml Bovine Serum Albumin (66.2 kDa), 0.2 mg/ml IgG (158 kDa) and Ferritin (440 kDa).

2.6. Enzyme assays and kinetic studies

Enzyme assays were carried out at 25 °C in 1 ml volume containing 20 mM Tris–HCl (pH 8.0), 2 mM $MgCl_2$ or $MnCl_2$, 0.5 mM trisodium DL-isocitrate, 0.5 mM $NADP^+$ or 8 mM NAD^+ [15,17]. The increase in NADPH or NADH was monitored at 340 nm with a thermostated Cary 300 UV–Vis spectrophotometer (VARIAN, CA, USA) using a molar extinction coefficient of 6220 M^{-1} cm^{-1}. One unit (U) of activity was defined as 1 μmol NADPH or NADH formed per minute. All kinetic parameters were obtained from at least three measurements. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad) with bovine serum albumin as a standard.

2.7. pH and temperature effects

The enzyme was assayed in 20 mM Tris–HCl buffer between pH 7.0–10.0. The optimal temperature was determined by the standard activity assay at various temperatures from 25 °C to 56 °C. To estimate thermal stability, enzyme aliquots were incubated for 20 min in a water bath at 25–50 °C, after which aliquots were cooled and assayed for activity.