Journal of Molecular Catalysis B: Enzymatic 134 (2016) 340–346

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

A more polar N-terminal helix releases MBP-tagged *Thermus thermophilus* proline dehydrogenase from tetramer-polymer self-association

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ARTICLE INFO

Article history:
Received 5 August 2016
Received in revised form
12 September 2016
Accepted 14 September 2016
Available online 14 September 2016

Keywords:
Flavoprotein
Molecular self-association
Proline dehydrogenase
Protein oligomerization
Thermus thermophilus

ABSTRACT

Proline dehydrogenase (ProDH) is a ubiquitous flavoenzyme involved in the biosynthesis of L-glutamate. ProDH is of interest for biocatalysis because the protein might be applied in multi-enzyme reactions for the synthesis of structurally complex molecules. We recently demonstrated that the thermotolerant ProDH from *Thermus thermophilus* (TtProDH) is overproduced in *Escherichia coli* when using maltose-binding protein (MBP) as a solubility tag. However, MBP-TtProDH and MBP-clipped TtProDH are prone to aggregation through non-native self-association. Here we provide evidence that the hydrophobic N-terminal helix of TtProDH is responsible for the self-association process. The more polar MBP-tagged F10E/L12E variant exclusively forms tetramers and exhibits excellent catalytic features over a wide range of temperatures. Understanding the hydrodynamic and catalytic properties of thermostable enzymes is important for the development of industrial biocatalysts as well as for pharmaceutical applications.

1. Introduction

Protein oligomerization is a commonly observed cellular process. Dimers (about 40%) and tetramers (about 20%) are the most common protein species in the cell, of which the majority is homo-oligomeric [1,2]. *In vivo,* protein oligomerization might be advantageous for many reasons, such as catalysis, cooperativity, improved stability and a reduction of surface area [1,3–6].

The oligomeric state of an *in vitro* produced enzyme does not necessarily represent the *in vivo* situation. Many intrinsic and extrinsic factors can influence the *in vitro* oligomeric state of proteins [7] and there are different types of 'non-native' oligomerization pathways [7,8]. One such pathway concerns aggregation through self-association, which means that proteins can associate into aggregates directly from the native state. This process can lead to soluble aggregates and is not always reversible.

Interacting protein surfaces are more hydrophobic than free protein surfaces. Furthermore, the subunit interface in oligomers is on average more hydrophobic than the average protein–protein interface [9–12]. Hydrophobic regions in proteins can also indicate regions sensitive to aggregation [13–15]. Knowing the location and properties of regions that are sensitive to aggregation can help in rationalizing what kind of effects sequence changes can have on the aggregation behavior of the protein [15]. Introducing so-called sequence breakers, for example an amino acid that disrupts a hydrophobic region in a sequence, can decrease the aggregation propensity of a protein [14,16,17]. By using negative design, formation of undesirable non-native structures can be avoided [18–20].

We are investigating the molecular properties of *Thermus thermophilus* proline dehydrogenase (TtProDH; EC 1.5.99.8) for potential application in multi-enzyme reactions. TtProDH is an extremely stable flavin adenine dinucleotide (FAD)-dependent enzyme involved in proline catabolism (Fig. 1). It has a conserved distorted $(\beta\alpha)_8$ TIM-barrel fold and an N-terminal arm that consists of three helices: αA, αB and αC [21] (Fig. 2A). The C-terminal helix α8 fits into the cleft that is formed by helices αA, αB and αC. Together, these four helices form a hydrophobic patch that is thought to be involved in channeling Δ^1-pyrroline-5-carboxylate (P5C)/glutamic semialdehyde (GSA) between TtProDH and its partner enzyme Δ^1-pyrroline-5-carboxylate dehydrogenase (TtP5CDH) [21,22]. Applying the selectivity of TtProDH in tandem reactions with other enzymes might be a powerful tool to efficiently produce structurally complex molecules from L-proline.

Abbreviations: BOG, *n*-octyl β-d-glucopyranoside; DCPIP, dichlorophenolindophenol; GSA, glutamic semialdehyde; MBP, maltose-binding protein; ProDH, proline dehydrogenase; P5C, Δ1-pyrroline-5-carboxylate; P5CDH, Δ1-pyrroline-5-carboxylate dehydrogenase.

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http://dx.doi.org/10.1016/j.molcatb.2016.09.014
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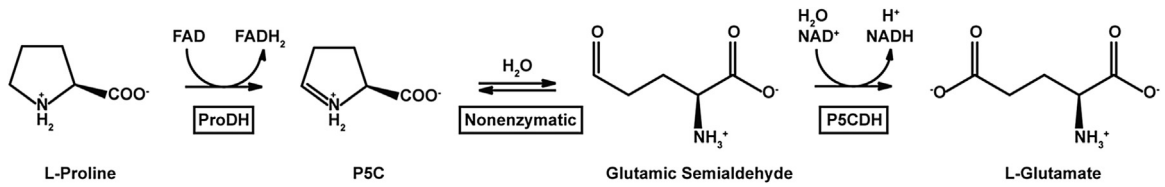

Fig. 1. Conversion of L-proline to L-glutamate by ProDH and P5CDH.

Several ProDHs from various sources have been described as dimers [23–25]. However, the oligomeric state of TtProDH remains ambiguous. N-terminally His-tagged TtProDH treated with *n*-octyl β-D-glucopyranoside (BOG) was described as a mixture of dimers and monomers [21]. We produced TtProDH with maltose-binding protein (MBP) as a solubility tag and found that, both in the absence and presence of BOG, MBP-TtProDH and MBP-clipped TtProDH consist of oligomers and soluble aggregates [26]. The non-native self-association observed might be related to the hydrophobic nature of the N-terminal helix of TtProDH. To address this issue, we increased the polarity of helix αA by introducing sequence-breakers via site-directed mutagenesis. Phe10 and Leu12, located at the protein surface (Fig. 2B), were replaced by glutamates, generating the F10E/L12E variant of MBP-TtProDH. The biochemical properties of MBP-TtProDH wildtype and MBP-TtProDH F10E/L12E (further referred herein as WT and EE, respectively) are compared in this research letter.

2. Materials and methods

2.1. Cloning and site-directed mutagenesis of TtProDH variants

In our previous research, we obtained two forms of MBP-TtProDH [26]. With peptide mapping and mass spectrometry, we showed that one form corresponded to full-length MBP-TtProDH while the other form corresponded to an elongated form of the enzyme. This elongated form was due to a read-through of the stop codon. Here, the stop codon was changed from TGA to TAA, resulting in the production of a single form of WT. This was done in a one-step PCR and ligation reaction, using the pBAD-MBP vector containing the synthetic ProDH gene from *Thermus thermophilus* [26] as template plasmid. A single primer (5′ G GTT AGC GGT CTA GAA TAA AAG CTT GGG CCC GAA C 3′ (nucleotide change underlined) was used to introduce the mutation. The PCR product was treated with DpnI and directly transformed to *E. coli* dh5α cells. Positive constructs were identified through automated sequencing of both strands (Macrogen). Subsequently, the correct plasmid was transformed to *E. coli* TOP10 host cells for recombinant expression.

The gene encoding the F10E/L12E variant of WT was constructed by using the same one-step PCR and ligation reaction as was used for the stop codon change. pBAD WT with the corrected stop codon (TAA) was used as template DNA, and the primer used was 5′ GCT TAC CGT AGC GAA GTT GAA GGT GTT GCA GGT C 3′ (nucleotide changes underlined).

2.2. Purification of MBP-TtProDH variants

WT and EE were purified according to a protocol described previously [26]. After the amylose column (New England Biolabs, 80 mL in XK 26/10), an additional polishing step was performed. 100 μM FAD was added to the enzyme, which was subsequently loaded onto a Q-Sepharose column (GE Healthcare, 60 mL in XK 26/10), pre-equilibrated in 20 mM Bis-Tris pH, 7.4. Next, the column was washed with one volume of starting buffer and three volumes of 20 mM Bis-Tris, 100 mM NaCl, pH 7.4. Subsequently, the enzyme was eluted with a linear gradient of 0.1–0.5 M NaCl in the same buffer. Purified protein was concentrated to a protein content of 5–10 mg/mL using a 10 kDa cut off Amicon filter and subsequently dialyzed against 5 L of 50 mM sodium phosphate, pH 7.4 at 4 °C for 16 h. The purified enzymes were flash-frozen in liquid nitrogen and stored at −80 °C.

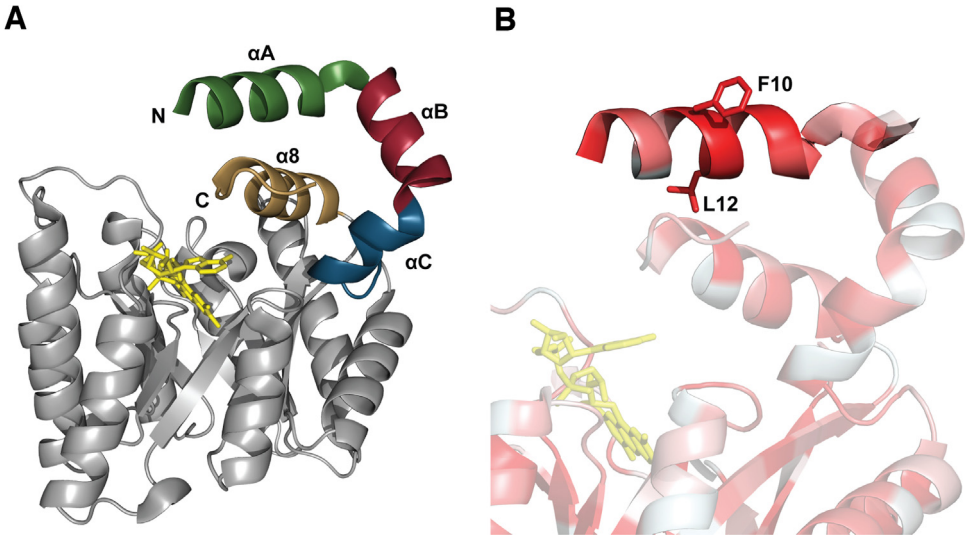

Fig. 2. Structural features of TtProDH. (A) Three-dimensional model of the crystal structure of TtProDH (PDB entry 2G37). The N-terminal helices αA (green), αB (red) and αC (blue) are indicated, as well as the C-terminal helix α8 (brown). The FAD cofactor is depicted in yellow. (B) Hydrophobicity of the N-terminal helix (5-LAYRSFVLGVAGHP-18) of TtProDH. The intensity of the red color is correlated to the hydrophobicity of the residue. Phe10 and Leu12 are shown in sticks. The N-terminal helix αA is shown bright, N-terminal helices αB, αC and the catalytic domain with the FAD cofactor are shown transparent. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)