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Cloning, expression and crystallization of VMA13p, an essential subunit of the vacuolar H^+-ATPase of *Saccharomyces cerevisiae*

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The expression and crystallization of the VMA13p subunit of the vacuolar proton-translocating ATPase (V-ATPase) of *Saccharomyces cerevisiae* is described. This 478 amino-acid subunit is essential for activity but not for the assembly of this multisubunit complex. The protein has been recombinantly overexpressed in *Escherichia coli* and purified. Diffraction-quality crystals have been obtained using the hanging-drop vapor-diffusion method with ammonium sulfate as precipitant. Several different crystal forms were obtained. The most suitable crystal form for crystallographic characterization belongs to space group $P3_121$ or its enantiomorph, with unit-cell parameters $a = b = 118.8$, $c = 119.3$ Å. Using an in-house X-ray source, the crystals diffract to about 3.5 Å resolution under rapidly frozen conditions.

1. Introduction

V-type ATPases are thought to facilitate the acidification of intracellular compartments in eukaryotic cells and therefore play an important role in receptor-mediated endocytosis, intracellular traffic processes and protein degradation (Forgac, 1999; Stevens & Forgac, 1997).

The vacuolar proton ATPase of *S. cerevisiae* is a multisubunit enzyme, consisting of two distinct functional domains V_1 and V_0. The V_0 domain is composed of five different integral membrane subunits totaling a molecular mass of 260 kDa. The 570 kDa V_1 domain consists of eight subunits. The V_1 domain, much like the F_1 domain in F-type ATPases, is responsible for the catalytic turnover of ATP, whereas the V_0 domain is involved in the translocation of protons across the membrane.

Unlike the F-type ATPases, little is known about the atomic architecture of the V-type ATPases or how the proton translocation is coupled to ATP synthesis or how activity and assembly are regulated.

Determination of the structure of ATP-generating domain of the bovine F-type ATPase provided the first atomic details of how the F_1 is assembled and binds nucleotides. The structure supported the proposal that ATP synthesis is catalyzed in a rotary catalytic fashion (Abrahams *et al.*, 1994; Boyer, 1997). Even though the α- and β-subunits of the F-type ATPases exhibit sequence homology to the VMA2p and VMA1p subunits of the V-type ATPases and the rotary motion of catalysis is presumed to be similar among these enzymes, the molecular architecture of the V-type ATPases appears to be more complex. The number of known subunits of the V-type ATPases exceeds that of the F-type ATPases. Also, recent evidence from electron microscopy suggests that the overall structure and shape of the two enzymes may be considerably different (Boekema *et al.*, 1997, 1999).

Several genes that code for various subunits in the V_1 domain as well as the V_0 domain of the vacuolar ATPase from yeast have been identified (Stevens & Forgac, 1997; Forgac, 1999; Graham & Stevens, 1999). VMA13p was first cloned by comparing the complementation of growth phenotypes and VMA13 mutations. It has been shown to be essential for catalysis but not for the assembly or the targeting of the enzyme (Ho *et al.*, 1992, 1993).

Here, we report the successful crystallization of the VMA13 gene product (also identified as subunit H) of the yeast vacuolar proton ATPase. The amino-acid sequence consists of 478 residues and does not display homology to any other sequence other than the subunits of the V-type ATPases in mammals, *Caenorhabditis elegans* and *Schizosaccharomyces pombe* (Lu *et al.*, 1998; Zhou *et al.*, 1998).

2. Cloning, expression and purification

The VMA13 gene from *S. cerevisiae* was amplified by PCR using the primers 5′ GGA TCC ATG GGC GCA ACC AAA ATT TTA ATG G 3′ and 5′ AGC GGC CGC TTA TTT GAA GGT ATA TCC AAT GAT TGC C 3′. The protein was cloned as a fusion construct with glutathione-S-transferase (GST-VMA13p). The PCR product of the correct size DNA fragment was gel-extracted, digested and subsequently subcloned into the *Bam*H1 and *Not*1 restriction sites of a pGEX-4T3 expression vector (Pharmacia Biotech).

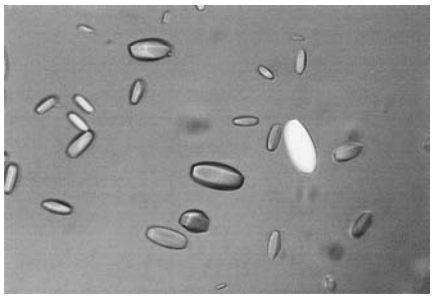

Figure 1
Crystals of VMA13p in polarized light. The crystals grow with the rounded shapes seen here and the extinction is sharp. The largest crystals are 0.12 mm in length.

Ligated vectors were transformed into electro-competent BL21-Gold cells (Stratagene) and selected on 100 µg ml^{-1} ampicillin-containing Luria–Bertani (LB) agar plates.

Positive clones were identified by analytical PCR of the *E. coli* cell plasmids, by DNA sequencing of the PCR fragments and the plasmids and by test expression of the protein.

For expression, selected clones were grown in 2 l LB medium and ampicillin to mid-log phase at 310 K and subsequently induced with 0.6 m*M* isopropyl-β-D-thiogalactopyranoside for about 3 h at a temperature of 301 K. Cells were harvested by centrifugation at 6370*g* for 15 min. The cell paste (~10–20 g for a 2 l preparation) was resuspended in 30 ml 100 m*M* Tris–HCl pH 8.0. After addition of DNAase 1 and a protease-inhibitor cocktail (one tablet of COMPLETE protease-inhibitor cocktail, Boehringer–Mannheim) the cells were ruptured using a French press. The homogenate was then subjected to centrifugation at 35 000*g* for 20 min.

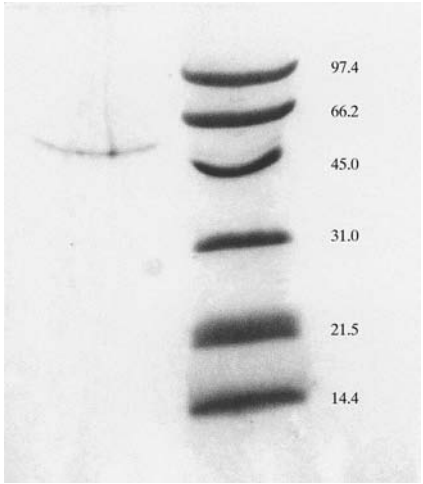

Figure 2
SDS–PAGE of a washed and dissolved crystal (left lane). The right lanes contain markers with molecular weights indicated in kDa.

The supernatant was passed over a 10 ml glutathione-Sephadex 4B column (Pharmacia Biotech) for affinity purification of the GST-VMA13p fusion protein. Unbound protein was washed off with 100 m*M* Tris–HCl pH 8.0 and 100 m*M* NaCl. Bound protein was then eluted with 100 m*M* reduced glutathione in 100 m*M* Tris–HCl pH 8.0. The eluted fusion protein was digested with 500 units of thrombin protease for 24 h at room temperature. Free VMA13 protein was harvested by incubation of the digest with glutathione-Sepharose 4B to remove cut GST protein and undigested fusion protein. SDS–PAGE and mass spectrometry were used to confirm the purity of the sample. Typically, a 2 l preparation yielded approximately 20–30 mg of purified protein.

3. Crystallization and X-ray data collection

Purified protein was dialyzed overnight into 50 m*M* Tris–HCl pH 7.5, 100 m*M* NaCl, 1 m*M* EDTA, 1 m*M* DTT. Subsequently, the protein was concentrated using a Centriprep 10 (Amicon) to a final concentration of about 10 mg ml^{-1}. Crystallization conditions were screened using the Hampton Fast Screen 2 crystallization kit with 48 initial trial conditions using the hanging-drop method. Various conditions appeared to result in crystalline protein material. The best crystals were obtained after three weeks with ammonium sulfate as a precipitant, 50 m*M* Tris HCl pH 7.2, 5 m*M* DTT.

Room-temperature data collection was carried out with crystals mounted in quartz capillaries.

In addition, crystals in mother liquor or crystals briefly washed in polyethylene glycol (PEG 4000, 50 m*M* Tris–HCl pH 7.2) were flash-frozen in a cold stream of nitrogen at 103 K. X-ray diffraction data were collected on an R-AXIS IIc or R-AXIS IV area detector (Rigaku, MSC) on a Rigaku RU-300 rotating-anode X-ray generator and were processed with *XDS* (Kabsch, 1988) or *MOSFLM* (Leslie, 1992). The distance between the crystal and the detector was set to 200 mm. The oscillation range per image was 0.8 or 1.0°. The Laue symmetry was characterized using the autoindexing routines on the strongest reflections. In addition, self-rotation Patterson functions (*GLRF*; Tong & Rossmann, 1990, 1997) and native Patterson maps (Collaborative Computational Project, Number 4, 1994) were calculated to confirm the symmetry and the number of molecules in the asymmetric unit.

4. Results

Crystals grew to a size of 0.1 × 0.07 × 0.07 mm within three to four weeks and were further improved by microseeding (Fig. 1). SDS–PAGE of extensively washed crystals (Fig. 2) confirmed that the molecular weight of the crystallized protein corresponded with that of the intact VMA13p subunit, *i.e.* 54.5 kDa. Amino-terminal sequencing (not shown) also confirmed the identity of the subunit.

At room temperature, the crystals diffracted to only about 10 Å resolution and appeared to decay rapidly in the X-ray beam. Rapidly frozen crystals diffracted to about 4.5 Å resolution, with a few reflections observed to 3.5 Å resolution. In order to reduce the formation of ice around or in the protein crystal, a variety of different cryoprotectants were tested. Washing the crystals in PEG 4000 25% and 50 m*M* Tris–HCl pH 7.2 prior to freezing reduced the number of ice rings in the diffraction pattern dramatically. A further improvement was made by immersing the crystals in paratone-N oil prior to mounting to further reduce the amount of liquid surrounding the crystal (Hope, 1988; Exxon Corp.).

The shape of the crystals indicated hexagonal symmetry, which was confirmed by the auto-indexing routines in *XDS* and *MOSFLM*. The unit-cell parameters were $a = 118.8$, $b = 118.8$, $c = 119.3$ Å, $\alpha = \beta = 90.0$, $\gamma = 120.0°$.

The systematic absences, together with intensity relationships, indicate that the space group is $P3_121$ or enantiomorph. This is also consistent with various rotation-function calculations (not shown). Assuming two molecules per asymmetric unit, the crystal solvent parameter V_m has a normal value of 2.23 Å^3 Da^{-1} (Matthews, 1968). Three-dimensional X-ray data to low resolution have been collected in-house. The resolution accessible with synchrotron radiation has not been determined.

A search for suitable heavy-atom derivatives and the preparation of selenomethionine-labeled protein are currently under way.

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