J Bioenerg Biomembr (2008) 40:569–576
DOI 10.1007/s10863-008-9193-3

Cloning and sequencing of V-ATPase subunit d from mung bean and its function in passive proton transport

Zhuqing Ouyang · Zhuo Li · Xujia Zhang

Received: 29 October 2008 / Accepted: 15 December 2008 / Published online: 5 February 2009

Abstract We have previously shown that vacuolar H+-ATPase subcomplex V_o from mung bean contains subunit d, however, its sequence and function were unknown. In the present study, we report the cloning and recombinant over expression of subunit d from mung bean in *E. coli*. To study the function of subunit d, two vacuolar H+-ATPase subcomplexes V_o from mung bean were purified-one containing subunits a and c(c',c") and the other containing subunits a, c(c',c") and d. After reconstitution of the purified V_o subcomplexes into liposomes, the proton translocation was studied. Our results show that the V_o subcomplex in the absence of subunit d is a passive proton channel, while the V_o subcomplex in the presence of the subunit d is not. Taken together, our data supports the conclusion that the subunit d of the plant vacuolar H^+-ATPase from mung bean is positioned at the central stalk and involved in the proton translocation across the tonoplast membrane.

Keywords Passive proton transport · cDNA sequence · Vacuolar H^+-ATPase · Vigna · RACE-PCR · Liposome · Fluorescence quench · d subunit · Central stalk

Z. Ouyang · X. Zhang (✉)
National Laboratory of Biomacromolecules,
Institute of Biophysics, Chinese Academy of Sciences,
100101 Beijing, China
e-mail: xjzhang@sun5.ibp.ac.cn

Z. Li
Division of Biology, California Institute of Technology,
Pasadena, CA 91125, USA

Introduction

Vacuolar H^+-ATPase(V-ATPase) a multisubunit proton pump,is essential for acidification of diverse intracellular compartments in eukaryotic cells, including lysosomes, endosomes, clathrin-coated and synaptic vesicles, chromaffin granules, and the central vacuoles of plants and fungi. In some specific cell types, V-ATPase also functions to pump protons across the plasma membrane, such cells include renal intercalated cells (Brown and Breton 2000), macrophages (Brisseau et al. 1996), osteoclasts (Li et al. 1999; Frattini et al. 2000), tumour cells (Martinez-Zaguilan et al. 1993) and midgut cells (Wieczorek et al. 2000).

In addition to sharing many structural and functional properties (Forgac 1989), the vacuolar (H^+)-ATPases also closely resemble the well characterized F_1Fo class of (H^+)-ATPases (Ysern et al. 1988). These two classes of ATPases share overall structural similarity (Arai et al. 1988; Adachi et al. 1990) in addition to sequence homology observed between certain subunits (Bowman and Bowman 1988; Bowman et al. 1988a; Mandel et al. 1988; Nelson and Nelson 1989; Puopolo et al. 1991).

Like the F-type ATPases,the V-ATPases are composed of two functional sectors,V_1 and V_o. The peripheral V_1 subcomplex, composed of eitht different subunits (A-H), hydrolyzes ATP and provides energy for protons translocation through the integral membrane V_o subcomplex (Nishi and Forgac 2002). V_o, which consists of five different subunits (a,d,c,c',c"), forms the proton-transport path. These two subcomplexes are connected with a central stalk composed of subunits D and F of V_1, and two or three peripheral stalks composed of subunits C, E, G and H together with the N-terminal domain of subunit a (Forgac 2007). ATP hydrolysis drives the rotation of the central stalk and the c subunit ring. Protons are pumped through a

pathway at the interface between the c subunit ring and subunit a (Forgac 2007).

Subunit d is an unique soluble subunit in V_o subcomplex. It may play an important role in embryonic development (Miura et al. 2003), in coupling ATP cleavage and proton transport (Nishi et al. 2003). It is also involved in the reversible association/dissociation of the V_1 and V_o domains *in vivo* (Momi Iwata et al. 2004). However, its position within the pump complex is still under debate. In this study, the whole cDNA sequence of subunit d from mung bean was for the first time cloned, and over expressed in *E. coli*. By reconstitution of the recombinant subunit d and the purified V_o without subunit d into liposomes, it was shown that the passive proton transportation was largely inhibited compared with that in the absence of subunit d. We also provide experimental evidence demonstrating that the d subunit from mung bean forms part of the pump's central stalk and is thus likely to be important in its rotary mechanism.

Material and methods

Materials

Fresh etiolated mung bean (Vigna radiata L. cv. Wilczek) seedlings were purchased from a local market. Lysophosphatidylcholine (egg yolk, typeI), L-phosphatidylcholine (soybean, Type II-S), ATP, Bafilomycin A and lysophosphatidylcholine were obtained from Sigma. BCA protein assay reagent was obtained from Pierce and *n*-dodecyl-b-D-maltoside from Calbiochem. 5′RACE kit, 3′RACE kit and PCR enzyme system were purchased from Clontech. pGEM-T Easy vector was from Promega. All other chemical reagents were A.R. grade available in P.R. China.

Purification of the Vo subcomplex with and without the 40 kDa band

The vacuolar membrane was prepared from mung bean hypocotyls as described previously (Matsuura-Endo and Yoshida 1990; Kawamura et al. 2000) with minor modification. Tonoplast vesicles were prepared from tissue homogenates by sucrose density ultracentrifugation (Matsuura-Endo and Yoshida 1990). Purification of mung bean Vo subcomplex with the 40 kDa band was according to (Li 2004). To purify Vo subcomplex without the 40 kDa band, The vacuolar membrane obtained above was treated with 5% Triton X-100 and 0.1 M KCl for 30 min. After diluting to 1 mg protein ml^{-1}, the vacular membrane was treated with 0.4 M KI plus 5 mM ATP for 1 h at 4°C to strip the soluble subunits and subsequently centrifuged for 30 min at 150,000 g (Zhang and Forgac 1992). The KI/ATP-stripped membrane was then solubilized with 2 mg ml^{-1} lysophosphatidylcholine for 10 min at 25°C. After centrifugation for 30 min at 150,000 g, the supernatant was immediately subjected to size-exclusion chromatography on a Superdex 200 column (Amersham Biosciences), which was equilibrated with the elution buffer (20 mM Tris–HCl, 1 mM dithiothreitol, 1 mM EGTA, 2 mM $MgCl_2$, 20% (*w*/*v*) glycerol and 0.05% dodecyl maltoside, pH 7.5). Fractions of 0.3 ml were collected. All steps were carried out at 4°C.

Polyacrylamide gel electrophoresis

Native gel electrophoresis of the purified V-ATPase (Li 2004) was based on a method described before (Schagger 1991). The band corresponding to the active protein complex was excised from the gel and subjected to Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). SDS-PAGE was carried out by the method of Laemmli (1970) in the presence of 0.1% SDS.

Cloning of subunit d from mung bean by RACE-PCR

To extract total RNA from mung bean, fresh etiolated mung bean seedlings were cut into pieces, frozen in liquid nitrogen, grinded into powder and then manipulated according to the Trizol reagent manual. Total RNA was then reverse transcribed with the SMART RACE cDNA Amplification Kit (Clontech) according to user's instructions. Specific 5′ and 3′ RACE cDNA ends were amplified with the universal primer mix provided by kit and gene specific primers (GSPs) with the advantage 2 PCR polymerase mix(Clontech). The full length of subunit d was amplified by four primer pairs:two primer pairs for 3′ RACE, and two primer pairs for 5′ RACE. Two degenerated primers 5′-RACE-GSP1(5′-DATNGCYTGRTAN GGNGG-3′) and 3′-RACE-GSP2(5′-GGMATGTTTGAC AGBATTGC-3′), together with adaptor primer(UPM) were used to get part of the conserved sequence. Then the other primers:5′-CAATMCCTGTCAAACATTCC-3′(for 5′ RACE) 5′-GAACTGACGAGAGATGACC-3′(for 3′RACE) together with adaptor primer (UPM), were used in the second round of 5′RACE and 3′RACE, respectively. A combination of touchdown PCR and hot-start PCR reactions was applied. The PCR products were subcloned into pGEM-T Easy vector (Promega) and several recombinant clones were isolated for complete sequencing.

Expression and purification of subunit d

The gene encoding subunit d was amplified by PCR using the following oligonucleotide primers: 5′-CGGGATCC ATGTACGGATTTGAAGCTC-3′(sense primer) and 5′-ATGGTCGACTCAAAATATGAAGACAACACTGTCA-

```
1   CAGAAATTTCCTTAAGGAGGAGCTTCTCATCCTTTATCTGGTTCCCTTTCCCTCAACCAATTTGAGATCCGCGACGCACATTAGATCCGA  90
91  TCCGAAATGTACGGATTTGAAGCTCTCACTTTCAACATCCATGGCGGCTTTCTCGAGGCTATCGTCCGCGGCCACCGCGCAGGTCTCCTC 180
          M  Y  G  F  E  A  L  T  F  N  I  H  G  G  F  L  E  A  I  V  R  G  H  R  A  G  L  L   28

181 ACTACCGCCGATTACAACAACCTCTGCCAATGCGAAACCCTAGATGACATCAAGATGCACCTCTCCGCCACCGAGTACGGCCCTTACCTC 270
29  T  T  A  D  Y  N  N  L  C  Q  C  E  T  L  D  D  I  K  M  H  L  S  A  T  E  Y  G  P  Y  L   58

271 CAAAACGAACCTTCTCCACTGCATACCACCACGATTGTGGAGAAATGTACTCTTAAGCTGGTTGATGATTACAAGAACATGCTATGCCAA 360
59  Q  N  E  P  S  P  L  H  T  T  T  I  V  E  K  C  T  L  K  L  V  D  D  Y  K  N  M  L  C  Q   88

361 GCCACAGAACCCTTGTCAACTTTTTTAGAGTATATCACTTATGGTCACATGATAGACAATGTTGTTTTGATTGTTACTGGCACTTTGCAT 450
89  A  T  E  P  L  S  T  F  L  E  Y  I  T  Y  G  H  M  I  D  N  V  V  L  I  V  T  G  T  L  H  118

451 GAGAGAGATGTTCAAGAATTACTCGAAAAATGCCATCCACTCGGAATGTTTGACAGGATTGCTACTCTGGCTGTTGCTCAGAATATGCGG 540
119 E  R  D  V  Q  E  L  L  E  K  C  H  P  L  G  M  F  D  R  I  A  T  L  A  V  A  Q  N  M  R  148

541 GAGCTTTACAGGTTGGTTCTTGTTGACACTCCTCTAGCTCCGTACTTCTCTGAGTGCATTACATCAGAGGACTTGGATGATATGAACATT 630
149 E  L  Y  R  L  V  L  V  D  T  P  L  A  P  Y  F  S  E  C  I  T  S  E  D  L  D  D  M  N  I  178

631 GAAATAATGAGGAACACTCTTTACAAGGCATACCTTGAAGACTTCTACAGATTTTGTCAGAAACTTGGTGGTGCCACAGCTGAGATAATG 720
179 E  I  M  R  N  T  L  Y  K  A  Y  L  E  D  F  Y  R  F  C  Q  K  L  G  G  A  T  A  E  I  M  208

721 TCTGACCTTCTAGCTTTTGAGGCTGATAGAAGGGCTGTGAATATTACCATAAATAGCATTGGTACTGAACTGACGAGAGATGACCGTAGA 810
209 S  D  L  L  A  F  E  A  D  R  R  A  V  N  I  T  I  N  S  I  G  T  E  L  T  R  D  D  R  R  238

811 AAATTGTACTCTAATTTTGGTTTGCTATACCCATATGGTCATGAGGAACTTGCTATCTGTGAGGACATTGATCAGGTTCGTGCTGTCATG 900
239 K  L  Y  S  N  F  G  L  L  Y  P  Y  G  H  E  E  L  A  I  C  E  D  I  D  Q  V  R  A  V  M  268

901 GAAAAATACCCCCCCCTATCAATCTATTTTTGCTAAGCTATCATATGGTGAAAGTCAGATGCTTGACAAGGCATTCTATGAGGAGGAGGTG 990
269 E  K  Y  P  P  Y  Q  S  I  F  A  K  L  S  Y  G  E  S  Q  M  L  D  K  A  F  Y  E  E  E  V  298

991 AAAAGGCTTTGCTTGGCGTTTGAACAACAGTTTCATTATGCTGTGTTCTTCGCATATATGAGGTTAAGGGAACAGGAGATCAGGAATTTA 1080
299 K  R  L  C  L  A  F  E  Q  Q  F  H  Y  A  V  F  F  A  Y  M  R  L  R  E  Q  E  I  R  N  L  328

1081ATGTGGATTTCAGAATGTGTTGCTCAGAATCAGAAGTCCAGAGTTCATGACAGTGTTGTCTTCATATTTTAGTCGGATTGCTCGTTATAG 1170
329 M  W  I  S  E  C  V  A  Q  N  Q  K  S  R  V  H  D  S  V  V  F  I  F  *

1171TTTGTATGTTCGTTGGGATAATTTGTTGACTTTCTGCATTCGCCATCTGGGTTGTCATTTGTAGACACTAGACAGCGACCGTTCAGAAGA 1260
1261TTGAGATAAAAATAATTAATCTTATGCTGATAAGATCTTCTGTAATATGGAAGGAAGATATTTATAGTCGACTTTGATTGAAATATGTTC 1350
1351AGTTTTAAAGATCTTAAGATTCAATTTGTATGTTGTGAATTTTACAGTAAATATTTCTATGCAATAAACCTGCTAGCTGGGTCTATGTAA 1440
1441AAAAAAAAAAAAAAAAAAAAAAAAAAAA  1467
```

Fig. 1 Sequence of V-ATPase subunit d from mung bean. The deduced amino acid sequence of the ORF is shown below the nucleotide sequence. Nucleotide numbers and amino acid numbers are given on the *left* and on the *right*, *up* and *down*, respectively

3′(antisense primer), possessing *Bam*HI and *Sal*I sites, respectively (underlined). Amplified DNA was digested with *Bam*HI and *Sal*I, followed by ligation between the *Bam*HI and *Sal*I sites of pGEX-6p-1 (Amersham pharmacia) to obtain pGEX-d. *E. coli* BL21(DE3) cells harboring pGEX-d were grown in Luriae Bertani (Frattini) agar plates containing 100 μg/ml ampicillin. Positive clones were identified by analytical PCR and by DNA sequencing. For

expression, selected clones were grown in LB medium containing ampicillin to mid-log phase and subsequently induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 18 h at a temperature of 289 K. The harvested cells were resuspended in 50 mM Tris–HCl (pH 8.0) and 1 mg/ml lysozyme, after centrifugation at 19,000 *g* for 30 min. The supernatant was passed over a 3 ml glutathione-Sephadex 4B column (Pharmacia Biotech) equilibrated with 1XPBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) for affinity purification of the GST-d fusion protein. Unbound protein was washed off with 30 ml 1XPBS. Bound protein was then eluted with 10 mM reduced glutathione in 1 XPBS. Desalting column was used for exchange with PreScission cleavage buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.5) The eluted fusion protein was digested with 500 units of PreScission Protease (Invitrogen) overnight at 277 K. Free subunit d was harvested by applying the sample to an equilibrated GST column to remove the GST moiety of the fusion protein and the PreScission Protease. For further purification,the fractions containing subunit d were collected and concentrated by ultrafiltration, then applied onto Superdex 75 HR 10/30 (Amersham Pharmacia) equilibrated with 50 mM Tris–HCl,0.1 M NaCl, pH 8.0. The

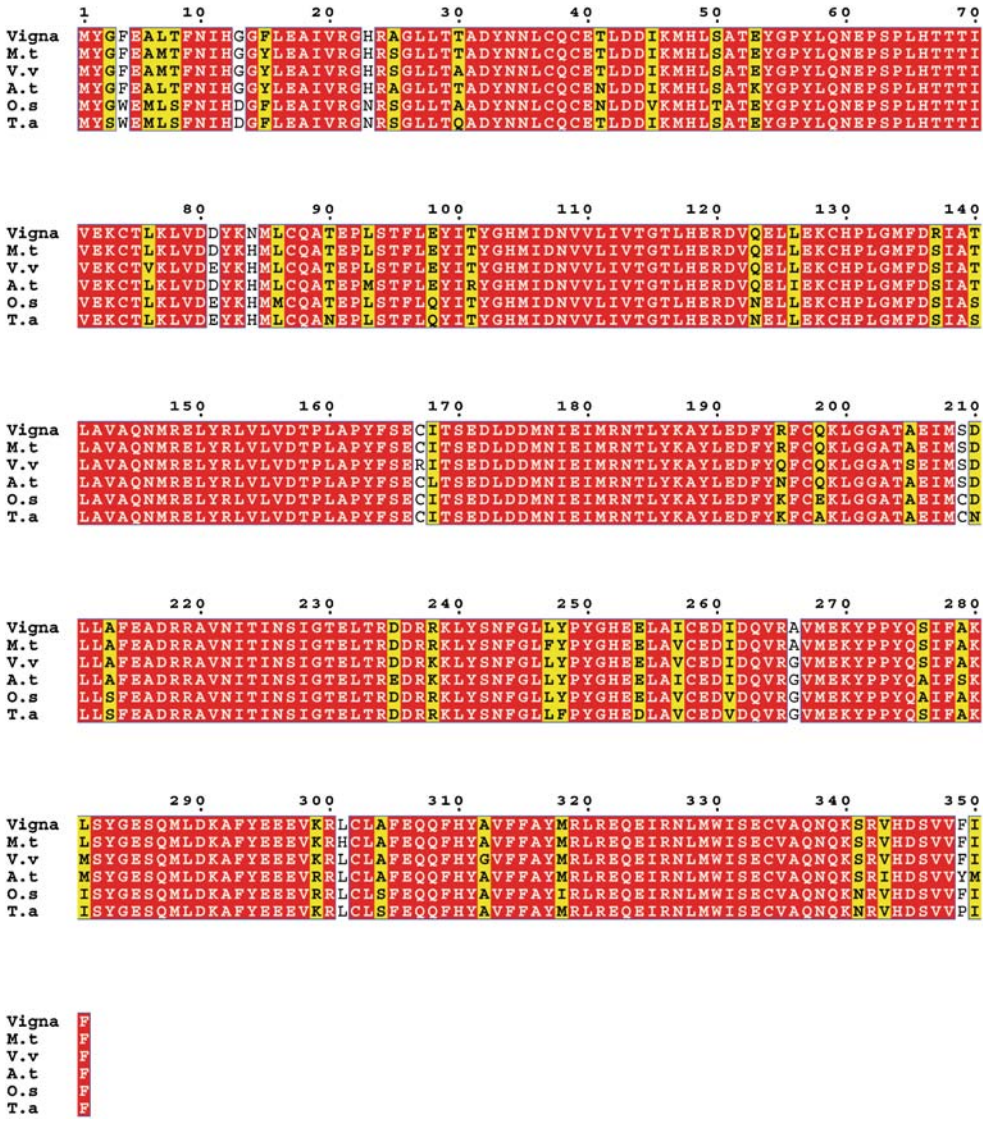

Fig. 2 Amino acid sequence alignment between subunit d of various sources. Calculated homology between vigna subunit d (GenBank accession number FJ360448) and that of *Medicago truncatula* was 97% (accession number ABN08957), *Vitis vinifera* 95% (accession number CAN79922), *Arabidopsis thaliana* 94% (accession number NP_189513), *Oryza sativa* 91% (accession number NP_001043429) and *Triticum aestivum* 91% (accession number ABG23315). Amino acids being identical in all selected specie are indicated in *red* and the most conserved residues are highlighted in *yellow*. Sequences were aligned using CLUSTALW (Thompson et al. 1994) and the figure was produced with ESPript (Gouet et al. 1999)

fractions containing subunit d were collected. SDS-PAGE and mass spectrometry were used to identify the recombinant protein.

Passive proton transport assay

L-phosphatidylcholine (soybean, Type II-S) was purified using organic solvent as described (Sone et al. 1977) and dissolved in diethyl ether at 10 mg/ml. For reconstitution, 10 mg L-phosphatidylcholine in diethyl ether was vacuum-dried and dissolved in resuspension buffer (2%Triton X-100, 0.25 M KCl, 10 mM MES/Tris, pH 7.0, 10% (*v*/*v*) glycerol) and sonicated to clarity. The reconstitution procedure was based on the method of Muller et al. (1997).

Wet Bio-Beads SM-2 (Bio-Rad), 0.4 g, were added to the mixture, which was incubated for 1 h at room temperature with gentle rocking. The beads were decanted, and the supernatant was recovered and mixed with another 0.4 g wet Bio-Beads. The same incubation process was repeated twice, and the supernatant was centrifuged for 30 min at 150,000 *g* (Beckman TLA-100.3). The pellet containing the reconstituted vesicles was resuspended in 300 µl of resuspension buffer containing 150 mM KCl but no Triton X-100. Proton pumping by reconstituted proteoliposomes was monitored by acridine orange fluorescence quenching. The reaction mix contained 10 mM MES/Tris, pH 7.0, 150 mM NaCl, 0.5 mM EDTA, 6% (*v*/*v*) glycerol and 6 µM Acridine orange. Fluorescence quenching (495 nm excitation, 530 nm emission wave lengths) was measured in a Fluorescence Spectrophotometer (Hitachi F-4500). The experiment was started with 20 nM valinomycin and terminated with the addition of the proton ionophore Gramicidin D(sigma) at a final concentration of 0.5 µg/ml.

Results and discussion

We have previously demonstrated that the 40 kDa protein band co-purified with V_o subcomplex is subunit d of V-ATPase from mung bean (Li 2004), but its sequence and function were not known. To understand its function, in the present study, we cloned and over expressed the subunit d from mung bean for the first time.

Molecular cloning of subunit d

The GSP sequences for the first round of 5′RACE and 3′ RACE are degenerate primers designed according to the amino acid sequence obtained from mass spectrometry and homology alignment of subunit d from several species respectively. Gene specific primers, together with the adaptor primer (UPM) for 3′ RACE and 5′RACE were used to obtain the full length cDNA sequence of subunit d.

The V-ATPase subunit d (GenBank accession number FJ360448) cDNA sequence is 1467 bp in length, including a 96 bp 5′ UTR (untranslated region), a 1056 bp open reading frame, and a 315 bp 3′ UTR (Fig. 1). The putative subunit d is predicted to be 351 amino acids long, with a calculated molecular mass of 40.7 kDa, agreeing well with that determined for subunit d purified from mung bean by SDS-PAGE, with an isoelectric point of 4.9. However, the deduced amino acid sequence "YPPYQSIFAK" is different from that obtained by mass spectrometry "YPPYQAIFSK"(Li 2004). This minor difference could be due to the fact that the database searched did not contain exactly the same peptide sequence as that deduced from the cDNA sequence at that time.

Sequence comparison of subunit d

The protein shows high amino acid identity with previously sequenced V-ATPase subunit d. Figure 2 aligns the sequence of *Vigna* with those of *Medicago truncatula*, *Vitis vinifera*, *Arabidopsis thaliana*, *Oryza sativa* and *Triticum*

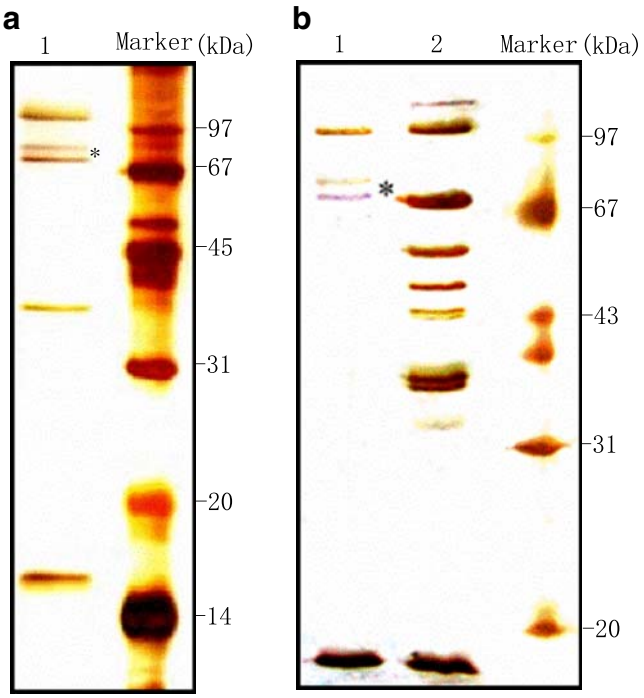

Fig. 3 Purification of V_o subcomplexes a) The V_o subcomplex containing subunits a, c and d; b) The V_o subcomplex containing subunits a and c (*lane 1*) and the intact enzyme (*lane 2*). The eluate of Superose 6 chromatography was electrophoresed on a native gel in the presence of 0.1% Triton X-100. After electrophoresis, the gel was stained with Coomassie blue. The band corresponding to the active V-ATPase was excised from the gel and subjected to SDS-PAGE (**b**, *lane 2*). The gel was stained with silver. Subunit positions are indicated in the silver stained gel. The marker is shown on the right. The 12 kDa subunit has run out of the gel. The molecular size standard is shown on the *right*. The two bands indicated by the *asterisk* are impurities

aestivum. Identity between the various d subunits ranged from 88% to 97%.

Subunit d plays an important role in passive proton transport

The V-ATPases are composed of a peripheral V_1 domain responsible for ATP hydrolysis and an integral V_o domain that carries out proton transport. The V_o domain consists of three subunits:subunit a, subunit c(c',c") and subunit d which is an unique soluble subunit. In this study, the V_o subcomplex with or without subunit d was purified respectively (Fig. 3). To study the function of subunit d in the proton translocation conducted by V_o, the purified V_o subcomplex with or without subunit d was reconstituted into liposomes. As shown in Fig. 4.a, fluorescence quenching traces conducted with liposomes in the absence of protein (negative control) and in the presence of V_o subcomplex containing subunit d decreased by about 25% (or in the presence of the holoenzyme, decreased by about 25%, data not shown), however Vo subcomplex without subunit d decreased by 65%. Similar to the purified V_o subcomplex containing subunit d, the incubation of the recombinant subunit d with the purified V_o subcomplex

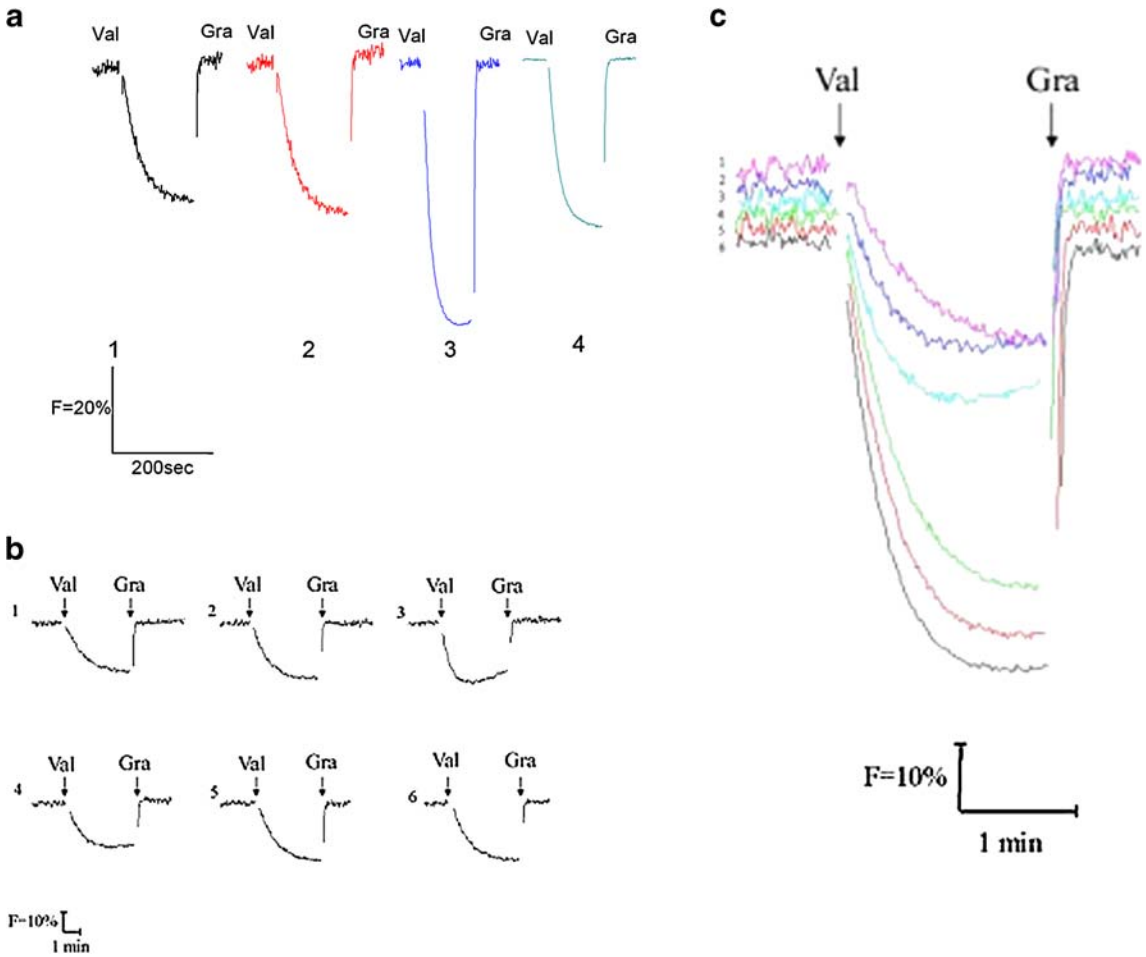

Fig. 4 **a** Proton-channel activities of Vo subcomplexes. *Panels 1–4* are acridine orange quenching traces conducted with liposomes without protein (negative control Trace 1), reconstituted V_o subcomplexes with subunit d (Trace 2), without subunit d (trace 3), V_o containing subunit a, c and the expressed subunit d (trace 4), respectively. In each *panel*, the experiment was initiated by the addition of valinomycin and terminated with the addition of proton ionophore Gramicidin D. **b** The inhibition profile of passive proton transport by reconstituted V_o containing subunits a and c. *Traces 2–6* show acridine orange quenching conducted with reconstituted V_o (0.6 μg) containing subunits a and c. The experiment of each trace was initiated by the addition of valinomycin and terminated with addition of the proton ionophore Gramicidin D. For *trace 2*, *4* and *5*, reconstituted V_o was pretreated with 5, 0.5 and 0.125 nM bafilomycin A1, respectively; For *trace 3*, reconstituted V_o was pretreated with 50 μM DCCD; For *trace 6*, reconstituted V_o was assayed in the absence of inhibitors; *trace 1* liposomes without protein as negative control. **c** Reconstituted V_o (a, c and d) is not a passive proton channel. *Traces 1–6* show acridine orange quenching conducted with liposomes without protein (*trace 1*) and reconstituted Vo (containing subunits a, c and d, 1 μg protein of each in *trace 2–6)*. For *traces 3*, *4* and *5*, reconstituted V_o was pretreated with 50 μM DCCD, 0.5 nM and 5 nM bafilomycin A1, respectively; For *trace 6*, reconstituted V_o was pretreated with 140 mM Mes; For *trace 2*, reconstituted V_o was assayed in the absence of inhibitors

without subunit d also resulted in the inhibition of the passive proton transport (Fig. 4.a4). The fluorescence quenching trace decreased by about 37%. Interestingly, fluorescence quenching traces conducted with V_o(a+c) and the recombinant atpC (Momi Iwata et al. 2004) reconstituted liposomes decreased by about 21% (data not shown). This result indicates that the V_o subcomplex without subunit d is sensitive to the K+/valinomycin mediated membrane potential.

Bafilomycin A1 is a V-ATPase specific inhibitor (Bowman et al. 1988b) and dicyclohexylcarbodiimide (DCCD) inhibits the proton translocation in F- and V-ATPases (Kaestner et al. 1988). To study the effects of the inhibitors on the proton translocation conducted by the V_o subcomplex, DCCD and bafilomycin A1 at different concentrations were added to the reaction mix, respectively. Figure 4.b shows that Bafilomycin A1 and DCCD are able to inhibit the passive proton transport conducted by the reconstituted V_o subcomplex without subunit d in a concentration dependent manner. However, neither Bafilomycin A1 nor DCCD had any effect on the passive proton transport by the V_o subcomplex with subunit d (Fig. 4.c). The results suggested that V_o subcomplex without subunit d is a passive proton channel driven by K^+/valinomycin mediated membrane potential while V_o subcomplex with subunit d is not.

It has been demonstrated by a number of laboratories (Aris 1985; Schneider 1985) that the F_o domain of the F_1F_o (H^+)-ATPases can act as a passive DCCD-inhibitable proton channel. Because of the similarity between the vacuolar and F_1F_o classes of (H^+)-ATPase, both in overall structure and in sequence homology, it was of interest to determine whether the V_o domain, like F_o, could conduct protons. But it has been demonstrated that the Vo domain is not a passive DCCD-inhibitable proton channel (Zhang and Forgac 1992). Interestingly, because it has been reported previously that the 17-kDa c subunit of the coated vesicle (H^+)-ATPase, when reconstituted into phospholipid vesicles, was itself competent to form a DCCD-inhibitable proton channel (Sun et al. 1987). Our results can explain the contradiction between both the results. As the V_o domain using by Zhang contained subunit d but the reconstituted subunit c did not. According to our results, the V_o domain without subunit d, like F_o, is also a passive DCCD-inhibitable proton channel, while V_o with subunit d is not. It should be noted that the Thermus enzyme, which contains subunit d (called subunit C in Thermus nomenclature) (Yokoyama et al. 2003) still passively conducts protons even in the presence of subunit d, suggesting that subunit d may not be sufficient to inhibit passive proton transport by free V_o.

Recently, The crystal structure of the subunit C of *Thermus thermophilus* V-ATPase, homologous to eukaryotic subunit d of V-ATPases, has been determined (Momi Iwata et al. 2004). It showed that the subunit C is serving as a "socket" to attach the V_1 central stalk subunits onto the L subunit ring (homologous to eukaryotic c subunit) of the V_o domain. Yokoyama also demonstrated that subunits C associates tightly with the L subunit ring (Yokoyama et al. 2003). Our results are consistent with their conclusions, because KI and DOC can not separate subunit d from the V_o subcomplex (Li 2004). Our electron-microscopy projection map of mung bean V-ATPases contained a protein density just above the proteolipid c ring that is likely to be subunit d (Li 2004). Very recently, Annabel and his colleagues demonstrated that the d subunit in human forms part of the central stalk of the H^+-ATPase (Smith et al. 2008). In the current study, we found that subunit d can be released from the V_o subcomplex by the combined treatment of Triton X-100, KI and ATP. The inhibition of passive proton translocation of the V_o subcomplex by subunit d indicated that subunit d blocks the passage of a "passive" proton translocation channel.

In summary, the subunit d of vacuolar H^+-ATPase from mung bean was cloned for the first time and over expressed in *E. coli*. By reconstitution of the purified V_o subcomplexes into the liposomes, we are able to demonstrate that the V_o subcomplex containing subunit a and c is a passive DCCD-inhibitable proton channel driven by K+/valinomycin mediated membrane potential and the d subunit in mung bean is centrally located within the proton pump.

Acknowledgment We thank Dr. So Iwata (Imperical College London, UK) for provision of subunit C of *Thermus thermophilus* V-ATPase, Dr. Fei Sun (Institute of Biophysics, Chinese Academy of Sciences, Beijing, China) for help in data processing, Ankita Roy (National Institute of Environmental Health Sciences, NIH, USA) and Elitza Tocheva (California Institute of Technology, Pasadena, USA) for reading of the manuscript. This work was supported by National Basic Research Program of China (2004CB720000 and 2006CB911001) and National Natural Science Foundation of China (30770492).

References

Adachi I, Arai H, Pimental R, Forgac M (1990) J Biol Chem 265(2): 960–966

Arai H, Terres G, Pink S, Forgac M (1988) J Biol Chem 263(18): 8796–8802

Aris JP, Simoni RD (1985) Biochem Biophys Res Commun 128(1): 155–162

Bowman EJ, Tenny K, Bowman BJ (1988) J Biol Chem 263(28): 13994–4001

Bowman EJ, Siebers A, Altendorf K (1988a) Proc Natl Acad Sci U S A 85:7972–7976

Bowman BJ, Alle R, Wechser MA, Bowman EJ (1988b) J Biol Chem 263(28):14002–14007

Brown D, Breton S (2000) J Exp Biol 203(Pt 1):137–145
Brisseau GF, Grinstein S, Hackam DJ et al (1996) J Biol Chem 271(4):2005–2011
Forgac M (1989) Physiol Rev 69(3):765–796
Forgac M (2007) Nat Rev Mol Cell Biol 8(11):917–929
Frattini A, Orchard PJ, Sobacchi C, Giliani S, Abinun M et al (2000) Nat Genet 25(3):343–346
Gouet P, Courcelle E, Stuart DI, Metoz F (1999) Bioinformatics 15(4): 305–308
Kaestner KH, Randal KL, Sze H (1988) J Biol Chem 263(3):1282–1287
Kawamura Y, Arakawa K, Maeshima M, Yoshida S (2000) J Biol Chem 275(9):6515–6522
Li Z, Zhang X (2004) Planta 219(6):948–954
Li YP, Chen W, Liang Y, Li E, Stashenko P (1999) Nat Genet 23(4): 447–451
Mandel MM, Hulmes Y, Pan JD, Nelson YC, Nelson H, Nelson N (1988) Proc Natl Acad Sci U S A 85(15):5521–5524
Martinez-Zaguilan R, Lynch RM, Martinez GM, Gillies RJ (1993) Am J Physiol 265(4 Pt 1):C1015–1029
Matsuura-Endo C, Maeshima M, Yoshida S (1990) Eur J Biochem 187(3):745–751
Miura GI, Froelick GJ, Marsh DJ, Stark KL, Palmiter RD (2003) Transgenic Res 12(1):131–133
Momi Iwata HI, Stambouli E†, Ikeda C, Tamakoshi M, Nagata K, Hisayoshi Makyio BH, Barber J, Yoshida M, Yokoyama K, Iwata S (2004) Proc Natl Acad Sci U S A 101:59–64
Muller ML, Irkens-Kiesecker U, Kramer D, Taiz L (1997) J Biol Chem 272(19):12762–12770
Nelson H, Mandiyan S, Nelson N (1989) J Biol Chem 264(3):1775–1778
Nishi T, Forgac M (2002) Nat Rev Mol Cell Biol 3(2):94–103
Nishi T, Kawasaki-Nishi S, Forgac M (2003) J Biol Chem 278(47): 46396–46402
Puopolo CK, Adachi I, Forgac M (1991) J Biol Chem 266(36):24564–24572
Schagger H, von Jagow G (1991) Anal Biochem 199(2):223–231
Schneider E, Altendorf K (1985) Embo J 4(2):515–518
Smith ANF, Sorrell RW, Karet SL, FE (2008) J Bioenerg Biomembr
Sone N, Yoshida M, Hirata H, Kagawa Y (1977) J Biochem 81(2):519–528
Sun S-Z, Xie X-S, Stone DK (1987) J Biol Chem 262(30):14790–14794
Thompson JD, H, iggins DG, Gibson TJ (1994) Nucleic Acids Res 22 (22):4673–4680
Laemmli UK (1970) Nature 227(5259):680–685
Wieczorek H, Gruber G, Harvey WR, Huss M, Merzendorfer H, Zeiske W (2000) J Exp Biol 203(Pt 1):127–135
Yokoyama K, Nagata K, Imamura H, Ohkuma S, Yoshida M, Tamakoshi M (2003) J Biol Chem 278(43):42686–42691
Ysern X, Amzel LM, Pedersen PL (1988) J Bioenerg Biomembr 20(4):423–450
Zhang J, Myers M, Forgac M (1992) J Biol Chem 267(14):9773–9778