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Regulation of enzyme activity of alcohol dehydrogenase through its interactions with pyruvate-ferredoxin oxidoreductase in *Thermoanaerobacter tengcongensis*

Qian Wang [a,1], Quanhui Wang [a,1], Wei Tong [a], Xue Bai [a], Zhen Chen [a], Jingjing Zhao [a], Jiyuan Zhang [a], Siqi Liu [a,b,*]

[a] Beijing Institute of Genomics, Chinese Academy of Sciences, Beijing 100029, China
[b] Beijing Protein Innovation, Beijing 101318, China

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ABSTRACT

Alcohol dehydrogenases (ADHs) from thermophilic microorganisms are interesting enzymes that have their potential applications in biotechnology and potentially provide insight into the mechanisms of action of thermo-tolerant proteins. The molecular mechanisms of ADHs under thermal stress *in vivo* have yet to be explored. Herein, we employed a proteomic strategy to survey the possible interactions of secondary-ADH (2-ADH) with other proteins in *Thermoanaerobacter tengcongensis* (*T. tengcongensis*) cultured at 75 °C and found that 2-ADH, pyruvate-ferredoxin oxidoreductase (PFOR) and several glycolytic enzymes coexisted in a protein complex. Using anion exchange chromatography, the elution profile indicated that the native 2-ADH was present in two forms, PFOR-bound and PFOR-free. Immuno-precipitation and pull down analysis further validated the interactions between 2-ADH and PFOR. The kinetic behaviours of 2-ADH either in the recombinant or native form were evaluated with different substrates. The enzyme activity of 2-ADH was inhibited in a non-competitive mode by PFOR, implying the interaction of 2-ADH and PFOR negatively regulated alcohol formation. In *T. tengcongensis,* PFOR is an enzyme complex located at the upstream of 2-ADH in the alcohol generation pathway. These findings, therefore, offered a plausible mechanism for how alcohol metabolism is regulated by hetero-interactions between 2-ADH and PFOR, especially in anaerobic thermophiles.

1. Introduction

Alcohol dehydrogenases (ADHs) are one of the most valuable enzyme classes in thermophilic microorganisms. They not only have significant potential for the generation of potable alcohol, solvents and acetic acid but are also important for elucidating the mechanisms of action, regulation, and evolution of thermophilic enzymes. According to the structures of carbon chains in alcohols, ADHs are generally divided into two groups, of which primary-ADHs (1-ADHs) prefer to generate primary alcohols, such as ethanol and propanol, while secondary-ADHs (2-ADHs) prefer to make secondary alcohols, such as isopropanol and isobutanol. A thermophilic bacterium may has multiple forms of ADHs that play different roles in alcohol synthesis and metabolism [1,2]. Bryant et al. proposed that the 2-ADHs functioned primarily to produce alcohol, whereas the 1-ADHs were used in alcohol consumption for nicotinamide cofactor recycling [3].

Several crystal structures of thermophilic ADHs have been carefully examined [4,5]. Analysis of the linear amino acid sequences and three-dimensional domains of ADHs reveals that thermophilic ADHs are comparable to mesophilic ADHs [6]. Therefore, it is unclear how thermophilic ADHs tolerate thermal stress. Peretz et al. postulated that the high content of proline residues in thermophilic ADH was a key factor in higher thermostability, as eight additional prolines were present in the anaerobic thermophilic *Thermoanaerobacter brockii* 2-ADH compared with the mesophilic *Clostridium beijerinckii* 2-ADH [7,8]. On the other hand, Bogin et al. observed that proline substitution in certain positions was able to increase the thermostability of mesophilic 2-ADH, whereas in other positions resulted in a reduction of the thermal stability [9]. Therefore, the thermostability of thermophilic ADHs is a result not only of major amino acid sequence differences from their mesophilic counterparts but also of stabilising factors, such as the

Abbreviations: ADHs, alcohol dehydrogenases; 2-ADHs, secondary alcohol dehydrogenases; PFOR, pyruvate-ferredoxin oxidoreductase; IP, immunoprecipitation; BN PAGE, blue native polyacrylamide gel electrophoresis; PTA, phosphate acetyltransferase; AK, acetate kinase; BLIP, β-lactamase inhibitory protein.

* Corresponding author. Fax: +86 10 80485324.
E-mail address: siqiliu@genomics.org.cn (S. Liu).

[1] These authors contributed equally to the work.

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hydrophilic exterior, the hydrophobic interior, the surface area, salt bridges and hydrophobic interactions [9,10]. The stability and activity of 2-ADHs may be regulated through intra- and intermolecular interactions both with other 2-ADHs and with other proteins. The thermal 2-ADHs are typically tetrameric pyridine dinucleotide-dependent metalloenzymes, and a large number of interactions occur between the subunits [11]. There is, however, a lack of any information regarding the hetero-interactions between 2-ADH and other proteins. The catalytic properties of thermal 2-ADH were similar to those of mesophilic 2-ADH in an *in vitro* assay with cysteine and histidine acting as ligands for the catalytic Zn^{2+} [12]. Although a variety of factors important for *in vitro* 2-ADH activity have been intensely studied, including temperature-dependent kinetics, metal ligands and selection of substrates and coenzymes, the *in vivo* regulation of 2-ADHs and the native state of these thermophilic enzymes have not been fully elucidated.

Thermoanaerobacter tengcongensis is a thermophilic eubacterium that is capable of survival from 50 to 80 °C [13]. Due to in-depth investigation into its genomics and proteomics, *T. tengcongensis* is becoming an ideal model thermophile for examining the molecular mechanisms of thermo-adaptation. In the *T. tengcongensis* genome, there are six genes annotated as ADHs: *TTE0695, TTE0696, TTE0313, TTE1591, TTE0722,* and *TTE2405*. Out of these, TTE0695 shares a high degree of sequence similarity with other well-known thermal 2-ADHs, including those from *T. brockii* and *Thermoanaerobacter ethanolicus*. Although Xue et al. reported that *T. tengcongensis* was able to generate a limited amount of ethanol, the functions of 2-ADH are not fully elucidated [13]. Recently, Meng et al. surveyed the temperature-dependent protein complexes in *T. tengcongensis* using proteomic approaches and demonstrated the involvement of protein interactions in temperature-adaptive mechanisms [14]. Intriguingly, several protein components in one complex in this bacterium belong to the same metabolic pathway, indicating that the enzymes within one metabolic pathway could form a stable complex for efficient regulation of activity in thermophiles. This discovery prompted us to inquire whether the alcohol metabolism pathway in *T. tengcongensis* is involved in a functional complex with which protein the *T. tengcongensis* ADH interacts *in vivo*.

In this communication, we screened for protein complexes containing 2-ADH with Blue Native PAGE (BN PAGE), Western blotting and MALDI TOF/TOF MS. To ensure 2-ADH complex formation, we used anion exchange chromatography to separate the complexed and free 2-ADH. Alternative approaches, such as immuno-precipitation (IP) and pull downs, were employed to examine the interactions between 2-ADH and PFOR. Finally, we measured the enzymatic activity of recombinant and native *T. tengcongensis* 2-ADHs and revealed that the interactions of 2-ADH with PFOR resulted in non-competitive inhibition of 2-ADH activity. For the first time, we found that PFOR could interact with 2-ADH and form a stable protein complex in *T. tengcongensis*. The interaction between 2-ADH and PFOR was likely a causal factor in the regulation of 2-ADH activity and alcohol formation in *T. tengcongensis*.

2. Materials and methods

2.1. Organisms and culture conditions

T. tengcongensis was cultured at 75 °C in TYE medium containing 1.0 g NH_4Cl, 0.3 g K_2HPO_4, 0.3 g KH_2PO_4, 0.5 g $MgCl_2{\cdot}6H_2O$, 2.0 g NaCl, 0.2 g KCl, 0.05 g $CaCl_2$ and 0.5% (w/v) glucose per litre. Cell cultivation and media preparation were performed under anaerobic conditions. The cells were harvested at log phase and stored at −80 °C after thorough washing with PBS.

2.2. Recombinant protein and antibody preparation

The *T. tengcongensis* genes, *TTE0695* and *TTE0445*, encoding 2-ADH and the PFOR α subunit were cloned into PET28a and expressed in *Escherichia coli* BL21. The two recombinant proteins were purified using Ni-NTA affinity chromatography and submitted to Beijing Protein Innovation (BPI, Beijing, China) for antibody generation. All of the generated antibodies were purified through protein A affinity chromatography (Bio-Rad, California, USA).

2.3. BN PAGE analysis of protein complexes in T. tengcongensis cells

Briefly, *T. tengcongensis* cell extracts were obtained by gently stirring the bacteria in 50 mM Tris–HCl buffer, pH 7.0, containing 100 μg/ml of lysozyme, 1% Triton X-100, and 2 μg/ml of DNase I for 30 min on ice. The lysates were then separated using 5–12% polyacrylamide gradient gels. The visible lanes on the gel were excised and transferred to a 10% SDS–PAGE gel. The presence of 2-ADH in each BN PAGE band was examined using Western blotting.

2.4. The interaction analysis of 2-ADH and PFOR by IP and pull down

For the IP analysis, the *T. tengcongensis* cell lysate was pre-incubated with protein A beads to remove non-specific binding proteins and then incubated successively with a 2-ADH polyclonal antibody and protein A beads. The IP pellet was collected and examined by Western blotting with monoclonal antibodies against 2-ADH and PFOR.

For pull down analysis, *T. tengcongensis* cell lysate was first incubated with 2-ADH recombinant protein and then with Ni-beads overnight. The proteins that interacted with 2-ADH were eluted with 300 mM imidazole. The eluted proteins were examined by Western blotting with the monoclonal antibody against PFOR.

2.5. Purification of native 2-ADH protein(s) from T. tengcongensis cells

T. tengcongensis cell lysates were first subjected to fractional precipitation by stepwise addition of ammonium sulphate. The precipitates at each step were examined by Western blotting using an antibody against 2-ADH. Precipitates containing 2-ADH were dissolved and loaded onto a DEAE column (2.5 × 10 cm). The proteins were carefully eluted with a 0–300 mM NaCl gradient and collected. The collected fractions were examined for the presence of 2-ADH by Western blotting. The concentration of 2-ADH in each fraction was determined using ELISA.

2.6. The enzyme activity analysis of purified native 2-ADH and recombinant 2-ADH

The enzymatic activities of the native 2-ADH purified from *T. tengcongensis* and of the recombinant 2-ADH expressed in *E. coli* were determined. The activity unit was defined as the oxidation of NADPH during the reduction of substrates. Acetyl-CoA, acetaldehyde, propan-2-ol and ethanol were used as substrates. Certain amounts of native 2-ADH or recombinant 2-ADH were added into a mixture containing1 mM substrate and 0.4 mM NADPH and then incubated at room temperature. The K_m and V_{max} values were calculated using the Michaelis–Menten equation.

2.7. The kinetic analysis of 2-ADH complexed with PFOR

Varying concentrations (0.01–1 mM) of acetyl-CoA, acetaldehyde, propan-2-ol and ethanol were added into a series of reaction mixtures containing 10 μg/ml of 2-ADH and different