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Enhanced catalytic properties of novel $(\alpha b\gamma)_2$ heterohexameric *Rhodobacter capsulatus* xanthine dehydrogenase by separate expression of the redox domains in *Escherichia coli*

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ABSTRACT

Post-translational proteolysis is usually necessary for the commercial production of xanthine dehydrogenases (XDHs), such as bovine $(\alpha\beta\gamma)_2$ XDH, to increase its catalytic activity. The proteolysis approach suffers from low controllability and inefficient purification. To obviate these disadvantages, we have developed a method that translates active *Rhodobacter capsulatus* $(\alpha\beta\gamma)_2$ XDH by directly expressing the iron-sulfur domain, the flavin adenine dinucleotide domain and the sulfurated molybdenum domain as three separate proteins in *Escherichia coli*. Two $(\alpha\beta\gamma)_2$ XDH variants, Split166 and Split178, which were designed by splitting the small subunit of *R. capsulatus* CGMCC 1.3366 $(\alpha\beta)_2$ XDH at the N- and C-terminal ends of the L_{167}-A_{178} peptide linking the iron–sulfur clusters and flavin adenine dinucleotide domains, respectively, were expressed in *E. coli*. Compared to the wild type, both split variants increased the thermostability by 11 °C and Split178 enhanced the turnover number and catalytic efficiency by 1.15-fold and 1.66-fold, while Split166 decreased these parameters by 3.2-fold and 5-fold, respectively. This study is the first successful trial to express an active split $(\alpha\beta\gamma)_2$ XDH directly by manipulating genes encoding redox domains, and the enhanced properties of the expressed $(\alpha\beta\gamma)_2$ XDH using the *in vivo* splitting strategy may be a promising technique for the commercial production of XDHs.

1. Introduction

Xanthine dehydrogenase (XDH; EC 1.17.1.4), a well-studied molybdenum-containing oxidoreductase, consists of three redox domains, one containing two distinct iron–sulfur clusters ([2Fe–2S] clusters), another including a flavin adenine dinucleotide (FAD), and the third incorporating a sulfurated molybdenum cofactor (Moco) [1]. XDH catalyzes the hydroxylation of sp^2-hybridized carbon atoms of heterocyclic compounds, including purines, pterins and aldehydes. For example, XDH catalyzes a two-step sequential oxidation of hypoxanthine to uric acid via xanthine with the concomitant reduction of nicotinamide adenine dinucleotide (NAD) in mammalian cells. This catalytic function of XDH is promising for use in the diagnosis of clinical diseases, the synthesis of nucleoside drugs and the detection and degradation of organic pollutants [1,2].

Numerous XDH gene sequences have been determined; however, only a few eukaryotic and bacterial XDHs have been well characterized [3]. Eukaryotic XDHs originate from animals, including cows, chickens and rats, and from plants such as *Arabidopsis thaliana*, whose subunit structures are organized in the α_2 form [4–8]. The bacterial XDH sources include *Rhodobacter capsulatus*, *Pseudomonas putida* and *Streptomyces cyanogenus*, in which XDHs are usually in the α_2, α_4, $(\alpha\beta)_2$, $(\alpha\beta)_4$ and $\alpha\beta\gamma$ forms [9–13]. Mammalian and bacterial XDHs catalyze the same reaction, and fold in highly similar ways despite the differences in subunit composition. The multimeric bacterial XDHs have higher levels of catalytic activity and thermal stability compared to the eukaryotic α_2 XDHs [5–8,14,15]. In particular, the $(\alpha\beta\gamma)_2$ heterohexameric form of bovine XDH, usually called the bovine xanthine oxidase (XOD; EC 1.17.3.2) with three redox center domains located in three separate subunits, is 10 times more active than its native α_2 form XDH [16]. Thus, limited proteolysis by trypsin or pepsin is employed to irreversibly convert the α_2 XDH to the $(\alpha\beta\gamma)_2$ form in the commercial

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production of bovine XOD [17]. However, such an *in vitro* splitting approach using limited post-translational proteolysis makes the XDH production process uncontrollable and inefficient, and it is also difficult to purify the end products [17,18]. To circumvent these problems, there is a need to develop methods to produce $(\alpha\beta\gamma)_2$ XDHs with separate redox domains in each subunit directly *in vivo*.

Although bovine $(\alpha\beta\gamma)_2$ XDH is the most extensively used commercial enzyme, *R. capsulatus* $(\alpha\beta)_2$ XDH has better excellent thermostability and a 5-fold higher level of catalytic activity [15]. *R. capsulatus* XDH is encoded by the *xdhA* and *xdhB* genes. The iron–sulfur clusters and the FAD binding domains of *R. capsulatus* XDH are located in the XDHA subunit, whereas the Moco domain is bound by the XDHB subunit [19]. The redox center domains of *R. capsulatus* $(\alpha\beta)_2$ XDH adopt very similar structures compared to the bovine split $(\alpha\beta\gamma)_2$ XDH [14,20]. Both the disulfide formation and partial proteolysis methods can transform the bovine XDH to the more catalytically active XOD form in a similar structural transition method [5,21]. However, the absence of cysteines that convert XDH to XOD disables the former method to produce RcXOD, as clearly demonstrated by the Truglio et al. [22]. The limited proteolysis cannot cut between FAD domain and iron-sulfur domain of XDHA of RcXDH, but only cleave the XDHB subunit, resulting in split variants with unchanged quaternary structure and catalytic activity [15]. The fused FAD domain and iron-sulfur domain in RcXDH split variant is fundamentally different from that of the bovine milk $(\alpha\beta\gamma)_2$ form XDH, which consists of separate FAD domain, iron-sulfur domain and Moco domain. To investigate such $(\alpha\beta\gamma)_2$ form RcXDH consisting of three separate redox center domains which can not be achieved by partial proteolysis, we carried out cloning as an alternative to directly split the XDHA of $(\alpha\beta)_2$ RcXDH between the iron–sulfur domain and FAD domain *in vivo*.

In this study, using *R. capsulatus* CGMCC 1.3366 $(\alpha\beta)_2$ XDH as a model, we established a new method to translate active $(\alpha\beta\gamma)_2$ XDH directly by separate expression of the redox domains in *E. coli* and characterized the engineered enzymes. Novel $(\alpha\beta\gamma)_2$ XDHs were functionally biosynthesized in *E. coli* by expressing the three redox center domains of *R. capsulatus* CGMCC 1.3366 $(\alpha\beta)_2$ XDH in separate redox proteins, which were the counterparts to the [2Fe-2S] clusters, FAD and Moco subunits of bovine $(\alpha\beta\gamma)_2$ XDH, respectively. The active $(\alpha\beta\gamma)_2$ XDH translated directly *in vivo* had an enhanced level of catalytic activity and greater thermostability compared to the wild type $(\alpha\beta)_2$ XDH.

2. Materials and methods

2.1. Bacterial strains, plasmids, media and growth conditions

The *R. capsulatus* strain CGMCC 1.3366 used for cloning the XDH gene was purchased from the China General Microbiological Culture Collection Center (CGMCC). The *E. coli* strain DH5α (Biomed Company, Beijing, China) and all the genetically engineered strains containing recombinant plasmids were grown aerobically at 37 °C in Luria–Bertani medium with 150 μg/ml ampicillin. Plasmid pTrc99A, used for heterologous expression of *R. capsulatus* XDH in *E. coli*, was purchased from the Thermo Fisher Scientific Corporation (Beijing, China). Ni-NTA agarose was purchased from Qiagen (Beijing, China); Q Sepharose, phenyl Sepharose and EAH-sepharose 4 B were bought from GE Healthcare Life Sciences (Beijing, China). An ultrafiltration spin-column with a molecular weight cut-off of 10 kDa was purchased from the Millipore Corporation (EMD Millipore, Billerica, MA, USA).

2.2. Design of split XDHs by structure-based sequence alignment

Split XDHs were designed according to structure-based sequence alignment by reference to the limited proteolysis of mammalian XDHs. Firstly, the 3D structures of bovine XOD, *i.e.* bovine $(\alpha\beta\gamma)_2$ XDH (PDB ID 3AX9) [5], chicken liver XDH (PDB ID 1WYG) [6] and *R. capsulatus* B10 XDH (PDB ID 2W54) [20] were retrieved from the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). Secondly, a homology model of *R. capsulatus* $(\alpha\beta)_2$ XDH used in this study was constructed via the on-line server (https://www.swissmodel.expasy.org/interactive) using the X-ray structure of *R. capsulatus* B10 XDH (PDB ID 2W54) as template, which has 91.3% amino acid identity with the XDH in this study [20,23]. Thirdly, a structural superimposition between the theoretical model and the four retrieved structures was done with Swiss-Pdbviewer (SPDBV, version 4.1) [23,24]. Finally, to minimize the effects of splitting on the structural integrity of the redox domains, the split sites for *R. capsulatus* $(\alpha\beta\gamma)_2$ XDH expression were chosen according to three principles: (1) located on the surface linker peptide connecting the [2Fe–2S] clusters and FAD domains; (2) not belonging to any secondary structure; and (3) equivalent residues eliminated by limited proteolysis or absent from the PDB structures because of the high degree of flexibility. Two ends of the $loop_{167-178}$, which are the sites between L_{166} and A_{167}, and A_{178} and P_{179}, respectively, were then chosen to split the XDHA of *R. capsulatus* XDH, yielding the two split variant XDHs designated Split166 and Split178.

2.3. Gene cloning, expression and purification of the wild type XDH and split variants

According to earlier work [15,19], the gene cluster *xdhABC* encoding the *R. capsulatus* CGMCC 1.3366 XDH was PCR amplified from genomic DNA. The Split166 was constructed by introducing a nucleotide sequence of TAA-***RBS***-**ATG** (5′-TAAGG***AGGA***AACAGACC**ATG**-3′**)** into the 498 and 499 bp sites of the *xdhA* gene, and the Split178 534 and 535 bp sites. All the gene clusters were inserted under the *trc* promoter of the plasmid pTrc99A to express the recombinant wild type XDH and split variants. An 18-bp nucleotide sequence (5′-CATCATCACCATCACCAT-3′) encoding a his_6-tag was inserted between the first start codon (ATG) and the second codon (GAA) of the *xdhA* of the gene cluster in all three recombinant plasmids. Recombinant XDHs were purified by sequential Ni-NTA, Q Sepharose anion exchange chromatography, phenyl Sepharose chromatography, and affinity chromatography on Sepharose 4B/folate gel as described by Leimkühler et al. [25,26] (see Supplementary materials for experimental details).

The purified proteins were assayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for purity and the protein concentrations were measured by the Bradford method with bovine serum albumin as the standard [27]. The molecular weights of the native XDHs were quantified by native-PAGE using urease (Sigma U7752, Shanghai, China) and bovine serum albumin (Sigma A8654, Shanghai, China) as reference.

2.4. Enzymatic characterization

Routine XDH assays were performed at room temperature using 1 μg/ml purified enzyme in a total volume of 2 ml of 50 mM Tris-ethylenediaminetetraacetic acid (EDTA) buffer (50 mM Tris, 1 mM EDTA, 0.1 mM oxonic acid, 0.1 mM xanthine, 0.1 mM NAD, pH 8.5), monitoring the change of absorbance at 295 nm caused by an increase of the uric acid product with an extinction coefficient of 12.5 $cm^2/\mu M$ by spectrophotometry (Ultraspec 3100 pro ultraviolet (UV)/visible spectrophotometer; Amersham Biosciences, NJ, USA). The ability to use molecular oxygen in the air as an elec-