Protein Expression and Purification 138 (2017) 76–80

Contents lists available at ScienceDirect

Protein Expression and Purification

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

Dissociation and purification of the endogenous membrane-bound Vo complex from *Pichia pastoris*

Sumei Li*, Tao Hong, Kun Wang, Yinghong Lu, Min Zhou**

School of Chemical Engineering, Nanjing University of Science and Technology, Nanjing 210094, People's Republic of China

ARTICLE INFO

Article history:
Received 29 March 2017
Received in revised form
29 May 2017
Accepted 11 July 2017
Available online 12 July 2017

Keywords:
Endogenous membrane-protein complex
FLAG affinity purification
Membrane-bound Vo complex
PCR-mediated gene modification
Pichia pastoris

ABSTRACT

Most proteins occur and function in complexes rather than as isolated entities in membranes. In most cases macromolecules with multiple subunits are purified from endogenous sources. In this study, an endogenous membrane-protein complex was obtained from *Pichia pastoris*, which can be grown at high densities to significantly improve the membrane protein yield. We successfully isolated the membrane-bound Vo complex of V-ATPase from *P. pastoris* using a fusion FLAG tag attached to the C-terminus of subunit a to generate the *vph-tag* strain, which was used for dissociation and purification. After FLAG affinity and size exclusion chromatography purification, the production quantity and purity of the membrane-bound Vo complex was 20 μg l^{-1} and >98%, respectively. The subunits of the endogenous membrane-bound Vo complex observed in *P. pastoris* were similar to those obtained from *S. cerevisiae*, as demonstrated by liquid chromatography-tandem mass spectrometry (LC-MS-MS). Therefore, successful dissociation and purification of the membrane-bound Vo complex at a high purity and sufficient quantity was achieved via a rapid and simple procedure that can be used to obtain the endogenous membrane-protein complexes from *P. pastoris*.

1. Introduction

Membrane proteins represent approximately one-third of the proteome of every cell. Most membrane proteins exist in complexes rather than as isolated entities [1]. It is essential to study intact membrane-protein assemblies to completely understand their biological functions. Expression and purification of many essential membrane-protein complexes offer considerable and often unsurmountable challenges. Although advanced DNA recombinant technologies and sophisticated overexpression strategies are available, many protein complexes that are involved in key biological processes are difficult to obtain using recombinant methods. In such cases, the only option to obtain these large, multi-subunit complexes is to extract them from endogenous sources. In the protein data bank (PDB) archive, the most deposited structures of high molecular weight complexes (>250 kDa) are from endogenous sources [2]. Additionally, the differences in multi-subunit conformation could be explained by changes in the conformation or subunit composition of multi-subunit complexes due to the partial loss of subunits during purification because of their loose association. Novel membrane-protein subunits of large hydrophobic protein complexes continue to be discovered even after many decades of study, including mitochondrial ATP synthase, complex I, and yeast V-ATPase [3–5]. Thus, preparations of macromolecular membrane-protein complexes from endogenous sources are crucial to understanding their biochemical structures and functions.

V-ATPases are large multi-subunit protein complexes that are found in the endomembrane systems of all eukaryotic organisms and are composed of a soluble V_1 catalytic region that has eight different subunits and a membrane-bound Vo proton translocation region that has at least six different subunits [6]. Dissociation and purification of the *S. cerevisiae* membrane-bound Vo region has led to better understanding of the internal construction, interactions between subunits and mechanisms of protonation of this region [5]. By contrast, variabilities in the intact V-ATPase complex structure have interfered in the efforts to construct atomic models of the membrane-bound Vo motor [7,8] and of other rotary ATPases [9–11]. The structure of the Vo complex is markedly more homogeneous when dissociated compared to in the intact V-ATPase form [5], and therefore, researchers have been able to construct a high resolution atomic map that provides insights into its biological function. The production of membrane-protein complexes would

* Corresponding author.
** Corresponding author.
E-mail addresses: smli321@163.com (S. Li), minzhou@njust.edu.cn (M. Zhou).

http://dx.doi.org/10.1016/j.pep.2017.07.003
1046-5928/

enable the determination of the stoichiometry, structure and cellular functions of large macromolecular cellular complexes.

Pichia pastoris has been widely used as a recombination expression host to produce a number of heterologous membrane proteins for structural studies [12]. However, dissociation and purification of endogenous membrane-protein complexes have rarely been reported. *P. pastoris* is a single-celled microorganism that can grow in shaker flasks and has the ability to grow to a high biomass without the need for complex media or additives. Therefore recombination tags were fused to the 3'-terminus of *P. pastoris VPH1*, the gene encoding subunit a of the membrane-bound Vo complex, to enable the target protein to be affinity purified. The endogenous membrane-bound Vo complex of *P. pastoris* was purified associated with the subunit a and its integrity was further assessed using LC-MS-MS.

2. Materials and methods

2.1. Strains and growth media

E. coli strain DH5α was cultivated in low salt Luria-Bertani (LB) medium (1% (w/v) peptone, 0.5% (w/v) yeast extract, and 0.5% (w/v) NaCl) supplemented with 25 μg zeocin ml^{-1} to select for transformants at 37 °C. The protease-deficient *P. pastoris* strain SMD1168H (*pep4*) was used as a DNA fragment cassette electroporation receptor for the tagged subunit a. Cells were cultured in rich yeast peptone dextrose (YPD) medium in a shaker flask under constant agitation at 250 rpm at 28 °C. 100 μg zeocin ml^{-1} and 2% agar were supplemented for the selection of transformants.

2.2. FLAG tag construct and DNA fragment cassette amplification

The *P. pastoris* vector pPICZA and the synthesized DNA sequence containing the 3 × FLAG peptide (DYKDHDGDYKDHDIDYKDDDDK) was used to construct the FLAG vector as shown in Fig. 1a procedure. The DNA fragment cassette for homologous recombination with 100 bp complementary sequences of target gene were conducted by two-step PCR (P1—P4 in Table 1). The first PCR step was amplified using the FLAG vector as a template, followed by 20 cycles. The second PCR step was conducted using the purified product from the first PCR step as a template (Fig. 1b). The resulting PCR product was subjected to DNA sequence analysis.

2.3. Yeast transformation and transformant identification

Approximately 5 μg of the purified DNA fragment was used to transform the competent SMD1168H cells according to the standard electroporation protocol [13]. The transformed cells were screened onto YPD plates containing 100 μg zeocin ml^{-1} and were incubated at 28 °C for 3 days. The colonies were selected and inoculated into liquid culture medium for genomic DNA extraction. The *VPH1*-specific forward primer and reverse primers within the insertion sequence or 3′ untranslated region (UTR) of *VPH1* (J1-J4 in Table 1) were used to verify chromosomal sequence insertion using PCR.

2.4. Membrane protein extraction

All steps were performed at 4 °C unless otherwise noted. Yeast cells were grown in shaker flasks until $OD_{600\ nm} = 10$ in YPD medium. Cells harvested (40 g) from the 2 l culture were resuspended in 200 ml of lysis buffer (25 mM HEPES, 8% (w/v) sucrose, 5% (w/v) sorbitol, 2% (w/v) glucose, 150 mM KCl, 2 mM $MgCl_2$, and 1 mM DTT, pH 8.0) containing protease inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF) and Complete™ Roche Protease Inhibitor Cocktail]

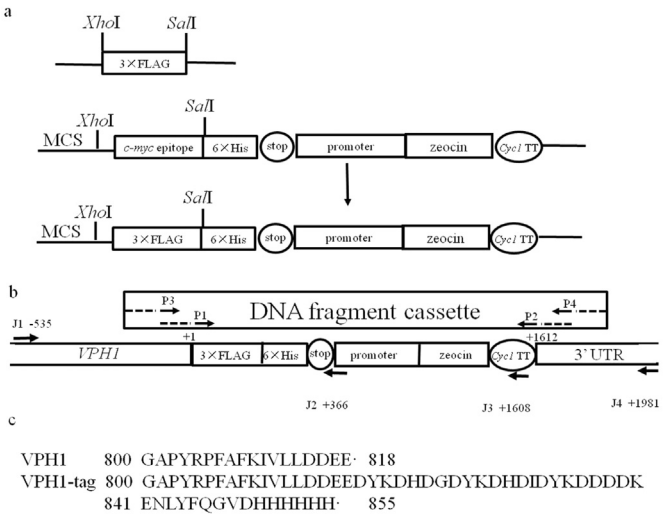

Fig. 1. Schematic of the FLAG vector, DNA fragment cassette and the amino acid sequences for homology recombination. (a) The pPICZA backbone was used to construct the FLAG vector. The sequence containing 3 × FLAG was introduced using the restriction enzymes *Xho*I and *Sal*I. (b) Schematic of the DNA fragment cassette fused to before TAA of *VPH1* 3'-terminator. P1-P4 are the primers used for PCR amplification, the solid arrow indicates the primer, and the dotted portion indicates the flanking homology sequence. J1-J4 are the primers used to identify the transformants. (c) The C-terminal amino acid sequences of VPH1 before and after the introduction of the tag.

by vigorous mixing. Cell lysis was accomplished with a homogenizer operated at 2000 psi. The lysate was centrifuged for 10 min at 5000 g to remove cellular debris. The membranes were pelleted by ultracentrifugation for 2 h at 100,000 g, and the resulting pellet was washed twice with lysis buffer. The membrane fraction was resuspended in TAP buffer (25 mM HEPES, 150 mM KCl, 2 mM $MgCl_2$, and 1 mM DTT, pH 8.0) containing 1% (w/v) dodecyl maltoside (DDM) and was shaken gently for 2 h. The insoluble fraction was removed by ultracentrifugation at 100,000 g for 2 h. The DDM-soluble supernatant contained the desired membrane-bound Vo complex.

2.5. Electrophoresis and western blotting analysis

SDS-PAGE analysis was conducted under reducing conditions in glycine buffer. The DDM-soluble supernatant was mixed with an equal volume of 2 × loading buffer, heated for 10 min at 65 °C and used for 12% SDS-PAGE. The gels were stained with Coomassie brilliant Blue R-250 or silver staining [14]. Proteins separated via 12% SDS-PAGE were transferred to a nitrocellulose membrane using a semi-dry transfer method for western blotting. Monoclonal mouse anti-FLAG primary antibodies (Abmart, Shanghai, China) and anti-mouse IgG secondary antibodies labeled with horseradish peroxidase (Abmart, Shanghai, China) were used for immunodetection. The subunit was detected using ECL western blotting reagent.

2.6. Affinity purification and size exclusion chromatography

Supernatants with the desired membrane-bound Vo complex were applied to 1 ml of anti-FLAG agarose (Biotool, Houston, USA) previously equilibrated with TBSD (50 mM Tris-HCl, 150 mM NaCl, and 0.025% (w/v) DDM, pH 7.4). The suspension of anti-FLAG agarose and membrane fractions was gently rotated at 4 °C for 2 h, and the gel suspension was subsequently loaded onto a 3 ml column container with a membrane to hold back the agarose. The column container was washed with 15 ml of TBSD, and the target