Process Biochemistry 47 (2012) 395–400

Contents lists available at SciVerse ScienceDirect

Process Biochemistry

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

High-level production of soluble adenine nucleotide translocator from *Schistosoma japonicum* in *E. coli* cell-free system

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ARTICLE INFO

Article history:
Received 31 July 2011
Received in revised form 28 October 2011
Accepted 14 November 2011
Available online 13 December 2011

Keywords:
Adenine nucleotide translocator
Escherichia coli cell-free system
Soluble expression
Schistosoma japonicum

ABSTRACT

Adenine nucleotide translocator is an important member of the mitochondrial carrier family, which is involved in the transportation of various metabolites. In the present work, the *Escherichia coli* cell-free system was chosen as an alternative way to express this highly toxic membrane protein. The expression level of insoluble sjANT from *Schistosoma japonicum* in *E. coli* cell-free system could attain 472 μg/mL, about 25.2 times improvement over the previous report. The obtained insoluble sjANT can be resolubilized with different detergents. Among them, Digitonin could effectively solubilize approximate 38% of the target membrane protein. Moreover, sjANT can be further expressed in the hydrophobic *E. coli* cell-free system as a soluble form with presence of different detergents. The results suggested that Digitonin and Brij 58 were two ideal candidates to support high expression of soluble sjANT, and the highest soluble expression level (182 μg/mL) was achieved with the supplementation of 0.4% (v/v) Digitonin in the cell-free system. The present work has provided a rapid and efficient procedure to express the complex and highly toxic membrane protein in the cell-free system, and will be beneficial to construct a novel drug-discovery model to screen the sjANT-based inhibitors for schistosomiasis treatment.

1. Introduction

Adenine nucleotide translocator (ANT) is a member of the mitochondrial carrier family (MCF) [1]. As a major site of energy production, mitochondria have already integrated with broad metabolism in cells. Several important metabolites such as nucleotides and cofactors need to be transported through the membrane of mitochondria to linking the internal and external metabolic processes [2], in which the member of mitochondrial carrier family (MCF) has played an important role. However, the outer and inner membranes of mitochondria are different in their permeability. The outer mitochondrial membrane is well permeable due to the presence of voltage dependent anion channels (VDAC) with large diameters, while the inner membrane is a permeability barrier without VDAC. The majority of MCF proteins is located mainly in the inner mitochondrial membrane, and is vital in the transportation of various metabolites between cytosol and the mitochondrial matrix [3]. As the prototypical MCF protein in eukaryotic bioenergetics, ANT is responsible for the exchange of ATP synthesized in the mitochondrial matrix by the F_0F_1 ATP-synthase and ADP produced by the energy consuming processes in the cytosol, which is the last step of the oxidative phosphorylation. The ATP/ADP transportation and the underlying carrier have been regarded as a paradigm of the MCF to understand the mechanism of biomembrane transportation [4]. ANT has a standard MCF structure suitable for the transportation of nucleotides and cofactors [5], which contains six transmembrane helices and three repeated domains each containing two helices (Fig. 1).

ANT could be expressed in yeast [6] and functionally detected by labeled ATP or ADP [7]. It was also reported that ANT could be expressed in the *Escherichia coli* C43 (DE3) and integrated into the bacterial membrane [8]. However, the expression level of ANT in the *E. coli* or yeast was very low because of its highly hydrophobic characteristics (Table 1). In *Schistosoma japonicum*, ANT is a key protein of the mitochondrion and can be applied as the effective target for the development of efficient medicine to treat schistosomiasis in humans [9]. However, the heterologous expression of this channel protein (sjANT) from *S. japonicum* was not reported so far. Therefore, it is necessary to establish

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doi:10.1016/j.procbio.2011.11.010

Table 1
List of ANT expressions in different hosts and *E. coli* cell-free system.

Host	Gene source	Plasmid	ANT (μg/mL)	Product form	References
E. coli BL21(DE3)	Bovine	pKN 172	Low	Insolube	[22]
BL21(DE3)	Bovine	pET series	N	N	[13]
C41(DE3)	Bovine	pET series	9.0	Insolube	[13]
C43(DE3)	Bovine	pET series	18.0	Insolube	[13]
DH1	*Neurospora crassa*	pJLA503	–	Insolube	[23]
BL21(DE3)plysS	*Neurospora crassa*	pET-3a	–	Insolube	[23]
C43(DE3)	*Plasmodium falciparum*	pET-14b	–	B	[8]
S. cerevisiae WB-12	*S. cerevisiae*	pYES-Ppic2-His6-aac2	–	B	[6]
E. coli cell-free system	*Schistosoma japonicum*	pSJ2-sjANT	472.0	Insoluble	This study
	Schistosoma japonicum	pSJ2-sjANT	197.0[a]	Soluble	This study
	Schistosoma japonicum	pSJ2-sjANT	182.0[b]	Soluble	This study

Note: N, not detected; –, the expression level was not indicated in the paper; B, bioactive with direct bioactivity determination.
[a] The precipitate solubilized with 2% Digitonin.
[b] Soluble expression by supplementing 0.4% Digitonin into the cell-free system.

an applicable strategy to effectively express sjANT for further studies.

Cell-free expression system has many advantages comparing to traditional in vivo protein expression. For example, it can directly use the PCR-generated templates to express proteins. As an open protein-synthesizing system, it enables researchers to control the reaction conditions and conveniently incorporate unnatural amino acids into the target proteins. Moreover, it avoids the inhibition of cell growth caused by the toxicity of accumulated membrane proteins [10]. A variety of membrane proteins have been successfully expressed in the *E. coli* cell-free system [11], but most of them are in insoluble form. Fortunately, various detergents can be applied to resolubilize the aggregated membrane protein in the cell-free system. It was also reported that suitable detergents could be directly added into this open system to obtain soluble and functional membrane proteins [12].

To our knowledge, the expression levels of ANT from different origins were very low in the in vivo expression systems [13], while the cell-free expression of sjANT has not been reported yet. In this study, *E. coli* cell-free system was used as an alternative method to express sjANT in vitro. With the advantages of cell-free system, high-level soluble expression of sjANT was effectively achieved with the aid of suitable detergents.

2. Materials and method

2.1. Chemicals

Digitonin, n-Octy-β-glucopyranoside (β-OG), polyoxyethylene-(20)-cetyl-ether (Brij 58), N-dodecyl-b-maltoside (DDM), polyoxyethylene-(20)-stearyl-ether (Brij 78), polyoxyethylene sorbitan monolaurate 20 (Tween 20), polyethylene glycol P-1,1,3,3 -tetramethyl-butylphenyl ether (Triton X-100) were all purchased from Sigma (St. Louis, MO). Sodium dodecyl sulfate was purchased from Amresco (St. Louis, MO). The restriction enzymes, BamHI and XhoI, and T4 DNA ligase, were purchased from Takara (Takara, Dalian, China).

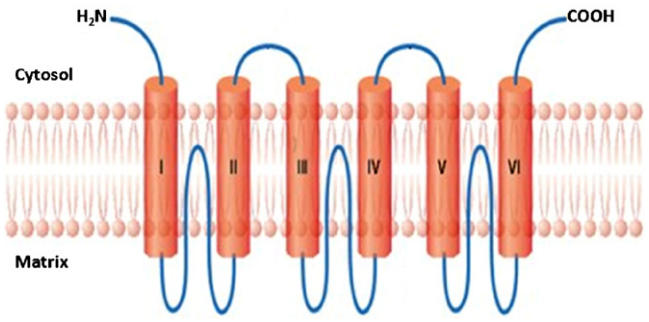

Fig. 1. A schematic diagram showing the proposed structure of mitochondria ANT.

2.2. The parasite and the total RNA

The adult parasitic worms used in this study were derived from Anhui strain (typical schistosome species found in Anhui provice, China) of *S. japonicum* and the life cycle was maintained in *Oncomelania hupensis* snails and C57BL/6 strain mice at the National Institute of Parasitic Diseases, Shanghai. The adult worms were harvested from the infected mice by mesenteric perfusion after 6 weeks infection. Total RNA samples from adult worms were extracted with TRIZOL Reagent (Invitrogn, USA) following the manufacturer's instruction. Total RNA of *S. japonicum* was transcripted into cDNA by using Rever Tra Ace (Toyobo, Japan). The reverse transcription (RT) was carried out in 20 μL reaction volume including 4 μL RT buffer, 2 μL dNTPs, 1 μL Oligo(dT)20, 1 μL RNase inhibitor, 1 μL Rever Tra Ace, 1 μg RNA. The RT reaction conditions were as follows: 30 °C for 10 min, 42 °C for 20 min, 99 °C for 5 min, 4 °C for 5 min.

2.3. Construction of the recombinant plasmid

According to the sequence of *Sj*ANT gene (GenBank acession number is AY813610), a pair of specific primers containing the endonuclease sites was designed to amplify the whole coding region of *Sj*ANT. The designed primers are: *Sj*ANT-F: 5′ CCG*GGATCC*ATGGGAGAAGGTGGTAAAGA 3′ *Sj*ANT-R: 5′ CCG*CTCGAG*GGGAGTTGGCTTCTTTGGTA3′. The obtained *Sj*ANT gene was cloned into pSJ2 via *BamHI* and *XhoI* site to generate the expression vector pSJ2-sjANT. The *Sj*ANT gene template and the pSJ2 plasmid were both obtained from National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention, China.

2.4. Expression of sjANT in E. coli cell-free system

The rapid translation system (RTS) 100 *E. coli* HY Kit was purchased from Roche Molecular Biochemical (Grenzacherstrasse, Switzerland). The plasmid pSJ2-sjANT was extracted from overnight culture using a QIAprep Spin Miniprep Kit (Qiagen, GmbH, Germany) and then added into RTS100 *E. coli* HY Kit to express the sjANT

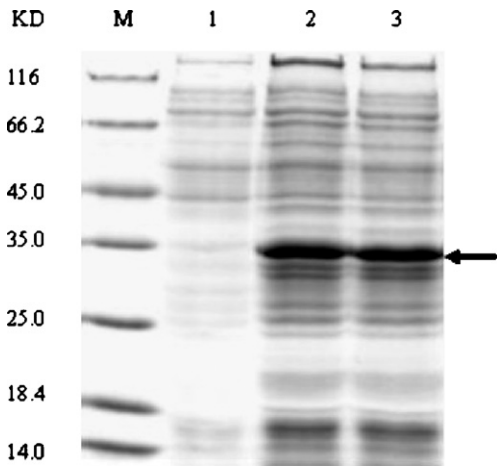

Fig. 2. SDS-PAGE analysis of ANT synthesized in the cell-free system. 1, the negative control without template DNA; 2, the cell-free ANT sample heated at 37 °C for 45 min before its SDS-PAGE analysis; 3, the cell-free ANT sample boiled at 100 °C for 5 min before its SDS-PAGE analysis.