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Characterization of acetohydroxyacid synthase from the hyperthermophilic bacterium *Thermotoga maritima*

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ABSTRACT

Acetohydroxyacid synthase (AHAS) is the key enzyme in branched chain amino acid biosynthesis pathway. The enzyme activity and properties of a highly thermostable AHAS from the hyperthermophilic bacterium *Thermotoga maritima* is being reported. The catalytic and regulatory subunits of AHAS from *T. maritima* were over-expressed in *Escherichia coli*. The recombinant subunits were purified using a simplified procedure including a heat-treatment step followed by chromatography. A discontinuous colorimetric assay method was optimized and used to determine the kinetic parameters. AHAS activity was determined to be present in several *Thermotogales* including *T. maritima*. The catalytic subunit of *T. maritima* AHAS was purified approximately 30-fold, with an AHAS activity of approximately 160 ± 27 U/mg and native molecular mass of 156 ± 6 kDa. The regulatory subunit was purified to homogeneity and showed no catalytic activity as expected. The optimum pH and temperature for AHAS activity were 7.0 and 85 °C, respectively. The apparent K_m and V_{max} for pyruvate were 16.4 ± 2 mM and 246 ± 7 U/mg, respectively. Reconstitution of the catalytic and regulatory subunits led to increased AHAS activity. This is the first report on characterization of an isoleucine, leucine, and valine operon (*ilv* operon) enzyme from a hyperthermophilic microorganism and may contribute to our understanding of the physiological pathways in *Thermotogales*. The enzyme represents the most active and thermostable AHAS reported so far.

1. Introduction

Hyperthermophiles are organisms that exhibit growth temperature optima of 80 °C or above [1,2]. During the last decade there has been enormous attention towards biotechnological and industrial applications of hyperthermophiles. One promising avenue is to use the organisms (or their metabolites) in bio-processing toward production of value-added commodities (*e.g.*, alcohols). However, advances are hindered by the relatively insufficient understanding of the physiology and metabolic pathways of these organisms [3].

Acetohydroxyacid synthases (AHAS, EC 2.2.1.6) are divided into two classes based on their metabolic/physiological roles, substrate specificity and cofactor (FAD) requirements: anabolic and catabolic AHASs [4,5]. The anabolic AHAS catalyzes the first common step in the biosynthesis of the branched-chain amino acids (BCAA, valine, leucine, and isoleucine) as well as the precursors derived from the same biosynthetic pathway (*e.g.* coenzyme A and pantothenate). The enzyme is relatively prevalent in archaea, bacteria, fungi, algae, and plants, but is absent from animals [6–8]. It catalyzes two parallel reactions during which one molecule of pyruvate is decarboxylated, and the resulting "active aldehyde" is condensed with a second molecule of either pyruvate or 2-ketobutyrate to produce acetolactate (precursor of valine and leucine) and 2-aceto-2-hydroxybutyrate (precursor of isoleucine), respectively (Fig. 1). The catabolic AHAS (also known as acetolactate synthase; ALS) has a single subunit with ~60 kDa in size, and is involved in channeling the excess pyruvate to the less inhibitory product 2-acetolactate (Fig. 1) which can be either decomposed to diacetyl (spontaneously) or be decarboxylated to acetoin by acetolactate decarboxylase [9]. This latter enzyme has only been described from certain bacterial species including *Klebsiella*, *Bacillus* species, and some lactic acid bacteria [5,10].

All of the anabolic AHASs studied so far are composed of two subunits: a larger active subunit known as catalytic subunit (generally 59–66 kDa) and a smaller (catalytically inactive) subunit known as regulatory subunit (generally 9–35 kDa). The regulatory

Abbreviations: AHAS, acetohydroxyacid synthase; BCAA, branched chain amino acid; CCE, crude cell extract; CFE, cell-free extract; HTCCE, heat-treated crude cell extract; IB, inclusion body; ilv, isoleucine, leucine, valine; IMAC, immobilized metal affinity chromatography; TmAHAS, *Thermotoga maritima* acetohydroxyacid synthase; TPP, thiamine pyrophosphate

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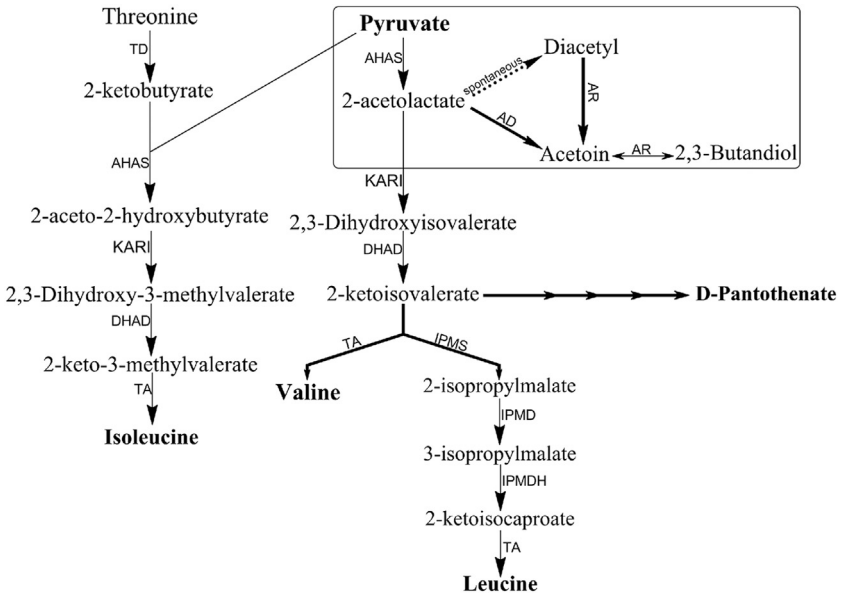

Fig. 1. Biosynthesis pathways of branched chain amino acids and the butanediol pathway (Boxed). TD, threonine deaminase (EC 4.3.1.19); KARI, ketol-acid reductoisomerase (EC 1.1.1.86); DHAD, dihydroxyacid dehydratase (EC 4.2.1.9) ; TA, transaminase (EC 2.6.1.42, EC 2.6.1.66, EC 2.6.1.6); IPMS, 2-isopropylmalate synthase (EC 2.3.3.13); IPMD, isopropylmalate dehydratase (EC 4.2.1.33); IPMDH, 3-isopropylmalate dehydrogenase (EC 1.1.1.85); AD, acetolactate decarboxylase (EC 4.1.1.5); AR, acetoin reductase (EC 1.1.1.4).

subunit mediates the regulation of the catalytic subunit through the feedback regulation by one or more of the branched chain amino acids [11,12]. Compared to the catalytic subunit, the holoenzyme (catalytic and regulatory subunits together) generally shows higher activity, which is also evident by the observation that combining separately purified subunits generally results in reconstitution of the holoenzyme with a several-fold increase of activity [13–17].

Several anabolic AHASs from various organisms have been characterized including different isozymes (isozymes I, II, and III) from *Escherichia coli* [17–19], and *Salmonella* [20], *Corynebacterium glutamicum* [21], *Klebsiella pneumoniae* [22], different pathogenic mycobacteria species [23–26], *Shigella sonnei* [27] and *Haemophilus influenzae* [28], *Saccharomyces cerevisiae* [16], and plants including Arabidopsis (*Arabidopsis thaliana*) [29], and tobacco (*Nicotiana tabacum*) [30]. Considering the absence of the branched chain amino acid (BCAA) biosynthesis pathway in animals, the anabolic AHAS has spurred significant interest as a potential candidate for development of new antimicrobial drugs and herbicides (reviewed in Refs. [31] and [32], respectively).

Very few AHASs have been investigated from extremophilic microorganisms. An oxygen-sensitive AHAS from the mesophilic archaeon *Methanococcus aeolicus* has been characterized [33]. A characteristically similar AHASs has been characterized from the halophilic archaeon *Haloferax volcanii* [34] and moderately thermophilic bacterium *Geobacillus stearothermophilus* [35]. However, so far no hyperthermophilic AHAS has been studied.

Thermotoga maritima is the model organism in the order of *Thermotogales* with a growth temperature range of 55–90 °C and an optimal growth temperature of 80 °C [36]. In the present study, AHAS activity was investigated in *Thermotogales*. To our knowledge this is the first report on the biochemical characterization of an *ilv* operon enzyme from a hyperthermophilic bacterium. The genes encoding the hypothetical catalytic and regulatory subunits of AHAS from *T. maritima* were heterologously expressed in *E. coli* and the properties of the purified recombinant enzyme were characterized.

2. Materials and methods

2.1. Microorganisms and plasmids

T. maritima , *Thermotoga hypogea* and *Thermotoga neapolitana* were obtained from German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and were grown under anaerobic conditions in 20 L glass carboys. *T. maritima* was grown anaerobically on glucose and yeast extract at 80 °C as described by Huber et al. [36] using a modified procedure as described elsewhere [37]. *T. hypogea* and *T. neapolitana* cell biomasses were grown on glucose at 70 °C and 77 °C, respectively using the procedure modified from Fardeau et al. [38] as described by Yang and Ma [39].

The growth was monitored by direct microscopic cell enumeration using a *Petroff-Hausser* cell counting chamber (1/400 mm^2, 0.02 mm deep; Hausser Scientific, Horsham, PA) and a Nikon Eclipse E600 phase-contrast light microscope (Nikon Canada, ON, Canada). The late log-phase cultures were cooled down in ice slurry and centrifuged at 13,000*g* using a Sharples continuous centrifugation system (Sharples equipment division, PA, USA) at 150–200 ml/min. The resulting biomass was snap-frozen in liquid nitrogen and then stored at −80 °C until use.

E. coli strains DH5α (BRL, CA, USA) was used for recombinant DNA propagation and *E. coli* BL21 (DE3) Rosetta 2 [F^- *ompT hsd*SB (rB^- mB^-) *gal dcm* pRARE27 (*CamR*)] (Novagen, WI, USA) was used for overexpression of AHAS subunits under the control of T7 polymerase of the plasmid pET30a (+) (Novagen, WI, USA). The recombinant *E. coli* strains were grown in LB broth (10 g Bacto-Tryptone, 5.0 g yeast extract, 10 g NaCl per liter, pH 7.5) supplemented with kanamycin (30 μg/ml) and chloramphenicol (34 μg/ml) for plasmid maintenance.

2.2. Preparation of cell-free extracts

The frozen biomass was thawed in a pre-degassed flask and then re-suspended in anaerobic lysis buffer (10 mM Tris–HCl, 5% glycerol, 2 mM sodium dithionite (SDT), 2 mM DTT, 0.01 mg/ml